EFFECTS OF UBIQUINOL WITH FLUID RESUSCITATION FOLLOWING HEMORRHAGIC SHOCK

BY

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Abstract

Hemorrhagic shock (HS) and fluid resuscitation triggers ischemia-reperfusion injury in cells and increases the production of reactive oxygen species (ROS) which are known to activate the intrinsic pathway of apoptosis and contribute to organ dysfunction.[1] Ubiquinol is a potent free radical scavenger which is produced endogenously and functions as part of the mitochondrial respiratory chain.[2] No study has been conducted to investigate the effects of ubiquinol related to HS. The overall aim of this study was to examine the effects of ubiquinol on leukocyte mitochondria and in the lungs, diaphragm, heart and kidneys as a supplemental treatment for HS.

A randomized experimental design was used for this study. Adult male Sprague-Dawley rats (n = 20) were anesthetized and HS was induced by withdrawing 40% of the rat's blood volume to maintain a mean arterial pressure of 45-55 mmHg for 60 minutes. Following HS the rats were resuscitated with blood and lactated Ringer's (LR) with or without ubiquinol (1 mg per 100 g of body weight). The rats were monitored for 120 minutes, the animals were euthanized and the organs harvested.

Leukocyte mitochondria superoxide ($O_2^{\bullet -}$) was measured by flow cytometry using MitoSOX Red, a mitochondrial-targeted variant of the fluorescent probe hydroethidine. Superoxide levels were measured at baseline, end of HS and 120 minutes following fluid resuscitation. Arterial blood values were also recorded at these times. At the end of experiment, diaphragms were evaluated for hydrogen peroxide (H_2O_2) using the fluorescent probe dihydrofluorescein-diacetate (Hfluor). The lungs, diaphragm, heart, and kidneys were examined for percent of apoptotic nuclear membrane damage using a differential dye uptake method with acridine orange and ethidium bromide.

No significant differences were found between groups with regard to the volume of blood removed, hemodynamic status or arterial blood values ($p > 0.05$). Ubiquinol decreased leukocyte mitochondrial production of $O_2^{\bullet-}$ at the end of the experiment by 35% compared to the control group (4687.2 ± 265.4 versus 7227.9 ± 534.5, $p < 0.001$). Similarly, the mean fluorescence intensity (MFI) of diaphragm H_2O_2 was significantly lower in the ubiquinol group compared to control (4193 ± 333 versus 23513 ± 5098, $p < 0.001$). The percent of apoptosis in the lungs, diaphragm, heart, and kidneys was significantly reduced in the animals treated with ubiquinol compared to the control group (6.0 ± 0.7% versus 39.2 ± 1.1%, 4.7 ± 0.5% versus 30.6 ± 2.4%, 2.9 ± 0.6% versus 23.6 ± 1.2%, 2.4 ± 0.3% versus 42.1± 1.9%, respectively, $p < 0.001$).

Ubiquinol was effective in decreasing leukocyte mitochondrial $O_2^{\bullet-}$ formation, which suggests that ubiquinol scavenged $O_2^{\bullet-}$ within the mitochondria. Since ubiquinol is a potent antioxidant, it also probably scavenged other free radicals outside the mitochondria. The increased concentration of ubiquinol within the mitochondria would assist in maintaining the activities within the electron transport chain during HS. In addition, the decreased mitochondrial $O_2^{\bullet-}$ would result in lower H_2O_2 production. The significant reduction in the percent of apoptosis in lungs, diaphragm, heart and kidneys between the control and treatment rats, suggests that decreased ROS production attenuated the activation of the intrinsic (mitochondrial) apoptosis pathway.[3] The findings could also be attributed to the stabilization of the mitochondrial membrane by ubiquinol, which has been demonstrated in a previous study.[4] In conclusion, ubiquinol may have application as a supplemental treatment to reduce free radical damage and apoptosis-related injury following HS and fluid resuscitation.

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Table of Contents

Abbreviations

2-OH-Et^+	2-hydroxyethidium
8-OHdG	8-hydroxy-2'-deoxyguanosine
ABG	Arterial Blood Gas
ABP	Arterial Blood Pressure
ADP	Adenosine Diphosphate
AO	Acridine Orange
Apaf-1	Apoptosis Activating Factor
ATP	Adenosine Triphosphate
Bcl-2	B Cell Lymphoma 2
BE	Base Excess
BHT	Butylated Hydroxytoluene
CAD	Cytoplasmic Dnase
CoQ_{10}	Coenzyme Q_{10}
CRNA	Certified Registered Nurse Anesthetist
DBZTC	2-chloro-1,3-dibenzothiazoline-cyclohexene
DCF	2′,7′-dichlorofluorescein
DHR	Dihydrorhodamine-123
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EB	Ethidium Bromide
ELISA	Enzyme-linked Immunosorbant Assay
ETC	Electron Transport Chain

FACS	Fluorescence-activated Cell Scanning or Sorting
FADD	Fas-Associated Death Domain
$FADH_2$	Flavin Adenine Dinucleotide-reduced form
FSC	Forward Scattered (Laser Light)
GABA	Gamma Aminobutyric Acid
Gpx	Glutathione Peroxidase
GSH	Glutathione
GSSG	Glutathione Disulfide
H_2DCF	Dichlorodihydrofluorescein
H_2O_2	Hydrogen Peroxide
HCO_3	Bicarbonate
HE	Hydroethidine
$HE^{\bullet+}$	Hydroethidine Radical
Hfluor-DA	Dihydrofluorescein-diacetate
HH	Hemorrhagic Hypotension
HOCl	Hypochlorous Acid
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
HS	Hemorrhagic Shock
IAPs	Inhibitors of Apoptosis Proteins
LR	Lactated Ringer’s Solution
MAP	Mean Arterial Pressure (MAP)
MDA	Malondialdehyde

MFI	Mean Fluorescence Intensity
MPTP	Mitochondrial Permeability Transition Pores
mtDNA	Mitochondrial DNA
MOF	Multiple Organ Failure
NMDA	N-methyl-d-aspartate
NADH/NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFκB1	Nuclear Factor kappa B1
$O_2^{\bullet -}$	Superoxide
$OH^{\bullet}$	Hydroxyl Radical
p53	Protein-53
$PaCO_2$	Partial Pressure of Arterial Carbon Dioxide
PaO_2	Partial Pressure of Arterial Oxygen
PE	Polyethylene
pH	Acidity
PMN	Polymorphonuclear Neutrophil
PS	Phosphatidylserine
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RR	Respiratory Rate
SOD	Superoxide Dismutase
SpO_2	Oxygen Saturation

SSC	Side-scattered (Laser Light)
TCA	Trichloroacetic Acid Cycle
TNF	Tumor Necrosis Factor
TRADD	TNF Receptor Associated Death Domain
U.S.	United States
VEGF	Vascular Endothelial Growth Factor
XO	Xanthine Oxidase

CHAPTER 1.

General Introduction

The United States (U.S.) military is frequently deployed in locations where troops are subject to hostile activity and combat injuries. Our most recent experiences in Iraq and Afghanistan have resulted in the combat-related deaths of more than 5,100 members of the armed forces with an additional 49,000 wounded in action.[5] From the U.S. military experience, hemorrhage is the single primary cause of death in battlefield casualties.[6] Injuries are also the fifth overall leading cause of civilian deaths in the U.S. and the primary cause of death for Americans less than 40 years of age. Independent of the specific mechanisms of injury, hemorrhagic shock (HS) is the second leading cause of death in the injured, accounting for as many as 40% of overall trauma deaths.[7] Hemorrhage accounts for the largest proportion of deaths in the first hour following trauma and 80% of operating room trauma deaths. In a recent Australian epidemiological study, researchers found the most common injuries to civilians were the result of automobile accidents, firearms and stabbings and suggested that hemorrhage is a significant determinant of outcome for these victims.[8] Post-injury organ failure also accounts for a high percentage of mortality from HS and represents another important opportunity in the care of injured patients to reduce morbidity and mortality.[9]

Our experiences with battlefield injuries have contributed to the development of trauma resuscitation regimens that include the use of hemostatic agents that can be administered or applied in the pre-hospital setting and fluid and blood replacement therapy in field hospital settings and emergency departments.[6] The development of trauma care and treatment in military settings has application for the management of injured civilian patients. In both military and civilian settings, the care of the hemorrhaging patient has led to the development of treatment designs that have focused

on identification and control of bleeding, the administration of fluids, management of coagulation problems and other supportive care.[10,11]

Hemorrhagic shock is defined as a loss of blood severe enough to induce a physiologic state where oxygen delivery does not meet metabolic demands.[12] It has been classified into three stages: 1) the compensatory or non-progressive stage, 2) the progressive stage and 3) the refractory or irreversible shock stage.[13] During the first stage, reflex neuroendocrine mechanisms are activated, resulting in an increase in heart rate and respirations, as well as peripheral vasoconstriction to maintain perfusion of the major organs. Stage two is characterized by tissue hypoperfusion, deteriorating circulation and the onset of metabolic imbalances (primarily acidosis from anaerobic metabolism in under-perfused tissues). In the third stage, the vital organs fail and the effects of shock can no longer be reversed, even if perfusion is restored. At this point, brain damage and cell death are sufficient to cause the death of the individual.[13,14]

Hemorrhagic shock involves the loss of a significant proportion of the circulating blood volume (typically greater than 20%), which, if not properly treated, will result in acidosis, cellular hypoxia, microcirculatory damage, multiple organ failure (MOF) and death.[14] The early effect of HS is a reduction in cardiac output leading to decreased delivery of oxygen to peripheral tissues. Initially, hypotension elicits a reflex that results in vasoconstriction in an effort to raise arterial pressure, but vasoconstriction may result in further damage to tissues by exacerbating peripheral hypoperfusion.[15] Persistent hemorrhage without resuscitation results in delayed hypotension, also called vasodilatory shock, which occurs when vascular smooth muscle is unable to constrict.[16] The loss of blood volume with decreased tissue oxygen delivery stimulates anaerobic production of

lactate with a reduction in plasma bicarbonate levels that results in metabolic acidosis. Increased acidosis, is predictive of complications such as coagulopathy, sepsis, organ failure and death.[7]

Death to the individual following HS may occur as a result of either the early or late effects of hemorrhage.[7] Death may occur quickly due to rapid exsanguination, even before resuscitation can be initiated.[8] Exsanguination may be present as the only lethal factor or in combination with other injuries that result in central nervous system demise or airway damage. Morbidity and mortality may also be delayed and occur secondary to these other injuries or as the result of life-threatening infection with sepsis and MOF following HS.[7,9,17] Post-injury MOF is the most significant cause of late trauma mortality with estimations of 51 to 61% of deaths.[9,18] The incidence of MOF has been reported to occur in a bimodal pattern with early and late peaks (during the first three days of hospitalization and between five to seven days).[7]

Systemic effects of HS, such as profound hypotension, increased respiratory rate and acidosis will direct clinicians' care in the immediate treatment of the trauma patient. Clinicians involved in resuscitation, monitor and treat the immediate signs and symptoms of HS. With shock, the decreased delivery of oxygen to the tissues is the result of more than one event. Although hypovolemia is the greater concern in the early stages of HS, the loss of circulating hemoglobin causes a reduction in oxygen-carrying capacity. The decrease in hemoglobin, circulating blood volume and cardiac output, combined with peripheral vasoconstriction and increased oxygen demand by organs, impairs peripheral perfusion, resulting in acidosis and further hypoxia.[13] Moreover, hypoxia at the cellular level initiates a cascade of events leading to the release of inflammatory cytokines,[19] a

decrease in the production of adenosine triphosphate (ATP) by the mitochondria and increased reactive oxygen species (ROS), causing a condition of oxidative stress.[20] Subsequent reperfusion during fluid resuscitation stimulates the release of cytokines into the mesentery from the previously ischemic gut. These cytokines enter the vascular circulation via the mesenteric lymph system and cause the "priming" of polymorphonuclear neutrophils (PMNs).[9] The PMNs migrate to end organs, especially the lungs, heart, liver and kidneys, and cause direct local cytotoxic damage by the release of ROS and pro-inflammatory mediators. Oxidative stress also initiates apoptosis or programmed cell death, resulting in widespread organ damage or patient demise that occurs during the later stages of HS.[21] The relationship between HS-induced oxidative stress and the incidence of MOF is one of the main issues addressed in this study.

It is well established that HS results in decreased tissue perfusion, and cellular hypoxia,[11,22] and can lead to cardiac dysfunction,[23] diaphragm muscle failure[24,25] and lung injury.[26,27] The severity of lung damage after HS has been shown to be predictive of patient outcome,[28] although the precise mechanisms for this finding are not clear. Inflammatory response and oxidative stress are two plausible explanations for the problem of organ injury following HS and may in fact be linked.[28-30] The role of oxidative stress and inflammation resulting from HS has led to speculation that exogenously administered antioxidants or anti-inflammatory substances may protect organ tissues from damage as a result of a HS state.[22,31-37]

Much of the emphasis on the management of HS has been on patient treatment in the early stages of trauma, with the development of strategies aimed at controlling bleeding, surgical repair, selection of resuscitation fluids and transfusion products and the

treatment of hypotension, hypothermia and coagulopathy.[6,10,38-40] Awareness of the detrimental effects of HS on organ systems has stimulated laboratory research in the last two decades. In most instances, following the induction of HS in animals, a therapeutic intervention targeting a specific pathway such as ROS-induced apoptosis, is measured for the effects on the protection of organ function or survival. In the search for treatments to reduce the damage from HS, investigators have focused on compounds that have anti-inflammatory characteristics or those that are scavengers of ROS.

One compound that has been used to reduce ROS is the nitroxide TEMPOL, a stabilized free radical with antioxidant characteristics. Mota-Filipe and colleagues explored the use of TEMPOL on post-HS dysfunction of kidneys, liver, lungs and intestine in a rat model.[31] After confirming an increase in the formation of ROS following controlled HS, the authors observed that rats treated with TEMPOL experienced significantly reduced levels of circulatory collapse and less injury to lungs, kidneys, liver and gut. In another study, Kentner et al used TEMPOL in combination with albumin and demonstrated improved rates of survival and higher antioxidant reserves in hemorrhaged rats, compared with rats that were resuscitated with only albumin.[35] A mitochondrial-targeted variant of TEMPOL was studied by Fink et al in the treatment of HS.[41] This compound was effective in prolonging survival times of rats subjected to HS in the absence of treatment with blood or fluid replacement.

Other antioxidants have been tested in animal models and found to reduce the damaging effects of ROS following hemorrhage. Tharakan et al reported that α-lipoic acid was useful in limiting the release of cytochrome-c from mitochondria.[32] A natural dietary antioxidant, curcumin, was effective in reducing vascular hyperpermeability after

HS.[42] Allicin, another natural derivative, decreased apoptosis in rat lungs, kidneys and intestine by interrupting apoptotic enzymatic pathways following HS with reperfusion.[43] Similar benefits in providing protection for rat hearts and kidneys following HS were reported from studies of the antioxidant, crocetin.[44,45] The use of platonin, a photosensitizing dye with antioxidant characteristics, was found to diminish acute lung injury in rats following HS.[26]

Several methods to reduce the inflammatory response to HS have also been reported. Administering an adenosine A_{2A} receptor agonist before the induction of HS appeared to have protective effects on the lungs, possibly related to alterations of either the inflammatory cascade or ROS production.[37] Yu administered a phosphodiesterase inhibitor (DSM-RX78) to rats following induced HS. After 24 hours, the experimental group had less evidence of shock lung injury, possibly as a result of attenuated neutrophil response.[46] In a recent study, Chima et al demonstrated that ciglitazone diminished apoptosis in rat lung cells following HS, via inhibition of an inflammatory pathway.[22]

Coenzyme Q_{10} (CoQ_{10}) is produced endogenously and functions as part of the mitochondrial respiratory chain. There are two types of CoQ_{10} supplements: ubiquinone (oxidized form) and ubiquinol (reduced form). Three studies have reported the use of ubiquinone in animal models of HS.[47-49] Aoyagi administered ubiquinone intravenously following one hour of HS and found that treated dogs had significantly improved cardiac and urinary output compared to untreated animals.[49] Hatano also evaluated the effects of ubiquinone on HS and reported that treatment did not influence coagulation but did suppress fibrinolysis.[48] Yamada evaluated the efficacy of ubiquinone on pulmonary function and chemical mediators in a canine model of HS. In animals that were treated

with 10 mg/kg of ubiquinone prior to hemorrhage, Yamada measured peak ventilated airway pressures and total lung compliance and found a smaller decrease in pulmonary compliance after HS than in those animals that did not receive ubiquinone. In addition, blood histamine levels increased in the non-treated dogs however histamine levels remained normal in dogs that received ubiquinone. Yamada also found increased levels of the inflammatory mediator leukotriene C_4 in the control group which was effectively attenuated with ubiquinone in treated animals. Plasma lactate levels increased sharply in both the treatment and control groups but were significantly attenuated in the CoQ_{10} group. Likewise, pyruvate levels also increased in both groups but returned to baseline two hours after reinfusion of blood in animals that received CoQ_{10}, while increased levels persisted in controls. These findings led Yamada to conclude that ubiquinone might be an effective treatment to reduce the complications of HS.[47]

Lim et al investigated the use of ubiquinol in a rat lung injury model not related to HS.[50] This group evaluated the protective effects of ubiquinol in rat lungs that were exposed to severe ischemia-reperfusion injury. Experimental rats were pretreated with high-dose oral ubiquinol for 7 days and then subjected to a lung transplantation protocol. Measures of peak airway pressures and arterial oxygen levels did not improve with oral ubiquinol compared to controls, however ubiquinol did significantly reduce levels of tumor-necrosis factor-α (TNF), a known activator of the extrinsic apoptosis pathway.[51]

Although studies suggest that ubiquinone or ubiquinol may reduce the complications of HS, a limited amount of research has been conducted to understand the mechanisms by which they protect cells from oxidative stress. Using mitochondria isolated from rat hearts, Yamamura et al demonstrated that ubiquinone exerts an

antioxidant function by inhibiting lipid peroxidation (a result of oxidative stress that damages cell membranes), increasing ATP synthesis and decreasing release of cytochrome-c from the mitochondria (a known signal that initiates apoptosis via an intrinsic pathway).[52] Although HS was not a condition examined by this group, their findings demonstrated the effects of ubiquinone as an antioxidant.

Papucci et al, in a study using rabbit corneal cells, suggested a possible function of ubiquinone in stabilizing the mitochondrial membrane by inhibiting the development of mitochondrial permeability transition pores (MPTP). The opening of MPTPs resulted in leaking of mitochondrial contents (cytochrome-c), to initiate apoptosis.[4] This activity of ubiquinone is separate from its effectiveness as a scavenger of ROS. The membrane-stabilizing characteristic of ubiquinone was confirmed by Somayajulu et al. These authors demonstrated that oxidative stress resulted in mitochondrial dysfunction of neuronal cells which led to increased ROS production and apoptotic cell death. Application of ubiquinone to neurons inhibited ROS generation and stabilized the mitochondrial membranes.[53]

Evaluation of the research to date reveals that there are no studies that have attempted to directly measure the effect of HS on lungs, diaphragm, heart or kidneys tissues as mediated by oxidative stress-apoptosis pathways. Further, only the three studies cited have been conducted using ubiquinone as a potential therapeutic agent for HS. The study by Yamada suggested possible benefits of the use of ubiquinone, however it was administered as a pre-treatment for HS rather than after HS had occurred.[47] Our increased understanding of the ROS/apoptosis pathway as a mechanism for tissue and organ injury following HS is one of the reasons this study was conducted. We investigated a HS

model of oxidative stress and apoptosis, comparing a treatment with ubiquinol to a standard treatment for HS. To our knowledge, this was the first study investigating ubiquinol in HS.

Ethical issues related to informed consent and research control issues would make it difficult to study HS in human subjects, therefore, rats have been extensively used in studies as models of HS.[25,28,36,54-56] Hemorrhagic shock in rats may be induced using one of two methods. One method is by the slow removal of blood to a pre-determined mean arterial pressure (MAP), often 40 mmHg or less (which represents severe shock).[57,58] An alternate technique is to remove 40% of the weight-estimated blood volume while ensuring that MAP does not fall below a pre-determined level that would result in the premature demise of the animal.[25,58] Removal of at least 33% of estimated blood volume is considered as a satisfactory means to induce severe HS.[28] Using a nuclear medicine red blood cell tag, Lee and Blaufox measured blood volumes in rats and suggest the formula 0.06 ml per gram body weight + 0.77 (a constant) as an accurate means of estimating volume.[59] Other sources suggest estimating blood volume in rats at 55 to 70 ml/kg.[60] For the purposes of this study, HS was defined as a MAP maintained between 45-55 mmHg which was induced by the removal of approximately 40% of the estimated blood volume.

Ischemia-Reperfusion Injury

Hemorrhagic shock represents a form of ischemia-reperfusion injury to cells.[12] The combination of diminished peripheral blood flow to tissues from hemorrhage-induced hypotension, decreased hemoglobin and vasoconstriction will result in cell hypoxia (the ischemia phase). This is followed by the restoration of blood and oxygen supplies during resuscitation using intravenous fluids and blood transfusions (reperfusion

phase). Tissues will sustain a loss of cells that are irreversibly damaged from hypoxia and also as a result of reperfusion.[13] Several mechanisms by which reperfusion causes damage to cells have been proposed. Damage is incurred as a result of the production of ROS in response to hypoxia.[61] This is followed by the extension of damage which occurs during reoxygenation, resulting from a further production of ROS from tissues as well as from infiltrating leukocytes. The increased supply of oxygen from reperfusion causes an increase in ROS. This is probably a result of damage to mitochondria, where ROS are produced, as well as by the action of oxidases in leukocytes where ROS are produced as an immune defense mechanism. The previous ischemic conditions may also have compromised the body's antioxidant defenses, favoring the accumulation of oxygen intermediates and the development of oxidative stress and inflammatory responses.[13]

Regardless of the cause of the initial hypoxic event, reperfusion injury appears to be primarily mediated in endothelial cells. These cells are sensitive to hypoxia and manifest damage by increased cell volume, loss of cytoskeletal organization and decreased membrane fluidity.[12] Early in reperfusion, endothelial cells exhibit damage in the form of cell swelling, loss of their attachment to the basement membrane and the adherence of infiltrating leukocytes secondary to an inflammatory response. The most common site of damage is in vascular endothelium at the level of the post-capillary venules, resulting in fluid leakage from the vessels. The increased adhesion of leukocytes to the vessel walls has been linked to the loss of endothelial integrity and leaking. These changes, accompanied by the increase in ROS production are part of a complex relationship that lead to cellular apoptosis or even necrosis.[61-64] It has been suggested that an over-production of ROS causes opening of the MPTP with the release of cytochrome c

into the cytosol which triggers the intrinsic apoptotic pathway.[65] In the case of ischemia-reperfusion injury to lung parenchyma, apoptosis has been found to peak at about two hours following reperfusion in experimental animal lung transplantation.[66]

Endogenous or exogenous antioxidants are important in limiting cell damage from ischemia-reperfusion injury. Endogenous molecules such as superoxide dismutase (SOD), catalase, glutathione and ubiquinone provide protection of heart and lung cells from the detrimental effects of ROS. Exogenously-administered antioxidants such as ascorbic acid (Vitamin C), β-Carotene (pro-Vitamin A) and α-tocopherol (Vitamin E), administered before or at the time of reperfusion, have also been beneficial as interventions to reduce cell death.[67]

Function of Mitochondria and the Production of ROS

As the primary source of ROS in cells, it is important to consider the role of mitochondria in cellular energy production and understand how mitochondrial activities lead to the production of oxygen species. Mitochondria serve as a primary source of energy for organisms by the production of ATP.

The matrix of the mitochondrion is the site of reactions that result in the production of ATP. As illustrated in Figure 1, pyruvate from cytoplasmic glycolysis and fatty acids are transported across the inner mitochondrial membrane and converted to acetyl CoA by enzymes in the matrix. The acetyl groups then enter the trichloroacetic acid (TCA) cycle (also known as the Krebs citric acid cycle), which generates ATP as well as the high energy electron carrier molecules nicotinamide adenine dinucleotide phosphate (NADH) and the reduced form of flavin adenine dinucleotide ($FADH_2$). The primary producer of ATP, the mitochondrial electron transport chain (ETC), is initiated

when the hydride ion is removed from NADH and converted to a proton and two high energy electrons. Figure 1 illustrates the relationship between the TCA and the ETC. As the electrons are passed to various carriers through the ETC complexes, their energy is gradually diminished and preserved until they are attached to oxygen and removed from the cell in the form of H_2O. The energy from the electrons is stored as a membrane gradient and is maintained with the assistance of a pH gradient across the inner mitochondrial membrane. A large protein in the inner membrane, ATP synthase, serves as a final step in the synthesis of ATP from adenosine diphosphate (ADP). The high energy phosphate bonds in ATP provide energy for cellular activity. Thus ATP moves out of the mitochondria to the locations in the cell where energy is needed. Typically, each molecule of ADP/ATP goes through this process at a rate of more than one time per minute.[68]

Oxidative damage to cells is implicated in the development of many pathological disease states as well as the aging process.[69] The molecules responsible for such damage are thought to be ROS such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), which are generated in the mitochondria as by-products of oxygen metabolism in the production of ATP. Energy for the production of ATP in the ETC is obtained as high energy electrons are passed from carriers by way of molecular complexes, creating an electrochemical gradient across the mitochondrial inner membrane and converting the electrons to lower levels of energy. As illustrated in Figure 2, pairs of electrons enter the chain at Complex I and ultimately reduce oxygen to water at Complex IV. At certain sites within the chain, electrons are passed singly to iron-sulfur cluster carriers (FeS), whose chemistry demands that the electrons be taken one at a time,

or to flavins or quinones that have stable one-electron reduced intermediates.[69,70] Single electrons may react directly with oxygen to form $O_2^{\bullet -}$.

Complex I is NADH-ubiquinone oxidoreductase and reactions at this complex may be one of the significant sources of $O_2^{\bullet -}$. Under normal circumstances, NADH produced in the TCA cycle is oxidized and ubiquinone is reduced to ubiquinol with the concurrent pumping of four protons across the inner membrane. The production of ROS appears to be dependent on the type of respiratory substrates that are present, with the highest rates of ROS production observed in the presence of succinate. Succinate concentrations are known to increase significantly in ischemic or hypoxic tissue, providing a connection between ischemia-reperfusion injury and dramatic increases in mitochondrial $O_2^{\bullet -}$ production.[70]

Complex II is succinate-ubiquinone oxidoreductase and is the simplest of the ETC complexes. In this step, electrons from the oxidation of succinate to fumarate are channeled through this complex to ubiquinone in a linkage to the TCA cycle.[71] Few ROS are produced at complex II.[69]

In Complex III (ubiquinone-cytochrome c oxidoreductase) the substrate ubiquinol, present in the lipid bilayer of the inner membrane, is oxidized and the electrons transferred to cytochrome c with an additional transfer of two protons across the inner membrane.[71] In certain circumstances, $O_2^{\bullet -}$ production at this complex may be substantial, with approximately half of the ROS being released into the matrix and half released into the inter-membrane space.[69]

Complex IV is referred to as cytochrome c oxidase. In this step molecular oxygen is the terminal electron acceptor, the carrier cytochrome c is re-oxidized and four

additional protons are transferred to the inter-membrane space.[71] Although oxygen receives electrons and protons at this step to form H_2O, complex IV is not considered to be a generator of ROS.

During periods of cell stress such as that created during ischemia-reperfusion, there is an increase in the "leakage" of ROS from the ETC.[71] Although periods of oxygen restriction during hypoxia would be expected to decrease the production of ROS, paradoxically it has been found to increase.[72] With reperfusion, there is a further increase in the production of ROS, placing the cell at risk of damage or destruction. Mitochondrial integration with the rest of the cell is established in the signaling pathways that lead to apoptosis in which mitochondria play a central role. When cells are injured, the complex cascade of apoptotic events is triggered to remove the cell and recycle the molecular components. Levels at which mitochondria may regulate apoptosis include: 1) cytosolic calcium homeostasis, 2) the generation of ROS and reactive nitrogen species (RNS), 3) mitochondrial membrane permeabilization and release of pro-apoptotic proteins, 4) altered provision of energy supply, and 5) caspase activation by cytochrome c.[73,74]

Function of Ubiquinol (CoQ_{10})

Antioxidants have the potential to block oxidative damage and exogenous $CoQo_{10}$ has been used for that purpose.[75-81] Coenzyme Q_{10} is a 1,4-benzoquinone where Q refers to the quinone chemical group at the head of the molecule and 10 refers to a tail of 10 five-carbon isoprenoid units.[2,82] The benzoquinone ring is synthesized from the amino acid phenylalanine and the isoprenoid chain is formed by a pathway in common with cholesterol biosynthesis.[83] Coenzyme Q_{10} exists in one of three oxidation-reduction states, fully oxidized (ubiquinone), the semiquinone radical (ubisemiquinone) and fully

reduced (ubiquinol).[84] Ubiquinone was isolated in 1955 and identified by Frederick Crane as a key component of the mitochondrial ETC in 1957.[81,85] It is a low molecular weight lipid constituent present in all human cell membranes and is the only lipid-soluble antioxidant synthesized endogenously.[82] In mitochondria, ubiquinone is synthesized in the lipid core of the inner membrane where it accepts two electrons from complexes I or II to be reduced to ubiquinol and then donates the electrons to complex III resulting in oxidation back to ubiquinone.[86] In addition to the bioenergetic role of ubiquinone in the ETC, it is also a component of the extra-mitochondrial redox chains that function to limit excess reducing power formed by glycolytic metabolism when mitochondrial activity is decreased.[83]

As ubiquinol, it is an endogenously produced antioxidant, an effect created by the donation of a hydrogen atom from one of its hydroxyl groups to a lipid peroxyl radical, thus decreasing lipid peroxidation within the inner mitochondrial membrane.[87] Ubiquinol also has protective functions in limiting and repairing damage to both proteins and deoxyribonucleic acid (DNA).[82,88] Ubiquinol has been available as a nutritional supplement, since 2007.[89] The activities of ubiquinol offer a possible therapeutic strategy in the treatment of human disease conditions including ischemia-reperfusion injury.[90,91] While ubiquinol is a potent antioxidant capable of scavenging $O_2^{\bullet-}$ radicals, concern exists that partially reduced and/or partially protonated intermediate forms, such as the ubisemiquinone radical, can act as pro-oxidants by interacting with oxygen to form $O_2^{\bullet-}$.[87] It has been suggested that there are benefits to this auto-oxidation as it may be a mechanism that preconditions cells and renders them less susceptible to subsequent oxidative stress.[92]

In addition to antioxidant benefits and proton translocation capabilities, several other important functions are associated with ubiquinone or ubiquinol: 1) regulation of cell growth and differentiation, 2) prevention of the opening of MPTP in apoptosis signaling, 3) anti-atherosclerotic properties provided by protecting low-density lipoproteins from oxidation, 4) the stimulation of an endothelial release of nitric oxide which counteracts production of the vasoconstrictor endothelin, and 5) exertion of anti-inflammatory effects by influencing the expression of Nuclear Factor kappa B1 (NFκB1)-dependent genes.[4,82,85] Increased understanding of the functions of ubiquinol have led to hypotheses that it may have therapeutic value in the treatment of various diseases through the mechanisms of antioxidant activity, stimulated oxidative phosphorylation and diminished lipid peroxidation as well as by other pathways.[92]

Although ubiquinol is used as an oral supplement in humans, its oral bioavailablility is low due to extreme hydrophobicity, with only small fractions of the ubiquinol reaching the circulatory system.[92] New ubiquinol supplements have been developed with the intent of offering improved bioavailability and pharmacokinetic properties over the oxidized form ubiquinone. In controlled human studies, ubiquinol had twice the bioavailability of the ubiquinone supplement.[89] Based on this information, we used ubiquinol instead of ubiquinone in our study. A mitochondrial-targeted ubiquinone compound (Mito-Q) has also been used in laboratory animal studies and in phase II human trials.[93,94]

Ubiquinol has been evaluated for its pharmacokinetics and safety in rats. It has been shown to have a half-life of seven to eight hours after intravenous administration in doses of 0.25 to 2.5 mg/kg.[95] Single doses up to 2000 mg/kg and repeated doses of 1000

mg/kg have not been shown to produce toxicity in rats.[96]

Researchers have demonstrated benefits of ubiquinol in the treatment of many diseases. Witting et al found ubiquinone to be of benefit in reducing atherogenesis in apolipoprotein E gene knockout mice.[97] Using pharmacologic doses of ubiquinone supplements, lipid hydroperoxides in atherosclerotic lesions decreased and the sizes of atherosclerotic lesions of the aorta were minimized. Oral ubiquinone was also useful in lowering blood pressure in humans[98] and in the treatment of endothelial dysfunction in patients with type II diabetes.[99] Pappuci and colleagues determined that ubiquinone enhanced stabilization of MPTP in keratocytes that had been exposed to laser irradiation, thereby preventing induction of the apoptosis cascade and ultimately cell death.[4]

Although research in the use of ubiquinol with HS is limited, its usefulness as a treatment has been evaluated in the management of cardiac and kidney diseases and to a lesser extent in lung disorders. In a review of the possible benefits of ubiquinol in cardiovascular diseases, Kumar and his colleagues found that there is evidence to support its use in the treatment of arteriosclerosis, ischemic heart disease, chronic heart failure, cardiomyopathy, heart surgery, hypertension, arrhythmias and valve disorders.[78] Several studies that address models of ischemia-reperfusion injury other than HS have supported the protective benefits of ubiquinone supplementation.[77,80,100,101]

In several studies, the effects of ubiquinone on ischemia-reperfusion have been evaluated in animals. Hano et al conducted a study using isolated rat hearts in which a 30 minute period of global ischemia was induced followed by 40 minutes of reperfusion.[100] Ubiquinone was administered at various times during the ischemia-reperfusion process followed by measurement of functional myocardial recovery and the recovery of the ATP

systems. The authors reported that ubiquinone, given prior to reperfusion, was the most beneficial in the recovery of the left ventricle to generate pressure while maintaining low diastolic pressure, and in the recovery of ATP production. Niibori et al demonstrated similar benefits in rat hearts, using an intravenously administered form of liposomal ubiquinone to improve myocardial function and efficiency and decrease the amount of injury.[77] Maulik and colleagues tested the hearts of swine that had been fed ubiquinone supplements for 30 days.[101] After subjecting the in-situ hearts to 60 minutes of ischemia and 120 minutes of reperfusion injury, the authors found that the experimental hearts sustained smaller areas of infarction and had lower biomarkers of oxidative stress, leading them to conclude that ubiquinone was a protective factor. Liposomal ubiquinone was also used to treat isolated rat hearts subjected to ischemia-reperfusion injury in a study by Crestanello et al.[80] These authors confirmed the benefit of ubiquinone for this purpose and suggested that cardioprotective effects were related to the preservation of mitochondrial function in the heart cells.

Cardiac surgery with cardiopulmonary bypass is a well-known source of ischemia-reperfusion injury and oxidative stress.[102] In a study by Rosenfeldt et al, oral ubiquinone given to patients prior to cardiac surgery was shown to provide significant protective benefits.[91] Intraoperative measurements of malondyaldehyde (MDA), a biological marker of lipid peroxidation and mitochondrial respiration were made following weaning from bypass. Patients who had been given ubiquinone had lower levels of MDA and improved mitochondrial efficiency in heart cells.[91]

Although research related to kidney ischemia-reperfusion has not been specifically addressed in the literature, studies of the effects of ubiquinol supplements on

renal disease have generated positive findings. Ubiquinol has been examined for the potential to attenuate the negative effects of type II diabetes on kidneys and appears to offer protection from diabetic nephropathies.[103,104] In a hypertensive rat model, developed by the excess administration of sodium, ubiquinol was also found to preserve renal function.[105] Renal toxicity is a concern with the use of drugs administered as chemotherapy to cancer patients. In evaluating chemotoxicity in mice, Fouad et al reported that ubiquinol significantly diminished the amount of injury to kidneys following the administration of cisplatin, a common chemotherapeutic agent.[106] Renal transplant patients are also known to be subjected to increased levels of oxidative stress. Oral ubiquinone has been successfully used in lowering multiple markers of oxidative stress in these individuals.[107]

Research on the use of ubiquinol for treatment of oxidative stress in lungs is limited. A study published in 1985 outlined the use of ubiquinone with guinea pig lung tissues.[108] The application of ubiquinone was reported to suppress the release of histamine. Yasumoto and Inada investigated the effects of ubiquinone pretreatment in dogs that were subsequently exposed to endotoxin shock. They found that ubiquinone was successful in inhibiting disturbances in peak ventilated airway pressures, lung compliance, and plasma levels of histamine, lactate and base excess.[109] Investigators who evaluated rat lung preservation with ubiquinone in in an isolated model of reperfusion demonstrated improved preservation of pulmonary artery pressures, airway pressures, gas exchange and wet/dry weight ratios when lungs were pretreated with ubiquinone.[110]

In human studies, ubiquinone has been evaluated in patients with chronic obstructive pulmonary disease and bronchial asthma. Fujimoto et al administered oral

ubiquinone to patients with chronic obstructive pulmonary disease who had hypoxemia at rest or with exercise and compared their baseline pulmonary function and arterial oxygen levels.[111] While pulmonary function was unchanged after eight weeks of ubiquinone supplementation, arterial oxygenation significantly improved and patient's heart rates were lower at exercise workloads that were the same as baseline, suggesting some possible benefits of ubiquinone. Asthma is a lung disorder related to disturbances in oxidant-antioxidant capacity of the lungs. Often patients with asthma are treated with corticosteroids, which can lead to mitochondrial dysfunction and oxidative damage to mitochondrial and nuclear DNA. Investigators in one study found that oral ubiquinone allowed patients to reduce their dosage of corticosteroids by approximately 70% in four weeks.[112]

Based on the research that supports the use of ubiquinol in disorders related to ischemia-reperfusion injury and other sources of oxidative stress, only minimal attention has been given to the treatment of HS-induced injury to the lungs, heart, kidneys and diaphragm with ubiquinol. We found this to be a significant gap in knowledge and therefore determined that there was a need for further research. This study was conducted to address that gap in the literature.

In our study, we used a liposomal form of oral ubiquinol, diluted and injected intra-vascularly. This decision was based on its superior bio-absorption profile in animal models and our own observations that it was better tolerated by rats than other forms of ubiquinol. Ubiquinol is not currently available in the U.S. in parenteral form. We therefore conducted a pilot study in the Pierce laboratory and tested several different mixtures of oral ubiquinol combined with liquid solvents for administration to rats. We

found that one ubiquinol product (QH Liposomal Ubiquinol, Tishcon Corporation, Westbury, NY), when dissolved in lactated Ringers' (LR) solution to a concentration of 1 mg/ml, was easily soluble and did not appear to injure the animals. For that reason, we used this formulation of ubiquinol in our study.

Statement of the Problem

Hemorrhagic shock following serious injury is a major problem in both military and civilian populations. In combat theaters, wounded U.S. personnel are at risk of rapid death from HS. Penetrating injuries to extremities frequently involve multiple sites and are often the result of mortar injury, other fragmentary explosive devices or high velocity weapons. These types of wounds cause significant tissue damage and subject victims to increased risk of death from HS.[113] The immediate treatment of hemorrhage is managed with fluid and blood infusions with vasopressor drugs to reverse hypotension.

Hemorrhagic shock patients are also prone to delayed effects in the form of severe organ damage especially to the lungs, diaphragm, heart, and kidneys. High levels of morbidity and mortality following HS in combat settings, especially due to MOF, highlight the need for new methods to reduce the consequences of traumatic injury.[114] The mechanisms of MOF include oxidative stress, initially from hypoxia, followed by reperfusion injury during blood and fluid resuscitation. This results in activation of apoptosis pathways and severe organ compromise or failure. Antioxidant adjuncts have been studied in animal models of HS as treatments to limit MOF. Ubiquinol is a component of the mitochondrial ETC and participates in the generation of ATP. Ubiquinol is also an antioxidant that has been used therapeutically. Few studies have investigated the use of ubiquinol or ubiquinone during HS. There have been, however,

numerous studies supporting the use of ubiquinol in the treatment of other diseases by reducing oxidative stress. Gaps in the literature in addressing the hypothetical benefits of ubiquinol to attenuate organ injury after HS, using the ischemia-reperfusion/oxidative stress model, made this a suitable and important problem for study.

Significance

Trauma accounts for a significant proportion of annual mortality in both the U.S. and the world. This is a particular concern for military personnel serving in hostile assignments in light of over 54,000 troops wounded or killed in hostile action since the U.S. invasion of Afghanistan in 2001.[5] The World Health Organization reports that almost 10% of global annual mortality in 2003 was due to injuries, representing 5 million deaths.[115] Multiple organ failure is listed as the cause of mortality in 9% of patients with trauma, and of the patients who develop MOF, approximately half do not survive.[7] The presence of MOF following injury results not only in an increase in mortality, but an increase in the length of stay in the intensive care unit and increased need for post-hospitalization assistance with activities of daily living.[9]

The World Health Organization has estimated that countries spend up to two percent of their gross domestic product providing medical care for victims of auto accidents alone.[7] Clearly, trauma that results in organ failure is costly in terms of human suffering and productivity and is a financial burden both to individuals and to society. Efforts aimed at reducing the disruption of the cellular integrity of organs resulting from HS may preserve patient function, reduce the need for disability support and help to control health care costs by decreasing expensive hospital stays. Thus, using ubiquinol as a novel treatment following HS may be a method to protect against ischemia-reperfusion

events, lipid peroxidation and DNA oxidation.[2,82]

There are many ethical and design issues with conducting a study using ubiquinol in humans with HS. Patients who have been severely traumatized and develop HS may be unable to give informed consent and the families of the patients would be under severe stress and probably should not be approached for consent. In addition, there would be many confounding variables related to timing and variances in treatment and care by health providers which would increase the complexity of data interpretation. Finally, a parenteral form of ubiquinol suitable for human use is not currently available in the U. S. Sublingual administration would be the only available method to administer ubiquinol to patients. Hence the use of rats to study the effects of ubiquinol following HS and fluid resuscitation was a reliable and useful model to use in this investigation.

Specific Aim

The aim of this study was to examine the effects of ubiquinol on leukocyte mitochondria and in the lungs, diaphragm, heart and kidneys as a supplemental treatment for hemorrhagic shock.

Research Hypotheses

Research Hypothesis 1: Administering ubiquinol will reduce leukocyte mitochondrial production of superoxide following hemorrhagic shock.

Research Hypothesis 2: Administering ubiquinol will attenuate hydrogen peroxide production in the diaphragm following hemorrhagic shock.

Research Hypothesis 3: There will be a decrease in the percentage of apoptotic nuclei in the lungs and diaphragm following ubiquinol administered post hemorrhagic shock.

Research Hypothesis 4: There will be a decrease in the percentage of apoptotic nuclei in

the heart and kidneys following ubiquinol administered post hemorrhagic shock.

Definition of Terms

Apoptosis/Apoptotic Cells

Conceptual Definition: An energy-dependent physiologic form of programmed cell death in which the cell responds to specific signals to begin the process of shrinkage, fragmentation and digestion by other cells for the purpose of "recycling" cellular materials.

Operational Definition: Nuclei of the lungs, diaphragm, heart and kidneys were stained with acridine orange (AO) and ethidium bromide (EB) and examined using a fluorescent microscope. Computer-assisted analysis of the nuclei allowed identification of apoptotic cells which are orange in hue (hue number between 89.9 and 26).[116]

Arterial Blood Analysis

Conceptual Definition: Arterial blood analysis included the measurement of acidity (pH), partial pressures of carbon dioxide ($PaCO_2$), oxygen (PaO_2), bicarbonate (HCO_3), base excess (BE), oxygen saturation (SpO_2), hemoglobin and hematocrit levels in arterial blood.

Operational Definition: The panel of blood tests listed above was measured using 0.1 ml of arterial rat blood using an ECG6+ cartridge with an i-STAT instrument (Abbott Laboratories, Abbott Park, IL).

Arterial Blood Pressure (ABP)

Conceptual Definition: The pressure of the blood in the arterial system, which depends on the heart's pumping pressure, the resistance of the arterial walls, elasticity of vessels, the blood volume, and its viscosity.[117]

Operational Definition: Continuous real-time measurements of systolic, mean and diastolic pressures using the carotid artery cannula were made with a Harvard Apparatus (Hollister, MI) monitor and transducer connected to a fluid-interface rigid plastic catheter.

CoenzymeQ_{10}

Conceptual Definition: A nutritional supplement of ubiquinone or ubiquinol available without prescription, usually in capsule or liquid form.[95] Coenzyme Q_{10} is a 1,4-benzoquinone with a quinone group at the head of the molecule and a tail of 10 five-carbon isoprenoid units.[2,82]

Operational Definition: A parenteral form of ubiquinol was not available commercially in the U.S. For this study, we used an unsterilized oral liquid preparation of QH Liposomal Ubiquinol (Tishcon Corporation, Westbury, NY). The stock solution is 100 mg/ml, from which we used a dose of 1 mg/100 g body weight and diluted the ubiquinol in LR solution. In a previous pilot study using this preparation of ubiquinol intravenously, we found the ubiquinol was well tolerated by the experimental animals.

Hemorrhagic Hypotension (HH)

Conceptual Definition: Low blood pressure that occurs as a result of hypovolemia secondary to blood loss.

Operational Definition: Removal of approximately 40% of blood volume by weight from study animals via the femoral arterial catheter to maintain a MAP of 45-55 mmHg for a period of 60 minutes.

Mean Arterial Pressure

Conceptual Definition: An arithmetic calculation of mean arterial pressure that takes into

account the approximate amount of time that the heart is in the systolic and diastolic phases. A common way to derive this is: MAP = Diastolic + 1/3(Systolic – Diastolic).[117]

Operational Definition: Mean arterial pressure was automatically calculated by the Harvard Apparatus and digitally displayed and recorded every two minutes throughout the experiment.

Mean Fluorescence Intensity (MFI)

Conceptual Definition: An arbitrary measure of the shift in fluorescence intensity of a population of cells calculated during flow cytometry.[118]

Operational Definition: Mean fluorescence intensity was a calculated relative value of leukocyte fluorescence provided by the BD FACS DIVA computer software during flow cytometry.

MitoSOX Red

Conceptual Definition: A red superoxide indicator (a variant of dihydroethidine dye) that facilitates uptake into the mitochondria.[119]

Operational Definition: A red molecular probe was used to detect $O_2^{\bullet -}$ production in leukocytes. MitoSOX Red was prepared according to the manufacturer's instructions and loaded into 20 μl samples of rat whole blood and incubated. After cell washing, mitochondrial $O_2^{\bullet -}$ was analyzed with MitoSOX Red and flow cytometry with excitation at 488 nm and emission readings at 576 nm.

Necrosis/Necrotic Cells

Conceptual Definition: A non-energy dependent means of cell death marked by cell swelling, lysis and activation of the inflammatory cascade affecting surrounding cells.[51]

Operational Definition: Nuclei from the lungs, diaphragm heart and kidneys were stained

with AO and EB and examined using fluorescent microscopy. Computer-assisted analysis of the nuclei also allowed identification of necrotic cells which were red-orange in hue (hue number 25 and below indicates necrosis).[116]

Non-apoptotic Nuclei

Conceptual Definition: Normal nuclei unaffected by either apoptosis or necrosis.

Operational Definition Nuclei from the heart, lungs, diaphragm and kidneys were stained with AO and EB and examined using fluorescent microscopy. Computer-assisted analysis of the nuclei allowed identification of normal cells which are green in hue (hue number between 120 and 90).[116]

Nuclei Count (apoptosis)

Conceptual Definition: The number of nuclei examined on a microscopic slide.

Operational Definition: The number of nuclei identified by the investigator during fluorescent microscopy using the analySIS computer software for interpretation of chromatin hue. Using statistical software, 300 total nuclei per tissue (lungs, heart, diaphragm, and kidneys) were examined in each experiment and classified as normal, apoptotic or necrotic. We considered a minimum of 200 nuclei to be sufficient for statistical analysis and elected to use 300 to ensure adequacy of sampling.

Oxidative Stress

Conceptual Definition: An imbalance between the systemic presence of ROS and a biological system's ability to readily prevent or repair damage from ROS.[20]

Operational Definition: Oxidative stress was quantified in relative terms using MFI data from flow cytometric analysis of rat whole blood prepared with MitoSOX Red. Determination of oxidative stress levels were made by statistical comparison of MFI

between treatment and control groups at the baseline, shock and treatment phases.

Reactive Oxygen Species (ROS)

Conceptual Definition: The free radical and non-radical derivatives of oxygen, including $O_2^{\bullet-}$, hydroxyl radicals and derivatives such as H_2O_2 and hypochlorous acid. Together, such radical and non-radical products of oxygen are termed reactive oxygen species.[120]

Operational Definition: For our experiments, we measured leukocyte mitochondrial $O_2^{\bullet-}$ in blood using flow cytometry and H_2O_2 in the diaphragm using confocal microscopy.

Resuscitation

Conceptual Definition: Emergency procedures or treatments, especially the administration of blood and fluids intended to restore circulation and oxygen delivery to tissues to prevent damage or death.[10]

Operational Definition: The resuscitation phase of this study was the administration of ubiquinol (diluted in LR) or control (LR only), followed by the return of most of the heparinized blood removed from the rat and LR given at twice the volume of the total blood removed.

Superoxide (mitochondrial)

Conceptual Definition: The production of $O_2^{\bullet-}$ radicals by leukocyte mitochondria (primarily neutrophils).[121]

Operational Definition: The presence of superoxide was quantified in relative terms using MFI data from flow cytometric analysis of rat whole blood prepared with MitoSOX Red. Determination of $O_2^{\bullet-}$ levels was made by statistical comparison of MFI between treatment and control groups at the shock and treatment phases.

Conceptual Design

A conceptual schema for this study relates to HS, oxidative stress and apoptosis and the administration of ubiquinol to diminish oxidative stress. Apoptosis is a series of well-controlled processes in which ligands binding to specific death receptors on the cell surface, or mitochondrial disruption with release of cytochrome c and other factors initiates programmed cell death via protease-dependent pathways.[122] Chapter 3 of this dissertation includes a detailed description of the apoptotic process. As depicted in Figure 3, the development of oxidative stress begins with the onset of HS which causes decreased oxygen supply to organs, resulting initially in hypoxic injury to cells. Following resuscitation with the administration of blood and fluids, reperfusion injury occurs either as a result of increased mitochondrial production of ROS in cells of organs that have been the target of ischemia-reperfusion, or by ROS produced by oxidases in activated phagocytic cells as described in Chapter two. The ROS produced by ischemia-reperfusion injury, as well as other mediators, are involved in activation of both the intrinsic and extrinsic apoptotic pathways, leading to cell death.[122] Heart[123] lungs,[124] diaphragm[125] and kidneys[126] have all been shown to be at risk of injury by this mechanism following an ischemia-reperfusion event. Lung alveolar endothelium is especially at risk of damage not only from apoptosis initiated by ROS produced by mitochondria within its cells, but also by ROS from respiratory burst generated by neutrophils that have been activated, resulting in their adhesion to the pulmonary microvasculature.[124,127]

Ubiquinol is a scavenger of ROS and has been shown in studies to reduce apoptosis, possibly by stabilization of the MPTP.[74] Yamada found that pre-treatment with ubiquinone prior to inducing HS in dogs was beneficial in maintaining lung compliance,

reducing peak airway pressures, decreasing plasma lactate and pyruvate levels and suppressing histamine release which has been associated with lung damage.[47] However, a connection between treatment with ubiquinol and the relationship of HS, oxidative stress and apoptosis had not been addressed. In response to this gap in knowledge, we hypothesized, as suggested by the model in Figure 3, that ubiquinol administration would decrease ROS production and apoptosis.

Assumptions

The assumptions for this study were:

1. The ubiquinol solution used in this study has been safely administered to rats in the Pierce laboratory in pilot studies. We assumed that the animals in the proposed experiments would not be killed by the intravenous diluted ubiquinol when administered following a one hour HS period.
2. We assumed the Sprague-Dawley rats purchased for this study were genetically homogenous.
3. Based on our preliminary experiments with HS in rats, we assumed that removal of 40% of estimated blood volume and maintenance of MAP of 45-55 mmHg would induce sufficient levels of shock to elicit oxidative stress. This is consistent with findings in preliminary pilot data produced in the Pierce laboratory.
4. We assumed that our resuscitation protocol of returning heparinized blood and LR would induce measurable reperfusion injury.
5. We assumed that parenteral administration of ubiquinol is superior to oral dosing of the compound and provided better bioavailability.
6. It was assumed that a small amount of oxidative stress would be induced in the rats at

baseline as a result of anesthetic and surgery to place the tracheostomy tube and arterial catheters.

7. Based on the preliminary study by Yamada[47] we assumed that 60 minutes of HS and 120 minutes of recovery time would be sufficient to allow for measurable effects of oxidative stress and apoptosis in rats.

Limitations

1. All four organs (lungs, diaphragm, heart and kidneys) cannot be examined in one animal. Therefore, only two organs were examined from each rat.
2. There was a set time for HS (60 minutes) and resuscitation (120 minutes) for the production of ROS and apoptosis.
3. Tissue analysis for H_2O_2 could only be performed on the diaphragm. This was due to the thickness of the other tissues (heart, lung and kidney). Originally, a laser scanning microscope was intended to be used for all four tissues, however it was not currently operational.
4. Use of the flow cytometer at KUMC was based on advanced reservation scheduling. If the cytometer was not immediately available for analysis of our MitoSOX specimens, there was a possibility that the results may be altered. Fortunately, no extended delays in gaining access to the flow cytometer were encountered in any of our experiments.
5. Working with fluorescent probes required that specimens be prepared in low light. Working in a darkened room makes some laboratory procedures, such as pipetting, difficult and there was a possibility for errors.

Figure 1. Citric Acid Cycle and the Electron Transport Chain in the Mitochondrial Matrix[68]

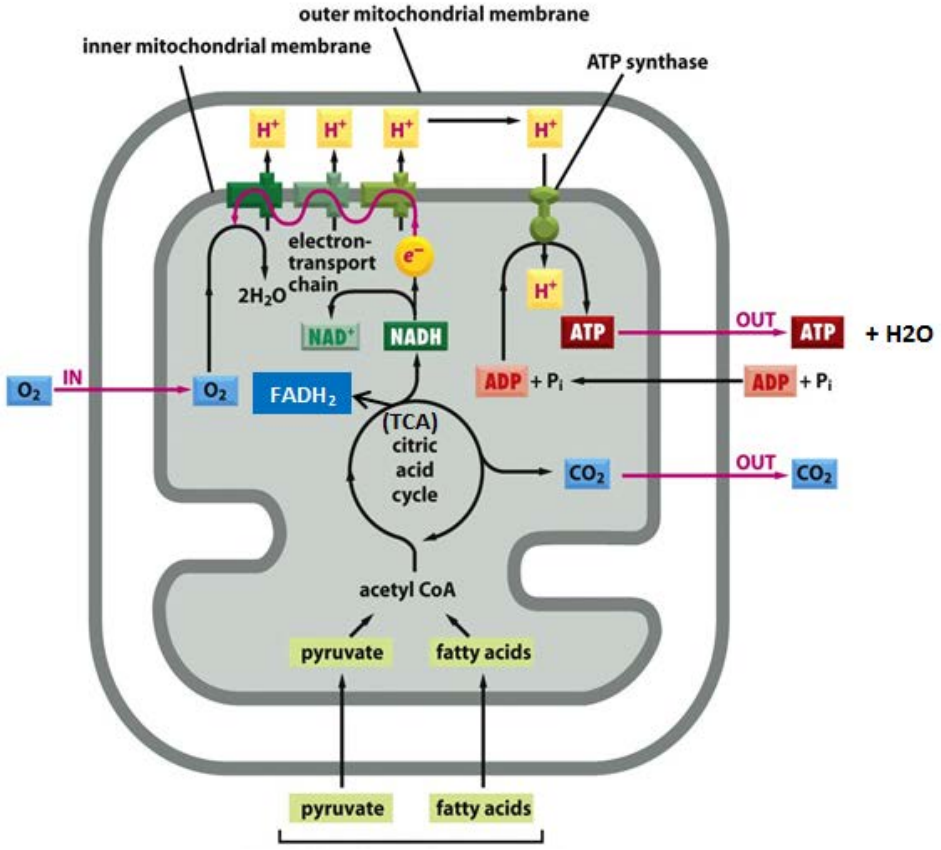


Figure 2. Mitochondrial Electron Transport Chain Complexes

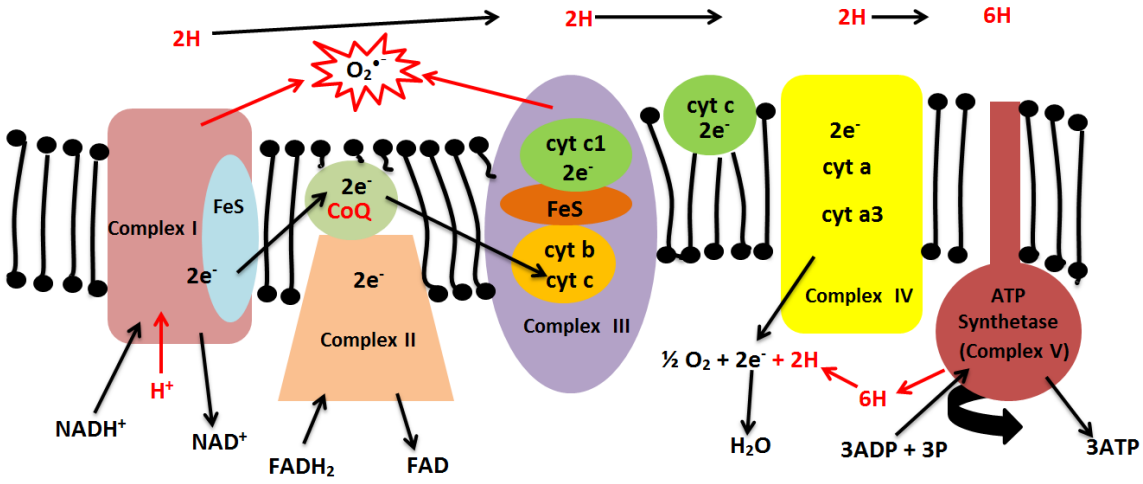
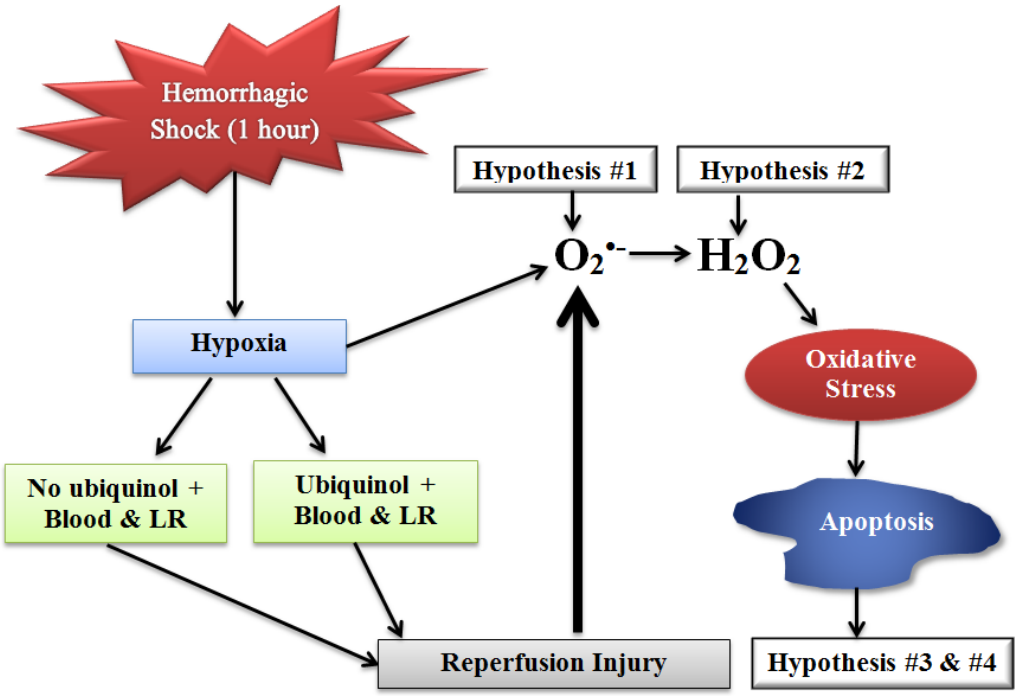


Figure 3. Study Conceptual Design



CHAPTER 2.

A Review of Reactive Oxygen Species and their Measurement in Nursing Research

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Biological Research for Nursing

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Abstract

The mitochondrial metabolism of oxygen to produce adenosine triphosphate (ATP) in mammals is essential to provide energy for cell functioning. Oxygen intermediates, termed reactive oxygen species (ROS) or “free radicals,” are also released as part of the metabolic chain and serve normal functions in cell signaling. Oxygen radicals are also produced by neutrophils and macrophages as a defense mechanism against potential pathogens. Previous research has established that excess amounts of ROS can damage cell membranes and deoxyribonucleic acid (DNA) and may play a role in the premature development of cell apoptosis. When the high levels of ROS overwhelm antioxidant defenses, a condition of oxidative stress exists, which has been theoretically connected to aging and the development of numerous human diseases. Nursing scientists and others seeking to understand the relationship of oxidative stress to patient illnesses should be knowledgeable of laboratory methods related to the detection and measurement of ROS. The purpose of this paper is to identify the biomarkers commonly used in clinical research of oxidative stress and to review the methods that have been developed to quantify ROS, using chemiluminescent and fluorescent techniques in conjunction with flow cytometry, confocal microscopy and high performance liquid chromatography (HPLC). Future directions using innovative technologies in ROS research are also presented.

Key Words

biomarker, fluorescent probe, hydrogen peroxide, oxidative stress, reactive oxygen species, superoxide

Introduction

The discovery of oxygen was claimed by three 18th century investigators, Carl Wilhelm Scheele, Joseph Priestly and Antoine Lavoisier.[128,129] The first observation of the detrimental effects of the oxygen molecule occurred in 1878 century with a report that prolonged breathing of increased concentrations of oxygen resulted in the deaths of laboratory animals.[130] The discovery of free radical reactions in 1894 led to the subsequent understanding that oxygen free radicals were the cause of harm to cells.[131] In health care, this phenomenon is frequently referred to as oxygen toxicity. An oxygen free radical is any oxygen-containing atom or molecule with an unpaired valence shell electron in its outer orbit, which may take an electron by the process of oxidation from another molecule.[120,132] The free radical derivatives of oxygen include superoxide ($O_2^{\bullet -}$) and highly reactive hydroxyl radicals ($OH^{\bullet}$). Non-radical oxygen derivatives such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are also biologically important. Together, such radical and non-radical products of oxygen are termed reactive oxygen species (ROS).[133]

In developing the free radical theory of oxygen toxicity Gershman and her colleagues described the formation of oxygen radicals as a common basis between "oxygen poisoning" and radiation injury.[131] This group recognized the effects of oxygen radicals in biological systems based on work using ionizing radiation that resulted in the dissociation of water into hydrogen ($H^{\bullet}$), $OH^{\bullet}$ and H_2O_2 and further determined that biological mechanisms of injury from oxygen radicals were related to the denaturation of enzymes, damage to nucleic acids and nuclear chromosomal derangement. Two years later, Harmon theoretically linked the effects of oxygen radical damage to the

degenerative processes of aging,[134] which led to a substantial body of work in an effort to understand the aging process.[135,136]

Between 95 to 99% of the oxygen consumption in cells involves the reduction of oxygen without the production of intermediate species. The reduction of the remaining oxygen (approximately one to five percent) generates intermediate species including ROS.[137] A major site of ROS generation is the mitochondrial electron transport chain (ETC) although other pathways, especially those important to the activities of phagocytic cells, contribute to ROS production.[138,139] Table 1 provides a list of known ROS including both radical (capable of oxidizing other molecules) and non-radicals. Reactive oxygen species at low or moderate concentrations have beneficial functions, including resistance to infectious agents and cellular signaling. In conditions where the overproduction of ROS occurs or endogenous antioxidant defenses are insufficient to provide protection, a condition of oxidative stress occurs, resulting in disequilibrium between pro-oxidant and antioxidant reactions. The presence of excess ROS in these circumstances exposes an individual to cellular injury as the result of lipid peroxidation, cell membrane disruption, damage to proteins and deoxyribonucleic acid (DNA) dysfunction.[133] The harmful effects of oxidative stress have been implicated in more than 250 human pathological conditions including heart disease, cancer, diabetes, neurological disorders, ischemia-reperfusion injury, as well as the aging process.[135,140] Given the relationship of oxidative stress to numerous human pathophysiological states, the development of reliable methods to measure the presence of ROS are of importance to scientists engaged in the study of aging and diseases.

Reactions involving the transfer of electrons resulting in a change of oxidation

status (either oxidation or reduction) of molecules are often referred to with the abbreviated term “redox.” In such a reaction, a reducing agent will relinquish one of its electrons and thereby become oxidized while the molecule that gains the electron in the process is reduced. In the case of the formation of $O_2^{\bullet -}$ oxygen has gained an electron and is reduced to the free radical form while the donor molecule is oxidized. Any molecule that oxidizes another by taking an electron is referred to as an oxidizing agent while a molecule that donates an electron in the reaction is the reducing agent.[141] In biological literature, references to the maintenance of a state of equilibrium between oxidation and reduction within the cell, such that ROS production and antioxidant scavenging capacity are relatively balanced, is referred to as “redox homeostasis”.[136]

According to the Committee on Biological Markers of the National Research Council,[142] biological markers are “indicators signaling events in biological systems or samples, classified according to exposure, effect or susceptibility.” As a measure of exposure criteria, the need for suitable clinical biomarkers for the detection of oxidative stress has received considerable attention from the research community. However, markers that are inexpensive, minimally invasive and meet the desirable characteristics of sensitivity and specificity have been elusive. Techniques to measure the biological products of oxidative stress include: 1) detection of protein carbonyls resulting from the oxidation of amino acids, 2) methods to measure the presence of oxidatively-modified DNA and 3) measures of end-products of lipid peroxidation such as malondyaldehyde (MDA) and F_2- isoprostanes.[143] While these methods have been useful, direct measures of ROS are also available and have been subjected to scientific scrutiny in an effort to fully define their usefulness in oxidative stress research. Such measures include

extracellular and intracellular analysis of $O2^{\bullet-}$ and H_2O_2. The reactivity and short half-lives of ROS make direct measurements of their presence technically challenging and results must be interpreted cautiously. Problems related to measuring ROS in the laboratory have been resolved with some success, but most of the methods developed to date are clinically impractical.[135] As we will present, numerous techniques have been devised in recent decades to quantify the presence of ROS. Advances in both methods and technology have placed the direct measurement of ROS within the grasp of researchers interested in understanding the relationship of ROS to human disease and developing therapeutic interventions. The purpose of this article is to review the cellular pathways of oxidative stress with emphasis on the production of $O_2^{\bullet-}$ and H_2O_2, and the methods currently being employed for their detection and measurement.

Reactive Oxygen Species and the Development of Oxidative Stress

Reactive oxygen species are produced endogenously in organisms by more than one mechanism which Chen and co-workers have labeled "intended" or "unintended."[144] Mitochondrial respiration produces the greatest amount of ROS by "unintended" leakage of electrons during the metabolism of molecular oxygen. The one-electron reduction of oxygen at mitochondrial Complex I and Complex III results in the first step of the ROS pathway, the production of $O_2^{\bullet-}$ which is almost immediately dismutated to a secondary ROS, H_2O_2, in the presence of manganese superoxide dismutase (SOD) by the reaction:

$$2\ O_2^{\bullet-} + 2H^+ \rightarrow \text{SOD} \rightarrow H_2O_2 + O_2.$$ [133,145]

As a result of the speed of this reaction, intra-mitochondrial levels of $O_2^{\bullet-}$ are low. Hydrogen peroxide, however, is able to leave the mitochondrial matrix and, when generated in substantial amounts, is even detectable extracellularly. Hydrogen peroxide

may undergo further conversion in the presence of Fe^{2+} (released as a result of the interaction of $O_2^{\bullet -}$ with iron-containing molecules) to form another secondary species, the $OH^{\bullet}$ radical, by the following reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}.^{133}$$

Hydroxyl radicals are short-lived, highly reactive and characterized as being very dangerous.[133] These radicals have been identified as important contributors to oxidative stress in biological systems due to their ability to attach to or transfer electrons from almost all compounds.[132]

The "intended pathway" of ROS production occurs in macrophages and neutrophils as a respiratory (oxidative) burst of $O_2^{\bullet -}$ capable of disrupting tumor cells or bacterial invasion.[136,144,146] The massive production of ROS in response to pathogens or inflammatory conditions provides a first line of defense for the individual. Production of $O_2^{\bullet -}$ occurs in the plasma membrane with the assembly and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in response to immune system stimulation.[138,147] It is believed that the activation of NADPH oxidase is stimulated by microbial products (bacterial lipopolysaccharides), lipoproteins and cytokines.[136] Mitochondria in neutrophils have not been demonstrated to be significant producers of ROS.[135,148] Nonphagocytic cells can produce ROS by NADPH oxidase as well, especially cardiomyocytes, cardiovascular endothelium and smooth muscle cells following stimulation by hormonal changes, hemodynamic forces or metabolic alterations.[136]

In addition to the mitochondrial- and NADPH oxidase-dependent production of ROS, other means of production of oxygen intermediates exist. Reactive oxygen species production occurs as a result of the catalytic activity of xanthine oxidase (XO) in the

cellular production of purines. In the XO-catalyzed conversion of hypoxanthine to xanthine and xanthine to uric acid, oxygen molecules are reduced to $O_2^{\bullet -}$ and H_2O_2. The breakdown or uncoupling of the cytochrome P-450 catalytic enzyme cycle also represents a source of ROS. Microsomes and peroxisomes are also both known to produce H_2O_2 under physiologic conditions.[149]

ROS Cell Signaling Function in Human Physiology

While the negative cell-damaging effects of ROS are most often focused upon, it is important to acknowledge that oxygen intermediates also have regulating functions that are beneficial to cells. Cells generate ROS as cell messengers[150] which may be used as second messengers in the signal transduction pathways involved in cell growth and differentiation as well as a factor of inflammatory responses. One important ROS signaling pathway also results in the expression of nuclear transcription factors which activate protective genes to repair damaged DNA, sustain the immune function and initiate the apoptotic pathways important in programmed cell death and recycling.[133,136] Other physiologic benefits of ROS include sensory monitoring of changes in oxygen concentration, especially during hypoxia. The carotid sinuses are the likely mediators of these ROS signals, which result in the stimulation of oxygenation-improving responses such as increasing the production of erythropoietin and stimulation of vascular endothelial growth factor (VEGF).[151] Additional ROS involvement has been proposed for neurological transmission, cell adhesion (including platelet aggregation), learning and memory, regulation of cellular calcium levels and the activation of T-lymphocytic immune responses.[133,149,152]

Oxidative Stress, Aging and Disease

The phenomenon of oxidative stress was conceptualized over two decades ago[153] and has been defined as a condition in which the presence of ROS exceeds the capacity of the body's defense systems.[154] This may be a result of the over-production of ROS or the loss of antioxidant defenses or both.[135] Reactive oxygen species may be endogenously produced or generated as the result of exposure to environmental stresses such as ionizing radiation, solar radiation or toxic pollutants.[155,156] Exposure to ROS made it necessary for organisms to have protective mechanisms to avoid cellular damage and resulted in the evolution of biological defenses against the development of oxidative stress or its effects. Such defenses are categorized as: 1) protective mechanisms (the development of mitochondria that are more efficient and produce fewer ROS as well as reduction in metabolic rates characteristic of larger organisms), 2) repair mechanisms (cell division, tissue renewal and remodeling and DNA replication), 3) physical barriers (the development of cell membranes and components with decreased numbers of peroxidizable fatty acid molecules) and 4) antioxidant defenses (the catalytic removal of free radicals).[140,157] Antioxidants are substances that, at low concentrations, are able to compete with other oxidizing substrates and thereby delay or inhibit the oxidation of those substrates.[141] Endogenous enzymatic antioxidant defenses include SOD, glutathione peroxidase (GPx) and catalase. Non-enzymatic antioxidants include glutathione (GSH), ubiquinol (Coenzyme Q_{10}), ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), as well as carotenoids and flavonoids.[133]

Oxidative stress has been frequently hypothesized as a causal agent in both aging and disease.[135,143,158-160] With the free radical theory of aging, Harman[134] suggested that oxygen free radicals cause progressive, random damage to enzymes and other proteins,

cell membranes, DNA and ribonucleic acid (RNA), resulting in the degenerative changes associated with aging. Based on this, the restoration of redox balance during conditions of oxidative stress has been suggested as the means to delay the aging process and has led to the widespread use of exogenous dietary antioxidants.[161] Many studies of the life-long use of non-enzymatic antioxidants have failed however, to demonstrate significant effects on the life spans of humans and have been suggested to cause harm to tissues and increase mortality.[162,163]

Dowling and Simmons[164] have evaluated the free radical theory of aging from the perspective of evolutionary biology. Using life-history theory, they suggest that while aging and ROS are related, other factors, particularly evolutionary "trade-offs" that impact physiological processes such as reproduction and metabolism, play significant roles in determining the levels of free radical production and oxidative stress to which organisms are subjected. In considering the potential benefits of calorie-restricted diets to improve longevity in organisms, they identify reduced metabolic rate, which limits the production of ROS, as a key factor.

Estimates of the number of disease processes in which oxidative stress has been implicated continue to grow.[132,149] A theoretical connection between the increased production of ROS and activation of apoptosis or necrosis pathways has been suggested as a basis for the relationship between oxidative stress and disease.[165] An increase in mitochondrial ROS production results in deterioration of the mitochondrial DNA (mtDNA) which is prone to oxidative damage due to a lack of protective histones. Mitochondrial DNA encodes for proteins essential to mitochondrial respiration so that its fragmentation results in deterioration of the respiratory chain and an increase in ROS

production.[3,166] Oxidative stress is also implicated in the opening of mitochondrial permeability transition pores (MPTP) leading to leakage of cytochrome c and other pro-apoptotic molecules into the cytoplasm. If sufficient numbers of mitochondria are affected, programmed cell death (apoptosis) pathways are triggered. The loss of ATP production from the mitochondria of cells also produces damage that activates pathways leading to cell death by the necrosis pathway.[167]

Reactive oxygen species-induced oxidative stress has been linked with cardiovascular disorders such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathy and congestive heart failure.[133] Although the specific pathways are complex, the essential mechanism is that oxidative stress modifies phospholipids and proteins leading to cellular membrane and protein damage, resulting in diminished function of the cardiac myocytes. The role of oxidative stress in hypertension is also well established. Increased $O_2^{\bullet -}$ levels promote cell proliferation with vascular endothelial dysfunction and decreased formation of nitric oxide (a vascular dilator) resulting in vasoconstriction and eventually, hypertension. Vascular disease may well be associated with more than one mechanism of endothelial cell damage or death, depending on the severity of the oxidative stress insult by which it was initiated.[168]

Ischemia-reperfusion injury is important in heart disease, stroke, burns, organ transplantation and any surgery involving cardiopulmonary bypass procedures.[130,169] Moderate levels of ROS are produced during periods of tissue ischemia, however when reperfusion is established, a massive ROS burst results, placing the individual at risk of serious tissue injury or other complications.[133] The development of pharmacological antioxidant-based interventions and other strategies to deter the effects of ischemia-

reperfusion injury are areas of interest in modern health care.

Neurological disorders are another human health threat related to oxidative stress.[170] Free radicals have been reported as contributory to the loss of neuronal tissues in cerebral ischemia, seizure disorders, schizophrenia, Parkinson disease and Alzheimer disease. The nervous system is particularly susceptible to insult from free radicals from both the peroxidation of brain cell membranes and the brain's characteristic low levels of antioxidant activity. Neuronal cells, in comparison to other body tissues, are considered to be more sensitive to oxidative damage.[171] An additional problem associated with aging is the accumulation of metal (copper, iron and zinc) reduction-oxidation end products at the junctions between the neuronal environment and blood vessels resulting in damaging plaques characteristic of neurodegeneration. In addition to neuronal cell damage, oxidative overload also results in the production of byproducts that are toxic to blood lymphocytes and macrophages, thus paralyzing the organism's immune defense systems.[170]

Evidence of oxidative stress has been found to be present in various cancer cells (compared with normal cells) related to interference with protective genes, DNA mutation as well as other precursors of carcinogenesis.[149,172] The resulting activation of transcription factors leads to cellular proliferation, the hallmark of cancer tumor growth. Some cancers are related to the increased production of ROS due to exposure to toxic substances such as tobacco smoke.[173]

Oxidative stress is implicated in numerous other altered health states that affect humans. Included are diabetes mellitus, lung diseases[132,133,174,175] (chronic obstructive pulmonary disease,[176] asthma,[177] pulmonary fibrosis,[178] adult respiratory distress

syndrome), autoimmune disorders (lupus, rheumatoid arthritis, Crohn's), sickle cell and inflammation.[179]

Biomarkers of Oxidative Damage – Indirect Measures

Methods of measuring the activities of ROS have included techniques referred to by Halliwell and Gutteridge[141] as "fingerprinting" or "footprinting." In these instances, the ROS are not measured directly but the amount of damage incurred by their presence is evaluated indirectly by measuring biomarkers generated by injured tissues. Using experiments to measure the detrimental impacts of ROS in humans, researchers have been able to develop testable predictions about oxidative stress-related conditions and their treatment. Most ROS studies with human subjects are of this type, rather than the direct measurement of ROS. The criteria for suitable indirect biomarkers include: 1) detection of a major part or percentage of ROS-induced damage, 2) measurable with validated technology, 3) limited variability of different assays of the same sample, 4) not confounded by dietary factors, 5) stable for storage and 6) uses easily obtained samples of urine, blood, saliva, etc.[180] It is also desirable for oxidative biomarkers to reflect specific oxidation pathways and correlate with disease severity in order to serve as diagnostic tools[135]

Measures of lipid peroxidation are widely-used indicators of oxidative stress in vivo and in vitro. Tests that measure lipid peroxidation in blood, urine or tissues using detection of thiobarbituric acid-reactive substances such as MDA or other reactive aldehydes generated by lipid peroxidation are now deemed to be too non-specific and are subject to variability related to absorption of peroxides and aldehydes from dietary intake.[143,180,181] Prostaglandin-like derivatives of arachidonic acid, called F_2-isoprostanes,

reflect the peroxidation of polyunsaturated fatty acids by radical intermediates and may be more suitable choices for a study. With greater stability, F_2-isoprostanes are not affected by dietary intake and can be quantified in blood plasma and urine as well as most other body fluids, although their short half-life in plasma makes testing this medium challenging.[135] The F_2-isoprostanes are also detectable in exhaled breath condensate and sputum. The most reliable measurement method of F_2-isoprostanes is by mass spectrometry which is expensive and time-consuming. Enzyme-linked immunosorbant assay (ELISA) kits have been developed to test for F_2-isoprostanes however interference by other substances in body fluids has presented problems with this method.[181] Immunoassay and radioimmunoassay techniques have also been employed.[143]

Protein carbonylation is an irreversible oxidative modification which provides stable biomarkers for research.[182] Protein carbonyl groups produced by the oxidation of amino acids, in particular, lysine, arginine, threonine and proline or by secondary reaction with the oxidation products of sugars and lipids.[143] Proteins are believed to be targets of ROS in states of oxidative stress and detection of increased levels of protein carbonyls are associated with various diseases.[183] A favorably long blood plasma half-life enhances the study of protein carbonyls in oxidative stress states and samples can be maintained frozen for long periods. Common methods of detection include spectrophotometry, high performance liquid chromatography (HPLC), electrophoresis and Western blot immunoassay, however none of these allow for identification of specific amino acid residues that have been attacked by oxidation.[143] A technique derived from proteomics using a combination of two-dimensional gel electrophoresis for protein separation and mass spectrometry for protein identification has solved this problem.[182,184]

Oxidative disruption of DNA, which leads to increased cancer risk, has been used as another fingerprint biomarker of oxidative stress, with 8-hydroxy-2'-deoxyguanosine (8-OHdG) as the representative product of DNA damage. Gas chromatography with mass spectrometry and HPLC have all been employed successfully to isolate 8pOHdG in urinary samples.[140,143] Immunohistochemistry is useful for semi-quantitative measurements of DNA oxidation.[185]

Glutathione levels in blood plasma are believed to reflect GSH status in other body tissues. Measurement of reduced GSH and glutathione disulfide (GSSG) in blood samples has been used as an index of GSH status and oxidative stress in humans. Spectrophotometry, fluorometry and bioluminescence with HPLC are techniques that have been employed in the analysis of this marker. Correct sample handling is critical to avoid artifact that may skew measurements. Lowered levels of GSH or low GSH:GSSG ratios are associated with aging and with a number of pathologies including ischemic brain disease, cardiovascular disease, cancer and diabetes mellitus. Lower total GSH levels have been observed in the elderly with chronic illnesses, whereas high levels of GSH are associated with good physical and mental health in older individuals.[135,186]

Measures of indirect biomarkers have been useful in the evaluation of diseases related to oxidative stress however they often require costly technology and processes that diminish their clinical usefulness, especially in the acute care setting. Results may also be prone to artifact, which should be considered when selecting the appropriate method for testing. This type of information reflects the damage incurred from oxidative stress but little concerning the causal mechanisms. Access to reliable and cost-effective methods of the direct measurement of ROS would be beneficial to health care

practitioners. Although several decades of research have resulted in the development of some methods for ROS detection, these methods have largely been applied to laboratory animal models. In the next section, we present methods that have been commonly used in the attempt to measure directly the amount of ROS (specifically H_2O_2 and $O_2^{\bullet -}$) produced in cells during periods of stress.

Direct Measures of ROS

The ability to accurately measure the presence of ROS is highly desirable to evaluate the oxidation-reduction status of cells and to better appreciate their role in living systems.[137,187,188] The ability to quantify ROS would be especially useful in health-related diagnostic and therapeutic research. Techniques to measure ROS should ideally be specific for the molecule of interest, be very sensitive, distinguish where the ROS are generated, not require expensive or cumbersome equipment or methods and easy to standardize.[189-191] The development of molecular "probes" that would provide investigators with the ability to measure ROS has progressed over the past four decades but due primarily to the short life span of ROS, no single method has emerged to satisfy the criteria for a best technique. Probes with chemiluminescent or fluorescent properties employed using a variety of analytical techniques have provided the greatest promise.[191] Only a brief description of chemiluminescence technique will be provided for this article, however, fluorescence-based techniques used in the detection of $O_2^{\bullet -}$ and H_2O_2 will be presented in greater detail.

Chemiluminescence (production of light by a chemical reaction) has been used to detect radical species in biological specimens for more than 50 years.[192] When exposed to $O_2^{\bullet -}$, chemiluminescent probes release photons which may be detected by a scintillation

counter or luminometer.[193] Most chemiluminescent probes are cell-permeable and therefore able to detect both intracellular and extracellular $O_2^{\bullet -}$. Lucinogen (bis-N-methylacridinium) is most commonly used although the use of other compounds such as luminol and cypridina luciferin analogs has been reported. The mechanism of $O_2^{\bullet -}$ detection by lucinogen is based on the reduction of lucinogen by $O_2^{\bullet -}$ to its cation radical, which then reacts with a second $O_2^{\bullet -}$ to form a high energy molecule called lucinogen dioxetane. Dioxetane is responsible for emitting the photon marker.[193] The ability to accurately detect $O_2^{\bullet -}$ with lucinogen has been widely questioned because lucinogen is itself capable of donating electrons to oxygen molecules in a process known as redox cycling, resulting in the over-production of $O_2^{\bullet -}$ and falsely high measurements.[180,190] Minimizing the concentration of lucinogen has been proposed as a means of minimizing the extraneous production of $O_2^{\bullet -}$[193] however, not all investigators agree that this technique is useful.[181] Lucinogen is a reductant of H_2O_2, which can also result in the inaccurate estimation of $O_2^{\bullet -}$.[191]

Fluorescent Probes

Probes used frequently for the detection of ROS are fluorochrome dyes which are stable in a reduced state, but when oxidized by a species of interest, a change in bonding occurs causing the molecule to express fluorescence that can be detected by a variety of methods.[191,194] Fluorochromes absorb light at characteristic wavelengths causing an electron to temporarily assume a higher energy state. When the electron returns to its ground state, the energy is released as a photon of light with a wavelength that is characteristic of the molecule.[195] The fluorescent dyes are loaded into cells using specific protocols[145] and the dye is oxidized by the ROS of interest yielding a cell “fluorophore.”

The fluorophore is then bombarded with high intensity light at a wavelength that is capable of stimulating maximum fluorescence. Emission light energy is dependent on the amount of energy used to stimulate the fluorophore. Table 2 is a list of desirable characteristics of an ideal fluorescent probe.

Depending on the tissues or cell types being examined, emission fluorescence is commonly detected through a filter at a specified wavelength, using flow cytometry, spectrofluorometry or fluorescence microscopy (including confocal techniques).[196-198] If the targets are neutrophils or other ROS-producing leukocytes, fluorochromes are often conjugated with monoclonal antibodies which serve as labels by attaching to antigens on the surfaces of the cells of interest and analyzed by flow cytometry.[195,199,200]

Several methods of detecting fluorescence are commonly used. Spectrofluorometry allows evaluation of fluorescent compounds by bombarding a sample with high intensity light to stimulate molecules to a state of excitation. Emission fluorescence is collected and processed to provide quantified digital or analog output of the emission intensity. Fluorescence microscopy also employs the excitation characteristics of a fluorescent dye that has been applied to a tissue or cell specimen but in this instance the fluorescence is used to generate reflected microscopic images which are also analyzed for intensity.[201] Fluorescence and confocal microscopy may be used in combination to allow ROS analysis in thick tissue samples through multiple layers.[202,203] Fluorescence intensity has been reported as an estimation of the quantity of ROS.[197,204] Other methods of interpreting fluorescence have also been proposed, including: 1) measures of the percent of cells expressing ROS,[196,205] 2) comparisons of the fluorescent intensity of ROS-producing cells to non-ROS-producing cells[206] and 3) estimates

determined by the product of the percent of cells fluorescing and the mean channel fluorescence.[207]

Flow cytometry, also known as fluorescence-activated cell scanning or sorting (FACS) is a technique of rapidly counting cells as they are carried single-file through a fluid sheath stream. Excitation lasers are focused on the cells and the forward and side-scattered light (FSC/SSC) beams are measured, allowing the cytometer to classify cell types based on size and complexity. Forward scattered light emissions provide information about size, whereas SSC emissions analyze cell granularity and complexity.[195,208] Advantages of flow cytometry are its ability to sort hundreds of thousands of cells in just a few minutes and the capability of analyzing blood constituents, especially ROS-producing neutrophils and macrophages, as well as tissue cells grown in vitro.[121,204,209,210] Figure 3 is a presentation depicting the concepts of laser light scatter and the use of a fluorophore with flow cytometry to analyze cells for the presence of ROS.

Confocal microscopy provides the ability to image cell structures and physiology in thick biological specimens through its capacity to reject out-of-focus light to provide high-contrast images of cells and structures within thick samples.[203] Confocal microscopes have computer-controlled focus and image-capturing which allow the user to obtain a through-focus series of optical sections that are used to reconstruct a volume of tissue. This provides information on the 3-dimensional (3D) structure and relationship of cells and their components. This computer-assisted technique provides flexibility in processing, manipulation and quantitative analysis of specimens. Images are obtained by illuminating a single spot in the tissue at a time by using a confocal (pinhole) aperture

focused at the illuminated spot. The sample is then scanned to generate 2-dimensional images which can be created for multiple tissue layers and processed into a 3D image. Figure 3 is a diagram of the confocal microscopy process. Confocal microscopes may be equipped with lasers, which are used as sources of excitation for use in fluorescence microscopy. An advantage of confocal microscopy is the ability to image large regions of tissue at subcellular resolution, which clearly displays cellular structures in their natural spatial relationships to other structures. When used with fluorescent probes, confocal microscopes provide the ability to analyze physiological characteristics of cells as well as information about structure. The use of fluorescence confocal microscopy has been reported in studies of lung,[211] diaphragm,[212] heart,[213] and kidney[214] tissues.

Oxidized fluorophore molecules may also be detected with HPLC,[54] which is recognized as one of the most accurate measures of ROS currently available.[215,216] Using HPLC, chemists have been able to separate mixtures of compounds for the purpose of quantifying and purifying the individual components of the mixtures. When HPLC is used in conjunction with a fluorescent dye, one is able to analyze the presence of the oxidized fluorochrome, currently the best means to capture quantitative evidence for the presence of ROS. Several studies have been published outlining this method of detection[217-220]

Measurement of Superoxide

A fluorescent dye frequently used for the intra-cellular detection of $O_2^{\bullet -}$ is hydroethidine (HE). A variant of HE useful in detecting intra-mitochondrial $O_2^{\bullet -}$ is MitoSOX Red which has an added cationic triphenylphosphonium group that facilitates preferential diffusion of the dye into the mitochondrial matrix to allow for the rapid

detection of $O_2^{\bullet -}$ from the ETC.[145] The oxidation of HE or MitoSOX Red occurs by a two-step process in the presence of $O_2^{\bullet -}$. Initially a radical ($HE^{\bullet +}$) is generated followed by the oxidative formation of two positively charged fluorescent products, ethidium (Et^+) and 2-hydroxyethidium (2-OH- Et^+). Early investigators believed that Et^+ was the only molecule generated by the reaction[188] but work by Zhao's group[217] using HPLC analysis, led to our current understanding that the oxidation of HE by $O_2^{\bullet -}$ specifically results in the formation of 2-OH- Et^+.

This was an important finding because the reaction of HE with $O_2^{\bullet -}$ produces 2-OH- Et^+ whereas the production of Et^+ may occur as a result of oxidation by ROS other than $O_2^{\bullet -}$ or in the presence of cytochrome-c.[216,220] This phenomenon is illustrated in Figure 4. Both Et^+ and 2-OH- Et^+ exhibit fluorescence at similar excitation/emission wavelengths ($510_{ex}/580_{em}$ nm) which means that increases in fluorescence intensity may be due to the increased production of $O_2^{\bullet -}$, resulting in the formation of 2-OH-Et^+ or to the presence of artifactual reactions to produce Et^+. Therefore, it has been challenging using flow cytometric or laser microscopy techniques to quantify the production of $O_2^{\bullet -}$. The use of an excitation wavelength of 396 nm that specifically stimulates 2-OH- Et^+ but not Et^+ has been proposed as a solution to this problem.[145,221,222]

Hydroethidine and MitoSOX Red in conjunction with flow cytometry and fluorescence microscopy have been used for in vitro studies of cultured live cells to include vascular endothelium,[210] cardiomyocytes,[209] lymphoid cells[223] and neuronal cells.[224] In these studies $O_2^{\bullet -}$ production was generated and then measured by estimation of fluorescence intensity.

Hydroethidine has also been used to measure the production of $O_2^{\bullet -}$ by

phagocytes. In one study with HE, Rothe and Valet[225] attempted simultaneous $O_2^{\bullet-}$ detection in human phagocytes (neutrophils, monocytes and macrophages) by HE and detection of H_2O_2 by another fluorescent indicator, 2′,7′-dichlorodihydrofluorescein (H_2DCF). These authors hypothesized that HE was preferentially oxidized by $O_2^{\bullet-}$ and that their method provided a satisfactory examination of respiratory burst using flow cytometry. Also using the HE probe, Perticarari, Presani and Banfi[226] reported the successful detection of respiratory burst in phagocytes isolated by flow cytometry from small samples of whole blood from human volunteers. Walrand and colleagues compared the use of HE with H_2DCF and dihydrorhodamine-123 (DHR) for detection of respiratory burst activity in neutrophils and confirmed the earlier finding that HE was a specific indicator for $O_2^{\bullet-}$.[227] Based on this, they suggested that fluorescent probes be selected for analysis based on the specific ROS of interest. In one other comparative study of multiple methods of ROS estimation using bovine neutrophils, investigators concluded that HE fluorescence analyzed by flow cytometry was useful in the in the measurement of intracellular ROS.[207]

Potential limitations other than the spectral overlay of the two products of HE oxidation already mentioned have been identified and must be considered by the researcher who is planning the use of fluorescence detection methods for $O_2^{\bullet-}$. The initial ROS produced by mitochondria is $O_2^{\bullet-}$ and its accurate detection in live cells using HE is significantly affected by the level at which SOD is being expressed at the same point in time, such that SOD and HE are effectively “competing” to react with molecules of $O_2^{\bullet-}$.[216,228] Further, the exposure of HE to continuous sources of visible illumination will result in photo-oxidation of the probe to Et^+ and experimental procedures must therefore

be carried out in low light.[229] Ultrasound sonication, used in some cell-lysis techniques, has been found to increase the decomposition of HE as well as the enhanced formation of hydroxyl radicals, representing a potential disturbance of results. One other variable is introduced when using Mn(III)TBAP, a cell-permeable manganese-porphyrin complex widely used to inhibit the intracellular formation of products from the reaction of HE with $O_2^{\bullet -}$, resulting in the increased production of Et^+.[229] The development of experimental protocols should account for the impacts of these factors. If fluorescence techniques are to be employed, careful analysis of the data with an appreciation of the inherent problems in attempting a quantitative representation of the presence of $O_2^{\bullet -}$ should be conducted. High performance liquid chromatography has become the standard for detecting the oxidation of HE by $O_2^{\bullet -}$, but the costs, arduous preparation of specimens and time involved in this may limit its usefulness in some laboratories.

Measurement of Hydrogen Peroxide

Two fluorescent probes that are specific for H_2O_2 are H_2DCF and DHR. 2′,7′-dichlorodihydrofluorescein, which was introduced some 40 years ago, is a non-fluorescent and colorless molecule, capable of being oxidized in the presence of H_2O_2 in a two-electron reaction to the fluorescent 2′,7′-dichlorofluorescein (DCF).[145,215] 2′,7′-dichlorofluorescein can then be monitored by fluorescence confocal microscopy or flow cytometry with parameters for excitation at 485 nm and emission at 530 nm.[138] Disagreement over the correct nomenclature for this probe has resulted in the use of numerous abbreviations in the literature (DCFH, DCFH2, DCFH-DA among others), leading to confusion.[144] It is the most common of the fluorescent probes used in ROS studies,[191,230] possibly due to the preference of many researchers to study H_2O_2 (rather

than $O_2^{\bullet -}$ or $OH^{\bullet}$) because of its relative stability.[228] 2′,7′-dichlorodihydrofluorescein also has the advantage of low reactivity with $O_2^{\bullet -}$ and is not affected by the presence of SOD. The caveat with H_2DCF is that it does not react directly with H_2O_2 but must be oxidized enzymatically by a Fenton chemistry pathway in which H_2O_2 is cleaved into an oxygen singlet ($O^{\bullet}$) in the presence of Fe^{2+}. The $O^{\bullet}$ then reacts with bicarbonate to form carbonate radicals which initiates the reaction with H_2DCF.[230] The 2′,7′-dichlorodihydrofluorescein is applied to cells in its cell-permeable acetylated form, and the diacetate is cleaved off once the molecule has entered the cell. Previously, the intracellular conversion of H_2DCF to the fluorescence intensity of DCF has been assumed to approximate the level of oxidative stress within cells. Others suggest that fluorescence may instead be related to lysosomal leakage of iron or the mitochondrial leakage of cytochrome c into the cytosol.[231]

The limitations of the use of H_2DCF as a specific probe for H_2O_2 have been recently detailed.[215] 2′,7′-dichlorodihydrofluorescein should not be considered a specific measure of H_2O_2 because H_2DCF does not react directly with H_2O_2, but requires enzymatic activation. Several other reactive oxygen species as well as heme-containing substances, peroxidases and cytochrome c are also capable of oxidizing H_2DCF to DCF in the absence of H_2O_2, making the interpretation of results difficult.[181] A DCF radical may also be formed and is capable of reacting with oxygen to form $O_2^{\bullet -}$, thus risking the amplification of fluorescence intensity and the overestimation of oxidative stress. Even with these challenges, H_2DCF is considered a useful indicator of cellular redox status.[152]

A cell-permeant probe similar to H_2DCF also useful in the measurement of H_2O_2 is dihydrofluorescein-diacetate (Hfluor) which has been used to measure intra-cellular

levels of H_2O_2 in tissues.[25] Hfluor permeates tissues and in its unoxidized unesterfied form reacts with H_2O_2 resulting in the conversion of Hfluor to fluorescein which fluoresces intensely green with laser stimulation. Hfluor has demonstrated superior ability to detect H_2O_2 in mitochondria and cell-free environments.[232,233]

Dihydrorhodamine-123 is another cell-permeant, mitochondrial-avid analog of H_2DCF that is oxidized to the fluorescent molecule rhodamine 123.[188] Dihydrorhodamine-123 will fluoresce at 536 nm when excited at 500 nm.[180] As with H_2DCF, DHR is not directly oxidized by H_2O_2 but must be catalyzed by heme-containing peroxidases. Dihydrorhodamine-123 is also subject to reaction with the nitrogen radical peroxynitrate, HOCl and cytochrome c and therefore is not a specific indicator for H_2O_2 but rather a measure of overall cellular redox status.[145] To ensure that the ROS molecules of interest are reacting with DHR, careful control of these possible confounding compounds should be included. Dihydrorhodamine-123 has been used with flow cytometry to test for oxidative burst in phagocytes and has been assumed to reflect H_2O_2 production in artificially activated neutrophils and monocytes.[199,204,234,235]

Latest Methods of ROS Detection

Given the challenges and limitations of the fluorescence-based techniques used to detect ROS in recent years, efforts are underway to both improve both the chemical properties of fluorphores as well as the methods of their detection. Recent reviews of fluorescent indicator development are available and should be followed by nurse scientists interested in pursuing ROS research.[150,236] One promising development is from Dickinson and Chang[237] who synthesized a mitochondrial-targeted specific probe to image H_2O_2 in living cells. The probe, mitochondria peroxy yellow 1 (MitoPY1), uses a

boronate chemical "switch" that allows the fluorophore to target mitochondria. Benzil, a compound that reacts readily with H_2O_2, has been used in the development of 5-benzoylcarbonylfluorescein to provide live-cell imaging of H_2O_2 in epidermoid carcinoma cells using confocal microscopy.[238] Other recent probe modifications include the use of protection-deprotection chemistry to improve probe specificity by eliminating the need for oxidative reactions to induce fluorescence[239] and the probes designed to target selected organelles within cells using proteins labels tagged to fluorophores.[150,239]

Researchers at the University of Michigan have developed a technique that involves the encapsulation of fluorescence probes in a nanomatrix to provide a highly specific and rapidly reacting indicator of H_2O_2 in biological specimens. This team encapsulated a traditional probe 2′ 7′-dichlorofluoroscein into a "nanoPEBBLE" which protected it from interference by other ROS except the target of interest, H_2O_2.[240] Such nanoprobes may allow the use of traditional fluorphores but with enhanced specificity to selected ROS.

One other novel technique is the application of a new fluorescent probe specific for mitochondrial $O_2^{\bullet -}$ detection, 2-chloro-1,3-dibenzothiazoline-cyclohexene (DBZTC), with microchip electrophoresis. The authors reported the method to be "simple, fast, reproducible and efficient," desirable characteristics of any method used to examine biological ROS. In addition, the microchip electrophoresis process allows the use of very small biological specimens.[241]

Conclusions

The roles of ROS in aging and disease have led to considerable efforts to develop methods for their detection in easily obtainable biological specimens collected using

minimally-invasive techniques. Fluorescent dye techniques have historically been useful options, but they lack specificity and the methods can be expensive. Methods using flow cytometry offer greater accessibility as cytometers are currently used in most major medical centers. The use of new generation dyes should dramatically enhance our ability to measure not just the presence of oxidative stress but to quantify the specific ROS.

The challenge to develop probes that are sensitive, specific to an individual ROS, well characterized, non-toxic and have a verifiable intra or extracellular focus of distribution will continue to drive research in this area. Reactive oxygen species have characteristics that render them difficult to quantify, their short life span being the most challenging. There is a lengthy list of the ideal qualities of a useful fluorescent probe, primarily sensitivity, specificity, stability and minimal production of artifacts due to other variables. As fluoroprobes improve, experiments must continue to be meticulously designed with respect for the potential limitations of fluorophore technology. Methods should be executed with precision using multiple probes when possible and results should be interpreted carefully. Such an approach will ultimately advance research to measure the effects of ROS.

Table 1. Reactive Oxygen Species[141]

Radicals	Non-radicals
Superoxide, $O_2^{\bullet -}$	Hydrogen Peroxide, H_2O_2
Hydroperoxyl, $HO_2^{\bullet}$	Peroxynitrate, $ONOO^-$ [(a)]
Hydroxyl, $OH^{\bullet}$	Peroxynitrous acid, ONOOH [(a)]
Peroxyl, $RO_2^{\bullet}$	Nitrosoperoxycarbonate, $ONOOCO_2^-$
Alkoxyl, $RO^{\bullet}$	Hypochlorous acid, HOCL [(b)]
Carbonate, $CO_3^{\bullet -}$	Hypobromous acid, HOBr [(c)]
Carbon dioxide, $CO_2^{\bullet -}$	Ozone, O_3
Singlet oxygen, $O_2\Sigma g^+$	Singlet oxygen, $O_2\Delta g$

(a) Could also be called a reactive nitrogen species
(b) Could also be called a reactive chlorine species
(c) Could also be called a reactive bromine species

Table 2. Characteristics of an Ideal Fluorescent Probe

1.	Converts from nonfluorescent to fluorescent form in the presence of ROS
2.	Activity is well characterized
3.	High photostability
4.	Insensitive to environment: pH, thermal changes, light, solvents, use of sonication
5.	Established focus: extracellular, intracellular or intraorganelle
6.	Suitable rate of reaction with efficient intracellular trapping of ROS
7.	Low spontaneous oxidation
8.	Minimal effects on cell function; low toxicity

Figure 1. Basic Pathways of Reactive Oxygen Species

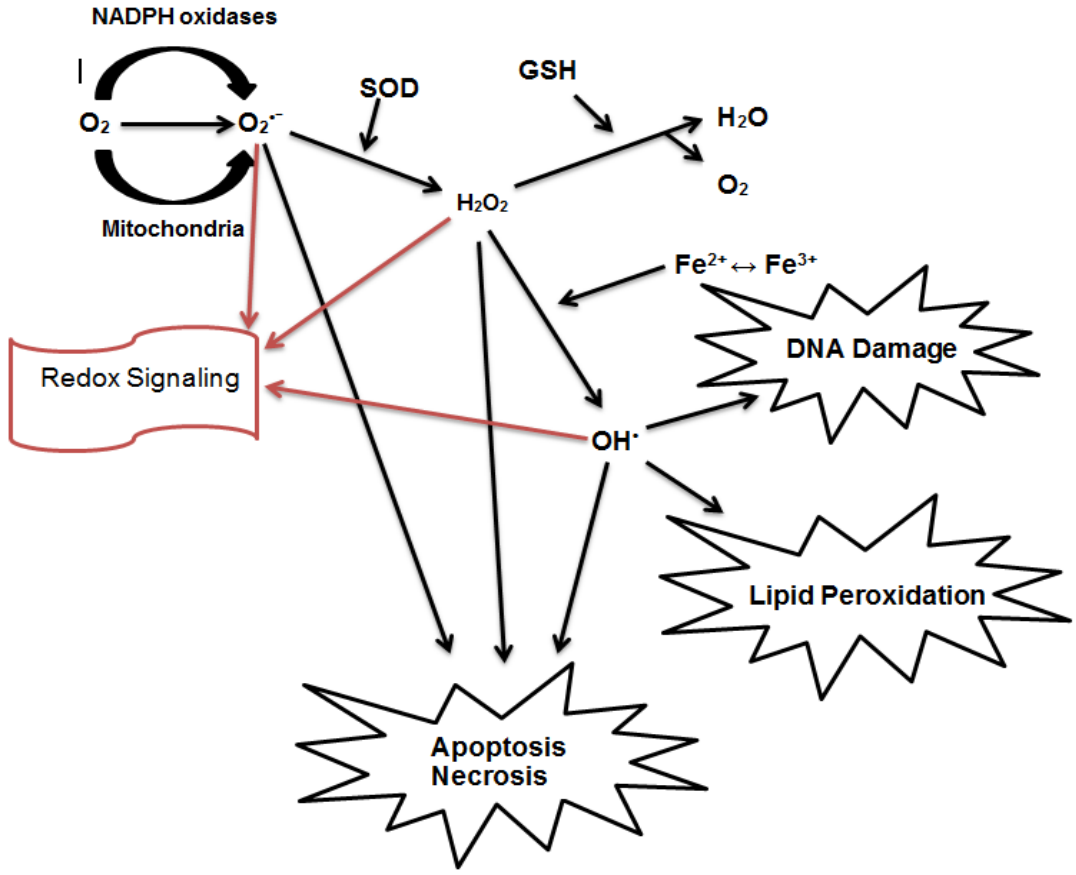
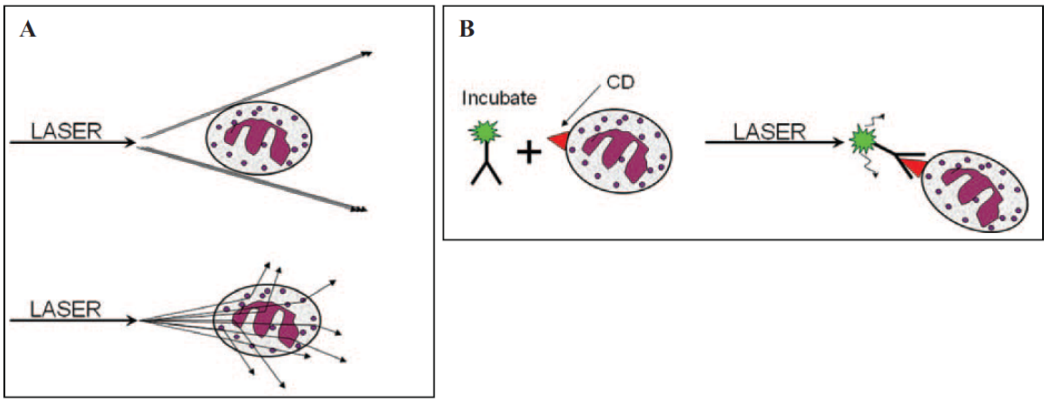


Figure 2. Using Flow Cytometry to Analyze Cells for the Presence of ROS[200]



A. As cells pass through the fluid sheath of the flow cytometer, they are directed into a single cell stream and pass through a laser. The laser light that passes over the cell and is analyzed for forward scatter (FSC) to determine size and side scatter (SSC) to measure intracellular organelles and cell complexity.
B. To measure the presence of extracellular markers, called cluster domains (CDs), cells are first incubated with a specific fluorophore–antibody conjugate during which they bind to their respective CDs. As the cell and attached antibody pass through the laser, the fluorophore is excited and energy is released at a specific wavelength. The resulting light emission (fluorescence intensity) is quantified and recorded for each cell.

Figure 3. Light Pathways of the Confocal Microscope[242]

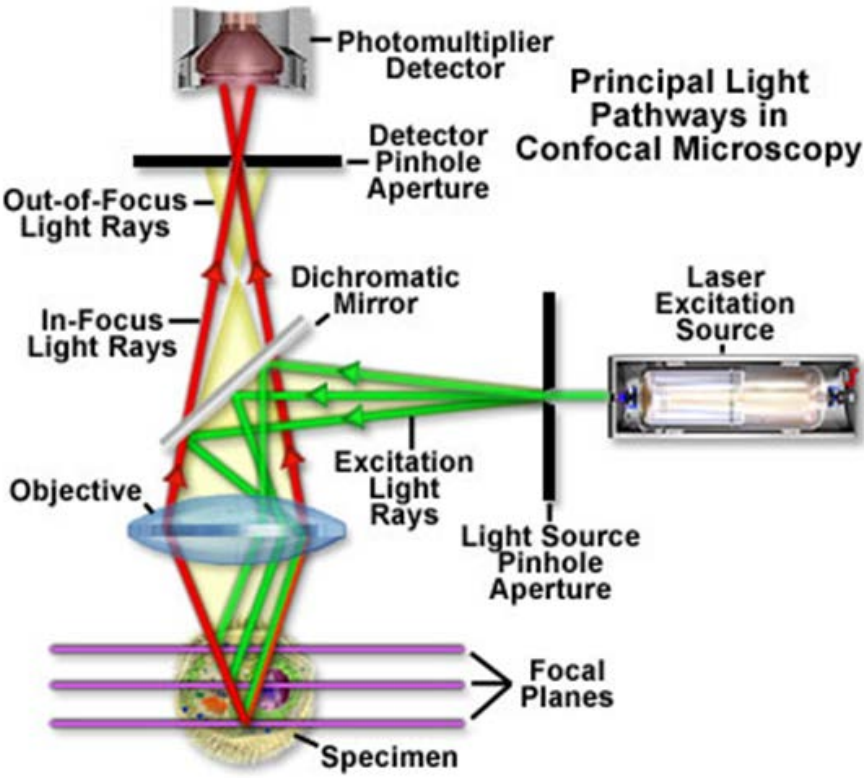
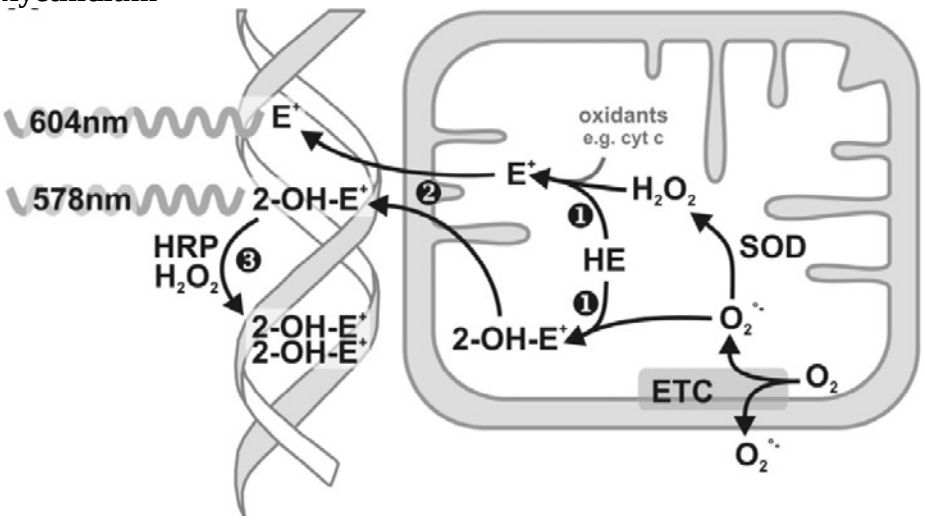


Figure 4. Oxidative Pathways of the Formation of Ethidium and 2-hydroxyethidium[243]



In the mitochondrial matrix, hydroethidium may be oxidized by superoxide to form 2-hydroxyethidium or by other reactants such as cytochrome c, myoglobin, or hydrogen peroxide by enzymatic reactions to form ethidium.

CHAPTER 3.

Apoptosis: Understanding Programmed Cell Death for the CRNA

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Abstract

Apoptosis or programmed cell death is a physiologic mechanism employed by most multicellular organisms to maintain homeostasis of body tissues. In balance with the production of new cells by mitosis, apoptosis provides for the orderly destruction and removal of cells that are no longer needed by the organism. Apoptosis occurs by complex pathways involving multiple biochemical signals and processes. Dysfunctional apoptotic mechanisms are the pathological basis for many human diseases including common disorders of the heart, lungs, brain, and endocrine systems. Researchers have demonstrated in animal models that neurodegenerative changes following the administration of anesthetic drugs are related to apoptosis. Anesthesia drugs have been found to induce apoptosis, perhaps through the production of reactive oxygen species. Propofol is a drug used in anesthesia that has unique antioxidant qualities which may be beneficial. The purpose of this article is to review for nurse anesthesia providers, current information about the process of apoptosis, the role of apoptosis in co-morbid diseases and the implications of the effects of anesthesia drugs on normal apoptotic mechanisms that need to be evaluated as a potential source of risk to surgical patients.

Key Words: apoptosis, programmed cell death, caspase.

Introduction

Apoptosis, or programmed cell death (PCD) is an energy-dependent biochemical process effectuated daily by some 50 to 70 billion cells in each adult human. The term apoptosis is derived from a Greek word which translates into the “dropping off” of leaves from trees. It is the mechanism that accounts for the change of color of autumn foliage from green to orange and yellow and ultimately leads to the death of the leaves. The human equivalent to falling leaves is the exfoliation of skin cells, and is but one example of the results of apoptosis. For virtually all multicellular organisms, apoptosis is a normal process, providing tissue homeostasis coordinated with mitosis to continuously balance cell production with cell death. Physiologic situations in which apoptosis plays an important role include embryological development, the hormonal mechanisms which control menstruation, lactation and menopause, the maintenance of a constant number of cells in proliferative cell populations such as the digestive tract, and the deaths of white blood cells that have served their useful purpose or failed to mature normally.[244,245]

Although apoptosis is a normal process, disrupted PCD pathways have been implicated in nearly all diseases that afflict humans. Co-morbid diseases frequently observed in surgical patients, for which a pathological basis in apoptosis have been established, include ischemic heart disease, cardiomyopathy, asthma, emphysema, cancer, diabetes mellitus, neurodegenerative diseases and sepsis.[246] In addition, a growing body of research has implicated some anesthetic drugs in the production of reactive oxygen species (ROS), including free radicals, which are associated with increased levels of apoptosis.[247,248] Studies have indicated that anesthetic drugs alter normal apoptotic mechanisms in the developing neural tissues of rat models, raising concerns over the

potential effects of these drugs on very young pediatric surgical patients.[249,250] Given these considerations, Certified Registered Nurse Anesthetists (CRNAs) should understand the mechanisms of apoptosis, the role of apoptosis in the diseases that may affect their patients, and the potential for anesthetic drugs to alter the apoptotic process. Apoptosis and alterations in normal apoptotic processes are important concepts in our approach to understanding both the mechanisms of disease and the safe delivery of anesthesia to the patient. This article is a review of apoptosis and how it relates to nurse anesthesia.

History

Medical use of the term apoptosis dates to the 4th century BCE when Hippocrates used it to describe the effects of gangrene resulting from the treatment of fractures.[251] The first observations of cellular death were reported in the scientific literature published by mid-19th century biologists; however it was not until 1914 that the concept of cell death as a mechanism of balance to cell proliferation was proposed.[252] In 1972, Kerr et al[253] published an article in which was detailed for the first time the processes of cellular death in healthy tissues, which they named apoptosis. Since then, there have been numerous studies related to apoptosis and there are several currently published journals specifically focused on apoptosis. In 2002, Brenner, Horvitz and Sulston were able to elucidate the genetic influences of PCD, for which they were awarded the Nobel Prize in Physiology or Medicine.[254] The importance of apoptosis in health care is evident with over 132,000 Pub Med citations for apoptosis in the last decade.

Pathways to Cell Death: Apoptosis and Necrosis

Apoptosis is a form of programmed cell suicide which allows for the orderly

removal of unwanted, improperly functioning, or injured cells, in balance with the production of new cells by mitosis. The apoptotic cycle is an energy-dependent process in which a genetically controlled sequence of events regulated by complex and numerous biochemical signals result in the condensation and fragmentation of an individual cell into small vesicles which contain the cellular components (organelles and nuclear fragments). These vesicles are phagocytized by macrophages and neighboring cells and recycled. One characteristic of apoptosis is that neighboring cells are not damaged during this process.[246]

Apoptosis is differentiated from necrosis, also referred to as passive cell death since necrosis is a non-energy dependent process. Necrosis is pathological or accidental cell death that occurs as a result of insult from harmful events such as hypoxia, toxicity or infection. With necrosis, cell death transpires marked by the presence of inflammation, resulting in damage to adjacent cells, however in the normal process of apoptosis, cells are usually affected individually.[246] Cell shrinkage in apoptosis is unlike what occurs in necrosis when enzymatic digestion results in deterioration of the cell membrane and leakage of noxious cellular contents into surrounding tissues, resulting in inflammation and wide-spread damage. In contrast, apoptosis generally does not induce inflammation or scarring. Apoptosis also requires activation of cell signaling, which is usually not a part of the necrotic process. Intracellular adenosine triphosphate (ATP) concentrations may be important in determining which path to cell death is taken. High ATP concentrations favor apoptosis, while low ATP shifts the cell toward necrosis. The nature or severity of an insult that precipitates cell death may result in the depletion of ATP or reduced ATP synthesis, thus favoring necrosis.[167]

In apoptosis, upon receiving a molecular signal, the cell identified for death, shrinks and separates from neighboring cells and the nucleus is condensed and fragmented. As the nucleus is broken apart, deoxyribonucleic acid (DNA) is divided into smaller pieces. In response to the consumption of cell matrix by protein enzymes (caspases), the cytoplasm and organelles condense and the cell eventually breaks apart into membrane-bound particles referred to as apoptotic bodies. These apoptotic bodies are then recognized and ingested by macrophages or other neighboring cells.[244,246] Figure 1 presents a diagram of the morphologic changes differentiating apoptosis and necrosis.

The Phases of Apoptosis

Apoptotic mechanisms may be conceptualized as a cascade of biochemical events comprising four explicit phases: 1) signaling, 2) control and regulation, 3) execution, and 4) removal of the dead cell.[255] During the *signaling phase*, a variety of stimuli are capable of triggering apoptotic pathways. These stimuli may include glucocorticoids, toxins, nitric oxide, nutrient deprivation, viral infection, hypoxia, ligand (molecule) receptor activation, ionizing irradiation or attack by cytotoxic lymphocytes. Activation of ligand-gated receptors on the cell surface (also referred to as “death receptors”) results in changes to these trans-membrane channels, triggering the sequences which lead to cell death. Two commonly identified cell surface death receptors are Fas and tumor necrosis factor (TNF). The activation of Fas receptors results in the formation of Fas-associated death domain (FADD) and activation of TNF receptors yields TNF receptor associated death domain (TRADD). The death domain molecules stimulate caspase activation that leads to apoptosis.[246,255]

During the second phase of apoptosis, *control and regulation*, the cell will either

commit to apoptosis or mechanisms are activated which interrupt the cascade of events initiated during the first phase and apoptosis is aborted. Two genes are important regulators at this stage of apoptosis, B cell lymphoma-2 (Bcl-2) and protein-53 (p53). The Bcl-2 gene family regulates apoptosis and exhibits both pro-apoptotic and anti-apoptotic influences. Bcl-2, referred to as a "cellular life or death switch"[256] will determine which cellular insults will ultimately result in apoptotic progression. Elevated levels of the Bcl-2 gene are known to inhibit apoptosis and are associated with the development of a number of human cancers, such as lymphoma, leukemia, adenocarcinoma, renal and lung cancers, neuroblastoma and melanoma.[257] The p53 gene is a nuclear phospho-protein which suppresses cell proliferation. Deficiencies of the tumor suppressor p53 gene are commonly found in half of human cancers and are also associated with resistance to treatment.[257,258] When apoptosis is allowed to proceed, the signaling phase activates a series of the caspase family of proteases. Caspases, a proteolytic central component of apoptosis, exist in an inactive form called procaspases. Procaspases must be activated by specific apoptotic signaling pathways in order for apoptosis to continue. Thus activated, caspases are destructive enzymes capable of protein cleavage leading to the morphologic changes in the apoptotic cell. At least 14 caspases have been identified in mammals and a number of these are present in the human apoptotic cascade.[246,256] Caspase activation also facilitates apoptosis by increasing permeability of the mitochondrial membrane which results in the release of mitochondrial proteins into the cytosol. These proteins bind inhibitors of apoptosis proteins (IAPs), allowing cell death to proceed.

Once fully committed to apoptosis, the cell enters into the *execution phase.* The

cell now will undergo an organized degradation of its matrix and organelles by the proteolytic caspases. Caspases will activate cytoplasmic Dnase (CAD) that enters the nucleus and affects the breakdown of DNA. Morphological changes associated with the execution phase include the condensation of chromatin (DNA and histones in the nucleus) and the blebbing and formation of vesicles containing the compacted cellular structures.[255,259]

Following the execution phase, the cell enters the *removal* phase of apoptosis. The apoptotic bodies present engulfment ligands on their surfaces, which are recognized by scavenger receptor sites in phagocytic cells.[260] Engulfment ligands may consist of lipid, sugar or protein markers. One phospholipid, phosphatidylserine (PS), is normally sequestered on an inner leaflet of the plasma membrane, however during apoptosis this lipid is translocated to the outer leaflet where it becomes accessible to receptors on macrophages. With the macrophage receptors activated, the binding and consumption of the apoptotic bodies is completed.[261]

The Pathways of Apoptosis

Two primary caspase-mediated pathways have been identified: 1) the *extrinsic* or death-receptor mediated pathway and 2) the *intrinsic* or mitochondria-dependent pathway.[167,246,251] The extrinsic pathway is activated when cell surface proteins (i.e. Fas or TNF) encounter the ligand activators to form the death domain molecules. Death domain molecules, (FADD and TRADD), in turn bind inactive forms of caspase-8 and caspase-10 to generate active forms of theses enzymes. Caspase-8 and caspase-10 activate other downstream caspases which in turn allow the apoptotic disposal of the cell. The extrinsic pathway plays an important role in tissue homeostasis especially in the

maintenance of lymphocytes. Patients with caspase deficiencies or Fas mutations are at risk for autoimmune or immunodeficiency disorders related to defective apoptosis of lymphocytes.[262]

In contrast, the intrinsic apoptotic pathway is regulated at the mitochondrial level and is sensitive to both extracellular stimuli and internal insults such as DNA damage. This pathway is initiated via alterations in mitochondrial membrane potential and increased permeability of the membrane, resulting in the leakage of cytochrome *c*, a protein important to mitochondrial respiration. In the cytosol, cytochrome *c* binds with apoptosis activating factor (Apaf-1) to form a complex known as an apoptosome, which activates caspase-9. At this point, if the balance between pro-apoptotic and inhibitory control is tipped in favor of cell death, then the caspase cascade is initiated and the cell is destroyed.[262] Figure 2 is a comparison of the extrinsic and intrinsic apoptotic pathways.

Though the extrinsic and intrinsic pathways of caspase activation are the cascades most often described, other pathways may also contribute to PCD. A pathway has been described that involves the exposure of target cell cytoplasm to a serine protease, granzyme B. Granzyme B initiates the caspase cascade through activation of caspase-3. Granzyme B may also initiate the pro-apoptotic gene Bcl-2, leading to cell destruction. The granzyme B pathway is used by T cells and natural killer cells for the elimination of virus infected cells from the body. Other pathways have also been suggested including a caspase-independent extrinsic pathway and intrinsic pathways dependent on other stimuli that are not yet understood.[262,263]

Apoptosis and Human Disease

Historically, tissue and cellular injury related to certain diseases was associated

only with necrosis, however recent studies have demonstrated that altered cellular regulation and damage by apoptosis are also important in understanding disease processes. Many of our patients are afflicted with acute or chronic diseases (or both) that are either the source of their need for surgical intervention or co-morbid conditions that complicate the delivery of anesthesia. Many nurse anesthetists may be either unaware of the role of altered apoptosis in disease or have limited understanding of this important physiological process. As most diseases are now associated with altered apoptosis, it would be a rational choice for the skilled anesthesia provider to have a foundational knowledge of these pathways. Table 1 is a list of common diseases known to be related to alterations in normal apoptotic processes.

Diseases involving aberrant apoptotic mechanisms may be categorized as related to: 1) excessive apoptosis or 2) insufficient apoptosis.[246] Numerous common diseases are related to excessive apoptotic activity. Surgical patients presenting with cardiac or pulmonary impairment present specific challenges to the anesthesia clinician and an appreciation of the role of up-regulated apoptosis in disorders of the heart and lungs is useful. Ischemia of coronary heart disease triggers apoptosis of cardiomyocytes and is a contributing factor in the evolution of myocardial infarction.[256,264] Ischemia followed by reperfusion (as in the instrumentation and re-opening of an infarct-related coronary artery) is a potent stimulus of cardiac cell death which persists even after perfusion is re-established. Other critical factors in cardiac disease appear to be the production of free radicals and depletion of growth factor and energy sources. Apoptotic cardiac insults may precipitate heart failure via the gradual loss of myocytes. The progressive thinning of ventricular tissues and loss of myocytes in heart failure resulting in ventricular

remodeling is known to have origins in perturbations of apoptosis although levels of apoptosis in heart failure are low in contrast to ischemia-reperfusion events.[265]

A focus on the suppression of cardiomyocyte apoptosis has led to interest in the development of cardioprotective drugs. Therapeutic drug interventions currently being used or investigated for this purpose include caspase inhibitors, antioxidants and angiotensin receptor antagonists.[256] CRNAs are familiar with the frequent use of beta adrenergic receptor antagonists in the operating room to control the impact of surgical and anesthesia stress in patients with known or suspected ischemic heart disease. Beta blockers such as carvedilol dramatically prevent myocardial ischemia and reperfusion-related apoptosis by inhibiting caspases, stress-activated protein kinases and by antioxidant mechanisms.[266]

Respiratory disorders are likewise correlated with increased levels of apoptosis. Respiratory diseases in which excessive apoptosis plays a central role include respiratory infections, bronchial asthma, smoking-induced chronic obstructive pulmonary disease (COPD), acute lung injury, hyperoxic lung injury, cystic fibrosis and interstitial pulmonary fibrosis.[267,268] Sphingolipids, key regulators of processes in biological membranes are known to affect apoptosis in lung tissue. Certain sphingolipids are known to be pro-apoptotic. The pharmacologic manipulation of these cell membrane lipids is generating new research that may eventually yield more effective treatments for lung disease than the typical bronchodilator, antibiotic and anti-inflammatory therapies currently available.[269]

Excessive apoptosis has also been demonstrated in neurodegenerative problems including Alzheimer disease, Parkinson disease, Huntington disease and amyotrophic

lateral sclerosis (ALS).[270] The progression of human immunodeficiency virus (HIV) to acquired immune deficiency syndrome (AIDS) marked by the depletion of CD4+ T-helper lymphocytes leads to immune system compromise. One of the important mechanisms by which the T-helper cells are depleted in HIV is by apoptosis.[271] Data also suggests that accelerated rates of apoptosis resulting in up to a ten-fold increase in the destruction of β-cells in the pancreas may contribute to the development of Type 2 diabetes mellitus. Apoptosis has been identified as the primary mechanism for accelerated pancreatic β-cell death in Type 1 diabetes, however the apoptotic pathways in Type 1 diabetes differ from those in Type 2. β-cell failure in Type 1 diabetes may be related to autoimmune-mediated apoptosis, however β-cell death in type 2 diabetes is likely related to increased apoptosis stimulated by chronic hyperlipidemia and hyperglycemia.[272] Interventions directed at reducing rates of β-cell apoptosis may offer future strategies for treatment of patients with Type 2 diabetes. Alterations in apoptosis have also been reported in other physiological abnormalities such as hemorrhage, stroke, sepsis, osteoarthritis, allograft rejection, and inflammation.[246,251,257,273] Understanding the impact of the apoptotic pathways in disease is currently an important stimulus in the search for innovative pharmacological treatments for these maladies.

The mechanism of disease in the presence of insufficient apoptosis is inappropriate cell survival and proliferation. Diseases of insufficient apoptosis include different types of cancer, autoimmune disorders, and viral infections where apoptosis is inhibited in order to preserve the host cell for viral replication.[274] In some cancers, the apoptosis initiator p53 gene is expressed abnormally favoring tumor growth and resistance to treatment. Papilloma virus and adenovirus are suspected of being able to

encode an inhibitory signal into the p53 gene, thus deferring apoptosis. Similarly, the Epstein-Barr virus has been shown to interfere with Bcl-2 gene activity resulting in cancers such as Hodgkin's Lymphoma and post-transplant lymphoma.[275] Cancer researchers have long been aware that many chemotherapeutic drugs exert their effects by promoting apoptosis. Genetic defects, however, in intrinsic cellular self-destruction programs often lead to chemoresistance. The apoptosis-resistant characteristics of some cancers have led to interest in the development of drugs that inactivate anti-apoptotic mechanisms, thus making cancer cells more susceptible to conventional treatments. Attractive targets for drug intervention are genes which express signals that inhibit apoptosis.[246,276]

Anesthesia Drugs and Apoptosis

We may assume that our standard monitoring of patients gives us the means to assess all possible effects of the anesthetic drugs being administered. We may also assume that the effects of anesthetic drugs are completely reversed once they have been removed from the patient or metabolized. We need however, to re-evaluate these assumptions. In the practice of nurse anesthesia, CRNAs administer drugs that affect multiple inter and intra-cellular mechanisms, to include receptor activation and the regulation of genes. Recent laboratory and clinical studies have demonstrated that most anesthetic agents are capable of activating the mechanisms that lead to premature cell death.[247-249] Other researchers have reported potentially beneficial effects of certain anesthetics on apoptotic pathways and related physiological processes.[277-280] A clear understanding of apoptotic pathways will enable the anesthetist to sort through seemingly conflicting research in order to be aware of potential implications stemming from the

effects of anesthesia drugs on this important process.

One area of particular concern is in regard to drug-related disruption of the normal apoptotic pathways. For example, neurotoxic effects have been demonstrated in rodent models as a result of anesthesia drugs administered during the developmental period referred to as synaptogenesis.[249,281,282] Synaptogenesis may be conceived of as a "growth spurt" of the brain synapses that, in humans, takes place from the sixth month of gestation until several years of age.[283] While synapse formation may occur throughout the lifespan of a healthy individual, synaptogenesis in early childhood is characterized by an explosion of activity important to normal neurological development. Anesthetic drugs that stimulate apoptosis in neural tissues and inhibit synaptogenesis, can impair synaptic function, possibly altering neurological development.[282]

Research then has established that drugs used in anesthesia result in widespread apoptotic neurodegeneration in the developing brains of animal models. Although there are undoubtedly multiple mechanisms by which intravenous and inhalation anesthetics drugs affect different cellular receptors, the two principal receptors involved are the N-methyl-d-aspartate (NMDA) receptor and the γ-aminobutyric acid (GABA) receptor. Ketamine and nitrous oxide are two known NMDA antagonists. All inhalation agents, as well as the intravenous barbiturates, propofol, etomidate, and benzodiazepines potentiate GABA receptors.[284] Studies have revealed that the neuroapoptotic effects of anesthetic drugs are both time and dose-dependent, such that greater levels of damage occur at higher doses and over increasing length of exposure. Peak vulnerability to the apoptotic effects of both NMDA antagonists and GABA-mimetics is during the sensitive period of brain growth when the surface area of the brain must be expanded to accommodate new

synaptic connections. This potential for long term neurological injury as a result of anesthesia administration to human infants during this critical developmental period is cause for concern.

Older adults may also be at risk for neuroapoptotic effects from anesthetics. The pro-apoptotic effect of isoflurane has been reported to induce the generation and aggregation of amyloid-ß protein in the brain. Alzheimer disease, the most common form of age-related dementia, is a rapidly growing health problem. Amyloid ß-protein production and accumulation are major pathological hallmarks of this disorder. Given this relationship, isoflurane-induced apoptosis and a subsequent transient increase in the production of amyloid-β protein in the geriatric patient is yet another concern for the anesthetist.[285] Although the preponderance of research has addressed the detrimental effects of anesthesia on neuroapoptosis, the apoptotic pathways in other cell types are also at risk.[286]

In an effort to understand the pro-apoptotic effects of anesthetics on cells, researchers are investigating the role of anesthesia drugs in the formation of reactive oxygen species (ROS). Reactive oxygen species are important regulatory molecules, produced under normal metabolic conditions by aerobic organisms. They have physiologic importance in use by macrophages to disable harmful bacteria by phagocytosis, however they cause cellular and organ damage either when produced endogenously in excess, accumulated from exogenous sources (smoking, pollution) or in the presence of insufficient antioxidant defenses.[102] When the body's antioxidant resources are overwhelmed by ROS, a condition of oxidative stress is said to exist. Injury from oxidative stress results in the lipid peroxidation of cell membranes, often causing

cellular injury or death and related to a wide range of acute and chronic conditions.[168] Inhalation agents have been demonstrated to increase ROS in human tissues, predisposing the patient to a condition of oxidative stress and increased apoptosis.[247,248] One detrimental effect resulting from this is the profound but transient reduction in blood lymphocytes in the immediate postoperative period, resulting in a period of immunosuppression, thus rendering the patient susceptible to localized infection and even sepsis.

Local anesthetics have also been studied for their effects on apoptosis. In-vitro studies have demonstrated the ability of lidocaine, in clinically relevant concentrations, to induce apoptosis via the mitochondrial pathway.[287] Higher concentrations of lidocaine were responsible for cell death by necrosis. This is offered as a possible explanation for lidocaine neurotoxicity. Zink and colleagues reported myotoxic damage related to continuous peripheral nerve blocks with bupivacaine in a pig model. Both apoptotic and necrotic cell death were identified in muscle cells examined after exposure to bupivacaine.[288] Human studies that further define the role of local anesthetics in altered apoptosis are being conducted.

In addition to the potentially harmful aspects of certain anesthetic drugs, some of the agents used in anesthesia, may provide protection against the detrimental pro-apoptotic effects of ROS. Animal studies have demonstrated potential benefits of volatile anesthetics in protecting the myocardium against ischemia-reperfusion injury, representing a possible therapeutic benefit of this phenomenon.[102,289] This activity appears to be mediated via a reduction of ROS in heart tissue by inhalation anesthesia during periods of induced ischemia. These agents also appear to protect myocardial cells

from the apoptosis death-signaling effects of norepinephrine.[277] Researchers, using *in vitro* methods, have also demonstrated the ability of isoflurane to reduce neuronal cell death in cortical cells that were subjected to hypoxic conditions, raising interest in the potential benefits of volatile agents for patients suffering from hypoxic brain injury.[290]

The intravenous anesthetic propofol is receiving considerable attention in studies related to ROS and apoptosis. Propofol, with structural similarities to phenol-based free radical scavengers such as butylated hydroxytoluene (BHT) and α-tocopherol (vitamin E), has well documented antioxidant properties and has been evaluated as a tool for reducing injury from oxidative stress in both animal and human tissues. For example, propofol has been shown to protect lung, heart, hepatic and vascular endothelial tissues from the negative effects of oxidative stress.[278-280] Blood components such as erythrocytes, also obtained antioxidant benefits in studies with propofol.[291] Hence, propofol does have important cellular and nuclear protective effect for cardiac and lung cells as well as other tissues.

Pharmacologic agents used daily in the administration of anesthesia have both favorable and unfavorable properties with regard to apoptosis. With potentially both desirable and detrimental effects of anesthesia drugs on the apoptotic process, the anesthetist is confronted with confusing implications about which pharmacologic techniques may be best in the anesthetic care of patients. Although the volume of research about the effects of anesthetics on apoptosis is growing at a rapid pace, clear recommendations are not yet available to guide the anesthesia provider in the selection of pharmacologic agents in this regard. It appears currently that certain conditions may favor the use of either intravenous propofol or volatile agents, depending on their known

effects on ROS and apoptosis. Controlled human clinical trials to further define the apoptotic and anti-apoptotic effects of anesthetics are needed to clarify the attendant risks versus benefits of our pharmacologic choices. Long-term follow up studies of both pediatric and aged patients would be especially important. In the meantime, a thorough understanding of the pathways of apoptosis will assist anesthesia providers in the correct interpretation of future research and its incorporation into practice. Above all, we must remember that the potent anesthetic agents we administer have multiple cellular effects not currently within our ability to monitor in the operating room.

Summary

The physiologic processes of apoptosis have been reviewed to provide foundational knowledge for nurse anesthetists about this important means by which the body's cells are eliminated. Disturbances of apoptosis are the pathological basis of many human diseases. Diseases frequently encountered in anesthesia practice that are mediated by apoptotic mechanisms were identified. Included among these are diseases of the heart, blood vessels, lungs, and nervous system as well as cancers and immune system disturbances. Nurse anesthetists are encouraged to be aware of the possible effects of anesthetic drugs in altering normal apoptosis. Researchers have identified numerous effects of anesthesia drugs, including local anesthetics, on apoptosis, which may impact patients requiring surgery. Practitioners are encouraged to maintain knowledgeable awareness about anesthesia drugs and inhalation agents that may possess apoptotic or anti-apoptotic properties.

Table 1. Apoptosis and Common Diseases

Excess Cell Death (enhanced apoptosis)	**Inappropriate Cell Survival (diminished apoptosis)**
➢ Cardiovascular Ischemia Myocardial infarction Heart failure Stroke ➢ Respiratory Asthma COPD / Emphysema Interstitial fibrosis ➢ Neurodegenerative Alzheimer disease Parkinson disease ➢ Type 1 and Type 2 diabetes ➢ Human Immunodeficiency Virus ➢ Sepsis	➢ Cancer Chemoresistance ➢ Autoimmunity ➢ Persistent infection

Figure 1. Morphologic Changes Associated with Apoptosis and Necrosis

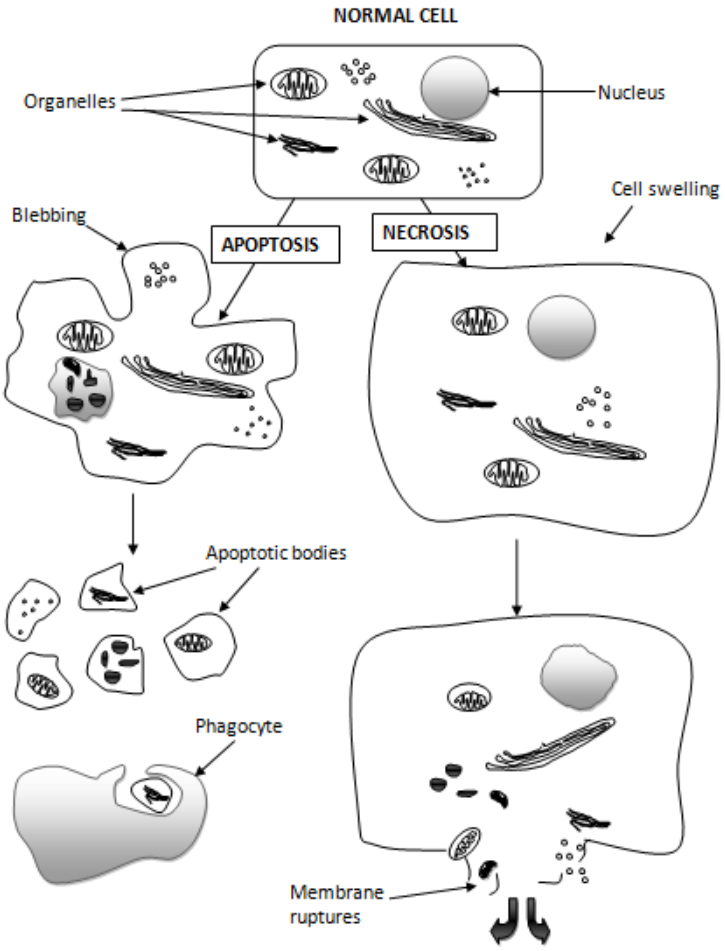
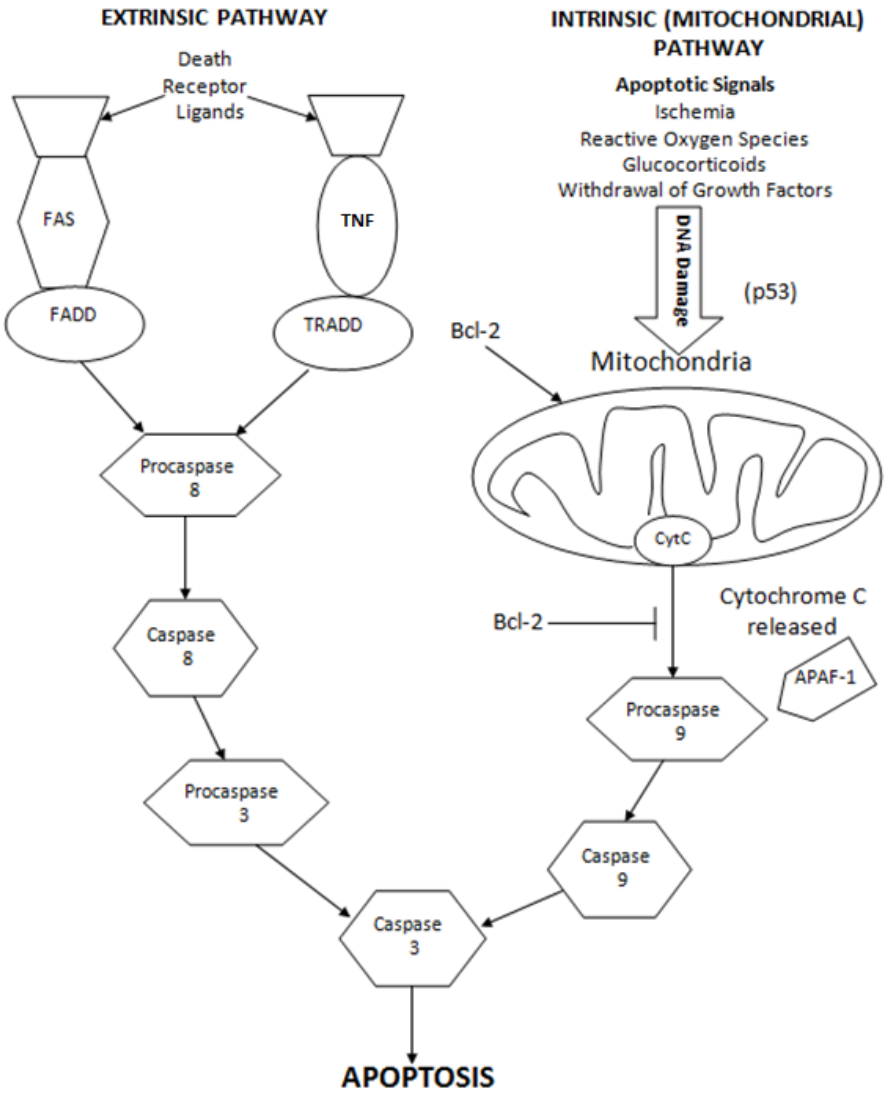


Figure 2. Simplified Diagrams of the Extrinsic and Intrinsic Apoptotic Pathways



TNF indicates tumor necrosis factor; FADD, Fas-associated death domain; TRADD, TNF-associated death domain; CytC, Cytochrome C, APAF-1, Apoptosis activating factor-1.

CHAPTER 4.

Effects of Ubiquinol on Leukocyte Mitochondrial Superoxide, Diaphragm Hydrogen Peroxide and Tissue Apoptosis following Hemorrhagic Shock and Fluid Resuscitation

This manuscript will be submitted for publication to the journal
Shock

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Abstract

With hemorrhagic shock (HS) and fluid resuscitation there is increased generation of reactive oxygen species (ROS) which leads to ischemia-reperfusion injury and organ damage. The aim of this study was to examine the effects of ubiquinol (the reduced form of Coenzyme Q_{10}) administered with fluid resuscitation following controlled HS. Adult male Sprague-Dawley rats were randomly assigned to treatment (ubiquinol 1 mg/100 g body weight) or control (no ubiquinol) groups. Rats were subjected to 60 minutes of HS by removal of 40% of the total blood volume to maintain mean arterial pressure between 45-55 mm Hg. At the end of HS, the animals were resuscitated with infusion of the removed blood and lactated Ringer's (LR) with or without ubiquinol and monitored for 120 minutes. At the end of the experiments, rats were euthanized and lungs, diaphragm, heart and kidneys were harvested immediately. Leukocytes were analyzed for mitochondrial superoxide ($O_2^{\bullet-}$) at baseline, end of the shock period and 120 minutes following fluid resuscitation using MitoSOX Red. Diaphragm tissues were examined for hydrogen peroxide (H_2O_2) using dihydrofluorescein diacetate and confocal microscopy. The percentage of apoptosis in lungs, diaphragm, heart and kidneys was measured using fluorescence microscopy with acridine orange and ethidium bromide. Leukocyte mitochondrial $O_2^{\bullet-}$ levels were significantly lower in rats who received ubiquinol than in the control animals. Production of H_2O_2 and the percent of apoptosis were significantly reduced in the organs of rats treated with ubiquinol. These findings suggest that ubiquinol, administered with fluid resuscitation after HS, attenuates ROS production and apoptosis. Thus, ubiquinol is a potent antioxidant that may be used as a potential treatment to reduce organ injury following hemorrhagic events.

Keywords

Reactive oxygen species, multiple organ failure (MOF), antioxidant, hemorrhage, apoptosis, MitoSOX Red

Introduction

Hemorrhagic shock (HS) is known to produce multiple life-threatening complications and accounts for the highest proportion of deaths in the immediate post injury period.[7] Delayed morbidity and mortality is also a significant problem even after HS has been appropriately treated.[9] Multiple organ failure (MOF) commonly precedes late trauma mortality, resulting in more than 50% of delayed deaths after severe injury.[9,18] Although improved fluid and blood replacement regimens and rapid surgical intervention have reduced the incidence of patients developing MOF, it continues to present challenges to the recovery of injured patients.[18]

Penetrating or blunt trauma can result in hemorrhage and lead to hypovolemic shock. Initially, hypotension elicits reflexes that result in vasoconstriction to increase arterial pressure, but vasoconstriction may precipitate further damage to tissues by exacerbating peripheral hypoperfusion.[15] This results in a hypoxic state where the delivery of oxygen and nutrients to cells does not meet metabolic demands. In profound, untreated HS, hypoxia may lead to irreversible injury and death. Even when timely resuscitation is initiated, cellular hypoxia and reperfusion stimulates a cascade of events leading to the release of inflammatory cytokines,[19] a reduction in the production of adenosine triphosphate (ATP) by the mitochondria and increased production of reactive oxygen species (ROS) capable of inducing oxidative stress.[20] Part of the cascade of ROS formation are the production of superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). Hydrogen peroxide is created from the dismutation of $O_2^{\bullet -}$ and is then converted to highly reactive hydroxyl radicals via Fenton and Haber-Weiss reactions. While not a free radical, H_2O_2 is a mild oxidant also capable of causing cell

damage. Levels of these oxygen species are known to increase following hemorrhagic events[292] and fluid resuscitation therapy.[25,293]

Hemorrhagic shock may result in injury to lungs, diaphragm, heart and kidneys.[22,23,25,294] The severity of lung damage after HS is predictive of patient outcome,[28] although precise mechanisms for this finding are not clear. Inflammatory response and damage from ROS-induced apoptosis are two plausible explanations for the problem of organ injury following HS.[28-30] Increased levels of ROS occurring as the result of ischemia-reperfusion events are known to initiate apoptosis via the mitochondrial pathway involving translocation of cytochrome c through mitochondrial permeability transition pores, resulting in caspase activation.[4,295] Increased ROS-related cell death following HS has led to studies of exogenously administered antioxidants to reduce organ tissue damage.[22,35,37] Ubiquinol (the reduced form of Coenzyme Q_{10}), is an endogenously-produced antioxidant that is also available as a non-prescription supplement. Three animal studies reported the administration of ubiquinone (the oxidized form of Coenzyme Q_{10}) before controlled HS and found that ubiquinone reduced systemic effects on the lungs and heart.[47-49] However, no investigation of the effects of ubiquinol and fluid resuscitation after HS on ROS production or apoptosis has been reported. Thus, the purpose of this study was to examine the effects of ubiquinol administered intravascularly immediately after a 60 minute period of controlled HS, followed by blood and fluid resuscitation, on leukocyte mitochondrial $O_2^{\bullet -}$ production, diaphragm H_2O_2 production and on apoptosis in the lungs, diaphragm, heart and kidneys.

Materials and Methods

Animal Preparation

Male adult Sprague-Dawley rats (n = 20) weighing 320-420 grams were used in these experiments which were conducted according to the procedures approved by the University of Kansas Medical Center (KUMC) Institutional Animal Use and Care Committee. The KUMC animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were ordered in lots of six and allowed to acclimate for at least 48 hours prior to experimentation. Rats were housed in pairs and maintained on 12 hour light/dark cycles. Standard rat chow and water was provided *ad libitum*.

Rats were anesthetized by intraperitoneal injection with sodium pentobarbital (50 mg/kg body weight). Atropine (0.04 mg/100 g body weight) was administered intraperitoneally to reduce respiratory secretions. A rectal probe was inserted to monitor the animal's body temperature and the animal was placed on a thermostat warming pad to maintain its temperature at approximately 37^{o} C. A tracheotomy was performed and the trachea cannulated with polyethylene (PE)-240 tubing and secured with suture. The right carotid artery was isolated and cannulated with a PE-50 catheter pre-flushed with heparinized saline and secured with suture. The carotid catheter was connected to a Harvard Apparatus (Hollister, MI) via fluid transducer and heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressures (MAP) were continuously monitored and recorded every 10 minutes. The left femoral artery was cannulated with a PE-50 catheter and used to withdraw blood to induce HS, administer ubiquinol and for fluid resuscitation. A number 4-French catheter was inserted orally and advanced into the esophagus to monitor respiratory rate (RR).

Experimental Protocol

This was a randomized experimental study with five stages (S1-S5). The timeline for the protocol is presented in Figure 1. At time S1 (baseline) following animal preparation, a 0.2 mL blood specimen was collected from the femoral artery catheter to measure baseline arterial blood values and leukocyte mitochondrial $O_2^{\bullet-}$. Arterial blood values were measured with an i-Stat Blood Analyzer (Abbott Laboratories, Abbott Park, IL) using an EG 6+ cartridge. At S2 (shock) approximately 40% of the total blood volume (approximately 10 mL) was removed via the femoral artery catheter to achieve a MAP of 45-55 mmHg which we maintained for 60 minutes. Removed blood was stored in a syringe flushed with 1:1,000U heparin. At the end of S2, 0.2 mL of blood was removed for arterial blood values and leukocyte mitochondrial $O_2^{\bullet-}$ analysis following HS.

At S3 fluid resuscitation was started, which included reinfusion of the removed blood and LR (twice the amount of the blood withdrawn) with or without ubiquinol (1 mg/100 g body weight) (QH Liposomal Ubiquinol, Tishcon Corporation, Westbury, NY). This stage lasted for 10 minutes. Stage S4 was a 120 minute period of post-fluid resuscitation during which SBP, DBP, MAP, HR, RR and rectal temperatures were monitored continuously and recorded every 10 minutes. During S5 (treatment) a final arterial specimen of 0.2 ml was drawn for blood values and leukocyte $O_2^{\bullet-}$ analysis following fluid resuscitation. The animal was then euthanized with sodium pentobarbital (150 mg/kg body weight) and the lungs, diaphragm, heart and kidneys were rapidly excised for analysis of H_2O_2 and apoptosis.

Leukocyte Mitochondrial Superoxide Protocol

Leukocyte mitochondrial $O_2^{\bullet-}$ was measured by mean fluorescent intensity (MFI)

of MitoSOX Red (Invitrogen, Carlsbad, CA) using flow cytometry (n = 20). MitoSOX Red is a mitochondrial-specific fluorescent probe that is oxidized by $O_2^{\bullet -}$ to form 2-hydroxyethidium. Whole blood samples were collected and analyzed at baseline (S1), shock (the end of S2) and treatment (S5). Three specimens for each time period were prepared and analyzed and the mean of the three measures of fluorescence was recorded for statistical analysis. All specimens were processed in a darkened environment to minimize ambient light interference with the fluorescent stains. For each sample, 20 μL of rat blood was added to 2 μL of leukocyte-specific monoclonal antibody CD45 (Alexa-Fluor-647, BioLegend, San Diego, CA). To the sample, 200 μL of 1X phosphate buffered solution (PBS) was added and the specimen vortexed for 15 seconds and incubated at 37^o C for 5 minutes. MitoSOX Red was reconstituted for each experiment with 13 μL dimethylsulfoxide (DMSO) to make a 5 mM stock solution. This was further diluted with Hank's balanced salt solution with calcium and magnesium (HyClone, Ogden, UT) to make a 5 μM MitoSOX Red solution. Following incubation 1 mL of the diluted MitoSOX Red solution was mixed thoroughly with each specimen and incubated for an additional 30 min at 37^o C. Next, 1 mL PBS was added to the specimen, mixed and then centrifuged at 1000 rpm for 5 minutes. Supernatant was aspirated from the specimen and discarded and the remaining pellet was re-suspended with 1 mL PBS. Specimens were analyzed using a flow cytometer (FACSCalibur LSR II, BD Biosciences, San Jose, CA) with FACS DIVA software gated for leukocyte analysis. The flow cytometer was calibrated daily prior to experimentation. Evaluation of MitoSOX Red fluorescence was measured with laser excitation at 488 nm and emission filter at 576 nm. Results are reported in arbitrary units as mean fluorescence intensity (MFI) for each specimen.

Diaphragm Hydrogen Peroxide Protocol

The presence of H_2O_2 in diaphragm tissue was measured by dihydrofluorescein diacetate (Hfluor-DA) using confocal microscopy. Hfluor-DA is oxidized to fluorescein by H_2O_2.[296] After harvesting, segments of both hemi-diaphragms (n = 16) were immediately placed in Krebs-Ringer solution, stretched with pins and loaded with Hfluor-DA for 30 minutes. After loading with Hfluor-DA, three strips were mounted on slides to measure MFI by laser confocal microscopy (Nikon TE 2000-U) using excitation 488 nm/emission 529 nm. Three consecutive images (~ 0.04 mm^2 of tissue) were obtained from each slide and a total of nine images were captured**.** Fluorescence intensity was directly proportional to the amount of H_2O_2 present in the tissue and was quantified by computer software (Metamorph V 7.1.6). Confocal microscope settings were: 1) magnification: 100X, 2) pixel dwell: 3.36 μsec, 3) fluorescence intensity: 8.15 pmt green, 4) pinhole: medium and 5) passes: 4 (average).

Apoptosis Protocol

Apoptosis was measured in the lungs, diaphragm, heart and kidney cortex (n = 20 for each organ) using fluorescent microscopy with differential dye uptake. The animal was euthanized, the organs were excised and the tissues minced into small pieces and added to a solution containing 2.5 mL Krebs solution, 200 μL antioxidant solutions and 300 μL each of collagenase and trypsin. The mixtures were vortexed for 30 seconds and incubated at 37^o C for 30 minutes with additional vortexing every 5 minutes. At the end of incubation, the supernatant from each specimen was transferred to a centrifuge tube and centrifuged for 30 minutes at 6000 rpm. Supernatant was discarded and the pellet re-suspended in 2 mL Krebs solution. A 250 mL aliquot was added to a tube containing 2

µL ethidium bromide (EB) (Sigma-Aldrich, St. Louis, MO; 10 mg/mL diluted with PBS for a final concentration of 0.1 mg/L) and 2 µL acridine orange (AO) (Sigma-Aldrich, St. Louis, MO; 10 mg/mL diluted with PBS for a final concentration of 0.1 mg/L). Acridine orange stain viable nuclei green while EB stain apoptotic nuclei orange.[297] Fluorescent microscopy (Nikon Eclipse TE 2000S, Melville NY) was employed at 400X to measure deoxyribonucleic acid (DNA) damage, an index of apoptosis. The images were analyzed with Boyce Scientific Analysis®, (St Louis, MO) software which reduces error by measuring fluorescent hue values for each nucleus, allowing for determination of relative amounts of each dye.[116] Approximately 300 nuclei from each specimen were analyzed, based on our prior experience that a minimum of 200 nuclei was needed.

Statistical Analysis

All data are presented as mean ± SEM. Statistical equivalence between groups for weight and total blood removed were ensured using non-parametric tests (Mann-Whitney U). Hemodynamic measures (SBP, DBP, MAP, HR and RR) and all arterial blood value data were compared with Mann-Whitney U for between group data and related samples for within group comparisons over time. Data for differences between groups in the leukocyte $O_2^{\bullet -}$, H_2O_2 and apoptosis studies were likewise tested using the Mann-Whitney U. Statistical significance for all tests was defined as $p \leq 0.05$. Statistical analyses were performed using Statistical Software Package for the Social Sciences, version 20.0 (SPSS, Inc., Chicago, Illinois).

Results

Hemodynamics, Respirations and Total Blood Removed

Total blood removed, hemodynamic and respiratory data are presented in Table 1.

There were no significant differences between the control and treatment groups with regard to total volume of blood removed to induce HS. There were also no significant differences between the groups for measures of SBP, DBP, MAP, HR or RR. The hemodynamic status of the two groups was comparable at the baseline (S1), shock (end of S2), and treatment (end of S4). Significant differences were observed within the groups relative to these three time periods. Blood pressures were significantly decreased at shock from baseline ($p < 0.001$) and recovered after fluid resuscitation in both groups. This confirms the intended hypotensive responses to HS with recovery after blood and fluid resuscitation. Heart rate and RR did not change significantly in either group between experimental stages.

Arterial Blood Values

Table 2 summarizes arterial blood values for both groups at the baseline, shock and treatment stages. There were no significant differences in pH, arterial carbon dioxide ($PaCO_2$), arterial oxygen (PaO_2), oxygen saturation (SaO_2), hematocrit (Hct) or hemoglobin (Hgb) between the control and treatment groups for any of the experimental stages ($p > 0.05$). Significant differences were found within groups with decreased $PaCO_2$ ($p < 0.01$) and Hct and Hgb ($p < 0.01$) at shock compared to baseline and significantly increased PaO_2 ($p \leq 0.01$) and SaO_2 ($p < 0.05$). These changes were consistent with the induced hemorrhage, hemodilution from fluid resuscitation and a compensatory increase in tidal ventilation related to the anticipated development of lactic acidosis during HS.

Leukocyte Mitochondrial Superoxide Production

In Table 3 are the MFI of MitoSOX Red for leukocyte $O_2^{\bullet -}$ for both groups. No significant difference in MFI was found between the control and treatment groups at

baseline or shock ($p > 0.05$). However, MFI at the end of S4 was significantly less in the ubiquinol group than control ($p < 0.001$). Within-group results indicated that levels of mitochondrial $O_2^{\bullet-}$ increased significantly in the control group between baseline and shock and between baseline and treatment. An upward trend was also noted in controls in $O_2^{\bullet-}$ levels between shock and treatment however, this did not reach statistical significance. In the ubiquinol group, $O_2^{\bullet-}$ levels were significantly higher at shock than at baseline, but then declined significantly between shock and treatment periods. In treatment animals, fluorescence after treatment was also lower than baseline measures, which may have been elevated from stress incurred during anesthesia and surgical preparation.

Diaphragm Hydrogen Peroxide

Results of diaphragm H_2O_2 measured by the MFI of Hfluor-DA are illustrated in Figure 2. The MFI of Hfluor-DA in the control group (23,513 ± 5098) was significantly more than five times higher than in the treatment group (4,193 ± 333, $p < 0.001$). Figure 3 is an example of H_2O_2 fluorescent images of diaphragm tissue for both control and treatment groups captured by confocal microscopy. The diaphragm image of the control rat has significantly brighter with greater green fluorescence than that of the diaphragm from the ubiquinol-treated rat.

Apoptosis Studies

Figure 4 shows the percent apoptosis in lungs, diaphragm, heart and kidneys with and without the administration of ubiquinol. Rats treated with ubiquinol had significantly less apoptotic nuclei ($p < 0.001$) than the controls in the lungs (6.0% ± 0.72 versus 39.2% ± 1.06), diaphragm (4.7% ± 0.46 versus 30.6% ± 2.37), heart (2.9% ± 0.57 versus 23.5%

± 1.32) and the kidneys (2.4% ± 0.27 versus 42.1% ± 1.91).

Discussion

In this study, we investigated the effects of ubiquinol administered following 60 minutes of HS, on the production of mitochondrial $O_2^{\bullet -}$ by leukocytes, the production of H_2O_2 in diaphragm tissue and on apoptosis of the lungs, diaphragm, heart and kidneys. There was significantly less leukocyte mitochondrial $O_2^{\bullet -}$ in the treatment group that received ubiquinol. Similarly, significantly less H_2O_2 in the diaphragm was also found in the treatment group. Examination of lung, diaphragm, heart and kidney nuclei using fluorescence microscopy revealed that apoptosis was substantially diminished in ubiquinol-treated rats suggesting that ubiquinol may offer protection against HS-induced organ injury.

Antioxidants have been used to minimize oxidative damage in a variety of diseases. Multiple compounds with antioxidant characteristics have been shown to reduce the negative effects of ischemia and reperfusion injury following HS and fluid resuscitation. These include dopamine,[25] α-lipoic acid,[32] curcumin,[42] and N-acetylcysteine.[298] In our study, we examined the effects of ubiquinol, a free radical scavenger and potent antioxidant known to inhibit lipid peroxidation.[82] Ubiquinol (or the oxidized form ubiquinone) has also been found to maintain mitochondrial membrane potential, stabilize mitochondrial permeability transition pores (MPTP) and limit the activation of apoptosis.[75,76,78,80] Ubiquinones have been applied therapeutically for use in atherogenesis,[97] endothelial dysfunction,[99] hypertension,[98] cardiac disorders[78] and ischemia-reperfusion injury.[100,101] Protective properties of ubiquinone administration have also been shown to attenuate oxidative injury from type II diabetes on kidneys[103,104]

and reduce tissue damage in renal transplantation and lung reperfusion.[110] Three animal studies have reported the use of ubiquinone in HS.[47-49] Aoyagi administered ubiquinone intravenously prior to inducing one hour of HS and found that treated dogs recovered cardiac output and urinary output significantly better than animals that did not receive treatment.[49] Hatano also evaluated the effects of ubiquinone on HS and reported that treatment did not influence coagulation but did suppress fibrinolysis.[48] Yamada pretreated dogs with ubiquinone 60 minutes prior to HS and observed that ubiquinone treatment decreased airway pressures, improved lung compliance and reduced the levels of plasma pyruvate, lactate, histamine, catecholamines and leukotriene C_4.[47] There have been no reported studies of ubiquinol on the generation of ROS or effects on the apoptotic mechanisms following HS and fluid resuscitation. Our findings support the earlier studies that CoQ_{10} is a potent antioxidant that protects oxidative phosphorylation and ATP-generation of the mitochondria. All three previous studies that have investigated ubiquinone found beneficial effects when it was administered prior to HS. We also found benefits when ubiquinol was administered after HS had already been induced.

Hemorrhagic shock produces cellular hypoxia that initiates a cascade of events leading to the release of inflammatory cytokines,[19] reduced production of ATP by the mitochondria and increased production of the oxygen intermediates $O_2^{\bullet -}$ and subsequently H_2O_2 and $OH^{\bullet}$,[20] leading to cellular injury.[61] In addition to the production of ROS by mitochondria, NADPH oxidases in polymorphonuclear neutrophils (PMN) membranes and vascular endothelium are also major producers of ROS.[122] With resuscitation and reperfusion, cytokines are released into the mesentery from the ischemic gut and enter the vascular circulation via the mesenteric lymph system. Neutrophils are primed and migrate

to organs including the lungs, heart and kidneys, and cause direct local cytotoxic injury by the release of ROS and pro-inflammatory mediators.[9] Kolamunne et al found under hypoxic conditions that ROS is increased from cardiac mitochondria.[299] They found that the generation of ROS during hypoxia disrupts mitochondrial homeostasis and membrane permeability. This leads to mitochondrial complexes I and III being fixed in a reduced state, decreasing the ability of the mitochondria to reduce ubiquinone to ubiquinol. Similar to Kolamunne et al, we found that hypoxia, induced by HS, increased ROS production as measured by MitoSOX Red. However, with the administration of ubiquinol following HS, mitochondrial levels of $O_2^{\bullet-}$ were lowered, which is likely from ubiquinol maintaining both mitochondrial membrane integrity and the activities of the complexes in the ETC.

In reperfusion injury, inflammatory pathways are responsible for cellular injury by leukocytes that release ROS and inflammatory mediators. Early in reperfusion, endothelial cells swell, lose their attachment to the basement membrane and adhere to the infiltrated leukocytes secondary to an inflammatory response. The most common site of damage is in vascular endothelium resulting in fluid leakage from the vessels. These changes, accompanied by the increase in ROS production are part of a complex relationship that lead to cellular apoptosis.[61-64] When ischemia occurs longer than 60 minutes, hypoxanthine is produced from ATP damage. With fluid resuscitation, blood flow is restored and cellular oxygen increases. The additional oxygen results in the production of xanthine dehydrogenase which results in the increased production of $O_2^{\bullet-}$ and H_2O_2.[300] In our study, when we restored blood flow with fluid resuscitation (blood and LR), oxygen was reintroduced to hypoxic cells, which increased the production of

free radicals that would damage plasma membranes, proteins and DNA. At 120 minutes after fluid resuscitation without ubiquinol, we found an increase in leukocyte mitochondrial $O_2^{\bullet-}$ as well as an increase in diaphragm H_2O_2. However, when ubiquinol was added to the fluid resuscitation regimen, ubiquinol scavenged ROS which resulted in lower levels of $O_2^{\bullet-}$ and H_2O_2. To examine vascular endothelial damage, we are currently conducting studies of the mesenteric microcirculation to evaluate the effects of ubiquinol administration after HS on leukocyte adherence and vascular permeability.

The increased production of ROS-activated phagocytes has been shown to be a mediator of apoptotic and necrotic organ injury, especially in the lungs.[124,301] We used the fluorescent probe MitoSOX Red to determine the levels of $O_2^{\bullet-}$ produced in leukocytes following fluid resuscitation. MitoSOX Red was used to determine if $O_2^{\bullet-}$ could be a potential biomarker to measure the effectiveness of ubiquinol administered prior to fluid resuscitation. It is well known that $O_2^{\bullet-}$ is produced in leukocytes via membrane-bound NADPH oxidase which catalyzes the production of superoxide in oxidative "bursts" from oxygen and NADPH.[190] However, the role of leukocyte mitochondria in $O_2^{\bullet-}$ production is not as clear. There is evidence that mitochondria in leukocytes participate in the generation of $O_2^{\bullet-}$.[302] MitoSOX Red is useful in detecting intra-mitochondrial $O_2^{\bullet-}$ as the result of an added cationic triphenylphosphonium group to facilitate preferential diffusion of the dye into the mitochondrial matrix. MitoSOX Red has been used to detect mitochondrial $O_2^{\bullet-}$ production in tissue cells.[209,210] However, only one study was identified in which MitoSOX Red was used to evaluate the mitochondrial production of $O_2^{\bullet-}$ in leukocytes.[303] In that study, Syu et al used MitoSOX Red to examine the effects of chronic exercise on ROS production and apoptosis in neutrophils following acute

strenuous exercise. In the present investigation, MitoSOX Red appeared to be able to measure the decrease in leukocyte mitochondrial $O_2^{\bullet-}$ when ubiquinol was administered following HS. The MFI of MitoSOX Red in the treatment group declined significantly 120 minutes after shock while the MFI for the control group continued to increase. We measured significantly lower levels of $O_2^{\bullet-}$ following fluid resuscitation in the ubiquinol group compared to the control group which suggests that ubiquinol is scavenging leukocyte mitochondrial $O_2^{\bullet-}$ following HS and fluid resuscitation. This may relate to organ survival by attenuating the activation of apoptosis.

Hydrogen peroxide is a mild oxidant involved in cell physiology and signaling.[150,237] It has also been implicated as a factor in aging, disease and cell toxicity[150] and is associated with detrimental effects to lung, heart and diaphragm function.[304-306] Reactive oxygen species are generated during reoxygenation following hypoxia in the diaphragm and have been observed to reduce the contractility and produce respiratory dysfunction.[296] Investigators have measured the production of H_2O_2 in tissues using the fluorescent probe dihydrofluorescein-DA (Hfluor-DA).[25,296] Hfluor-DA permeates the diaphragm and the unoxidized, unesterfied form, reacts with H_2O_2 to convert Hfluor to fluorescein. In previous studies, Hfluor demonstrated superior ability to detect H_2O_2 in both in mitochondria and in cell-free environments compared with other fluorescent probes[232,233] and has been demonstrated to be useful in measuring levels of H_2O_2 in diaphragm.[25] Pierce et al found that dopamine, which is an antioxidant, was effective in decreasing the level of diaphragm H_2O_2 in rats post-HS.[25] In this study, we also found significantly less diaphragm H_2O_2 in rats treated with ubiquinol compared to controls. The potential benefit of using ubiquinol following HS and fluid resuscitation would be to

reduce ROS in the diaphragm which would improve contractile function and potentially lower the risk of respiratory impairment.

A relationship between increased ROS and the development of apoptosis is well established.[124,307] Hemorrhagic shock and reperfusion injury has been associated with organ damage and apoptosis.[308] This may be related to an up-regulation of genes that affect oxidation and apoptosis.[309] Studies show that the administration of antioxidants is effective in reducing apoptosis following HS in animal models.[43,310] Other investigators have observed that production of H_2O_2 coincided with antioxidant depletion in cells undergoing apoptosis.[311] Following hypoxia-reperfusion events, elevated levels of ROS in organ tissues cause mitochondrial damage, initiating apoptosis which results in organ damage and failure during the later stages of HS.[21,312] Specifically, ROS precipitate damage to mitochondrial DNA leading to increased membrane permeability, loss of transmembrane potential and translocation of cytochrome c with activation of caspase signaling characteristic of the mitochondrial apoptotic pathway.[122] Kolamunne et al observed an increase in mitochondrial $O_2^{\bullet-}$ in cardiac myoblasts following a period of hypoxia which resulted in a significant increase in apoptosis.[299] They concluded that the increased exposure to $O_2^{\bullet-}$ was the major cause of cell death. In this investigation, mitochondrial $O_2^{\bullet-}$ in the ubiquinol group did not significantly increase and the percent of apoptosis remained low. Thus, similarly to Kolamunne's findings, the levels of $O_2^{\bullet-}$ corresponded to the levels of apoptosis.

Ubiquinone has been observed to reduce ROS activation of the apoptotic caspase cascade in neuronal tissues[313,314] and keratocytes.[4] Ubiquinol is a potent scavenger of ROS[315] that exhibits protective functions including reduction of DNA damage,

protection of lipid membranes and prevention of opening of MPTPs in the apoptosis mitochondrial signaling pathway.[4,85] In our study, administering ubiquinol at the beginning of fluid resuscitation was highly effective in reducing apoptosis in lungs, diaphragm, heart and kidneys. We suggest that this is related to the antioxidant effects of ubiquinol which limited the production of ROS, both in leukocytes as well as in tissues, thereby attenuating the activation of the apoptotic pathways.

Conclusions

In conclusion, this study demonstrates for the first time that ubiquinol attenuates damage to the lungs, diaphragm, heart and kidneys following HS and fluid resuscitation in rats. We provide evidence that ubiquinol: 1) reduces mitochondrial leukocyte $O_2^{\bullet-}$, 2) decreases diaphragm H_2O_2 and 3) diminishes DNA damage in tissue. The benefits of ubiquinol administration in either reducing the production of ROS from the mitochondria or scavenging of ROS suggests that it may be used as a supplemental treatment for hemorrhage and resuscitation injury. Future research should be conducted to examine the effects of ubiquinol following HS related to stabilization of the MPTP and ATP production. Translational studies using ubiquinol could be performed in cardiopulmonary bypass and organ transplantation patients to investigate its usefulness in reducing reperfusion injury. Our results support that ubiquinol may be useful in reducing the complications from HS.

Table 1. Comparison of Mean Total Blood Removed, Hemodynamic and Respiratory Measures for the Control and Ubiquinol Groups

	Control	Ubiquinol
Total blood removed (mL)	10.0 ± 0.8	10.9 ± 0.4
Baseline		
SBP	168 ± 6	168 ± 6
DBP	134 ± 5	130 ± 4
MAP	149 ± 5	148 ± 4
HR	375 ± 10	356 ± 15
RR	75 ± 4	86 ± 4
Shock		
SBP	64 ± 6†	63 ± 3†
DBP	40 ± 3†	38 ± 2†
MAP	49 ± 2†	48 ± 3†
HR	376 ± 13	359 ± 15
RR	74 ± 5	77 ± 6
Treatment		
SBP	132 ± 8†*	145 ± 5†*
DBP	98 ± 8†*	96 ± 7†*
MAP	114 ± 7†*	118 ± 7†*
HR	389 ± 11	379 ±14
RR	80 ± 4	80 ± 5

Data are presented as mean ± SEM; n = 10 per group. SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial blood pressure, HR: heart rate, RR: respiratory rate.
† $p < 0.05$ in comparison with baseline
* $p < 0.05$ in comparison with shock

Table 2. Comparison of Mean Arterial Blood Values for the Control and Ubiquinol Groups

	Control	Ubiquinol
Baseline		
pH	7.44 ± 0.01	7.43 ± 0.01
$PaCO_2$ (mm Hg)	42.4 ± 1.2	39.3 ± 1.1
PaO_2 (mm Hg)	74.3 ± 2.4	79.7 ± 2.1
SaO_2 (%)	94.8 ± 0.6	95.0 ± 0.4
Hct	41.3 ± 0.9	39.9 ± 1.4
Hgb (g/100 mL)	14.1 ± 0.3	13.7 ± 0.4
Shock		
pH	7.40 ± 0.04	7.45 ± 0.05
$PaCO_2$	23.9 ± 2.5†	24.2 ± 2.0†
PaO_2	96.8 ± 10.1†	91.0 ± 1.8†
SaO_2	97.2 ± 0.6†	97.5 ± 0.6†
Hct	19.7 ± 1.1†	20.8 ± 1.4†
Hgb	6.8 ± 0.3†	6.8 ± 0.4†
Treatment		
pH	7.43 ± 0.02	7.44 ± 0.01
$PaCO_2$	29.1 ± 1.9†*	31.7 ± 2.5†*
PaO_2	76.9 ± 5.0	77.6 ± 4.2*
SaO_2	93.9 ± 2.6	94.6 ± 1.0*
Hct	34.4 ± 2.6†*	33.3 ± 2.3†*
Hgb	11.7 ± 0.9†*	11.8 ± 0.6†*

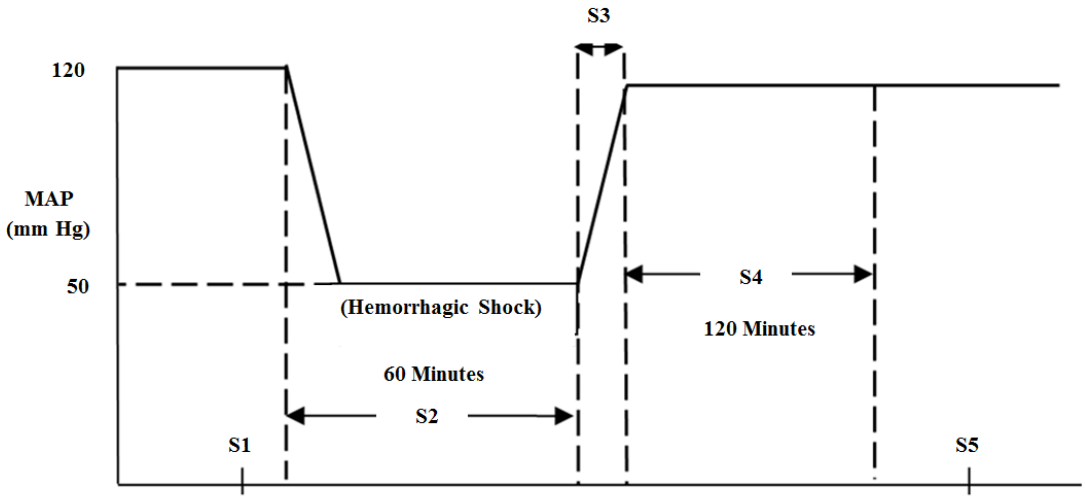
Data are presented as mean ± SEM; n = 10 per group. $PaCO_2$: partial pressure of carbon dioxide, PaO2: partial pressure of oxygen, SaO_2: oxygen saturation percent, Hct: hematocrit, Hgb: hemoglobin.
† $p \leq 0.05$ in comparison with baseline
* $p < 0.05$ in comparison with shock

Table 3. Comparison of MitoSOX Red Mean Fluorescence Intensity (MFI) Measured by Flow Cytometry for the Control and Ubiquinol Groups

	Control	Ubiquinol
Baseline	5653.5 ± 306.2	5617.5 ± 242.3
Shock	6593.0 ± 377.8†	6491.1 ± 265.8†
Treatment	7227.9 ± 534.5†	4687.2 ± 265.4‡†*
Difference (Shock - Treatment)	- 634.9 ± 401.2	1803.9 ± 389.4‡

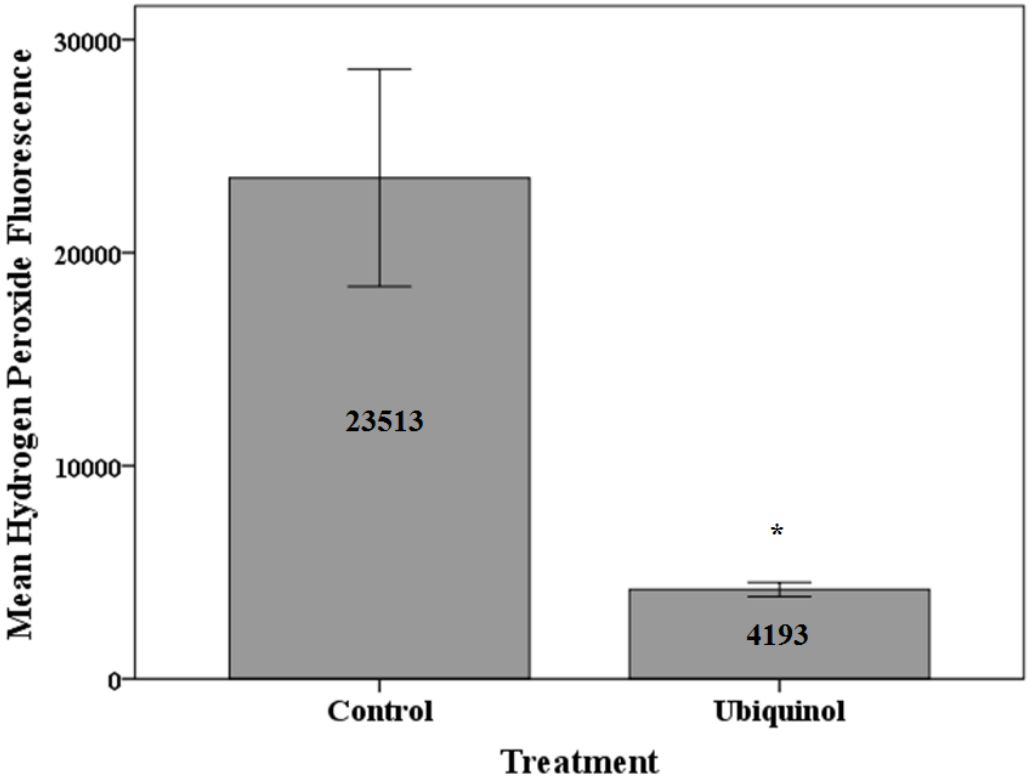
Data are presented as mean ± SEM; n = 10 per group.
† $p < 0.05$ in comparison with baseline
* $p < 0.01$ in comparison with shock
‡ $p < 0.05$ in comparison with control group

Figure 1. Experimental Stages



Time Periods S1: baseline; S2: 60 minutes of HS; S3: resuscitation with/without ubiquinol and with blood and LR; S4: Post-treatment monitoring for 120 minutes; S5: completion of experiment

Figure 2. Diaphragm Mean Fluorescent Intensity (MFI) of Hfluor for Hydrogen Peroxide in Control and Ubiquinol Groups



* = significantly different from control ($p < 0.05$).
Data are presented as mean ± SEM, n = 8 per group

Figure 3. Examples of Hydrogen Peroxide Fluorescence in Diaphragms using Confocal Microscopy for Control and Ubiquinol Groups

Control **Ubiquinol**

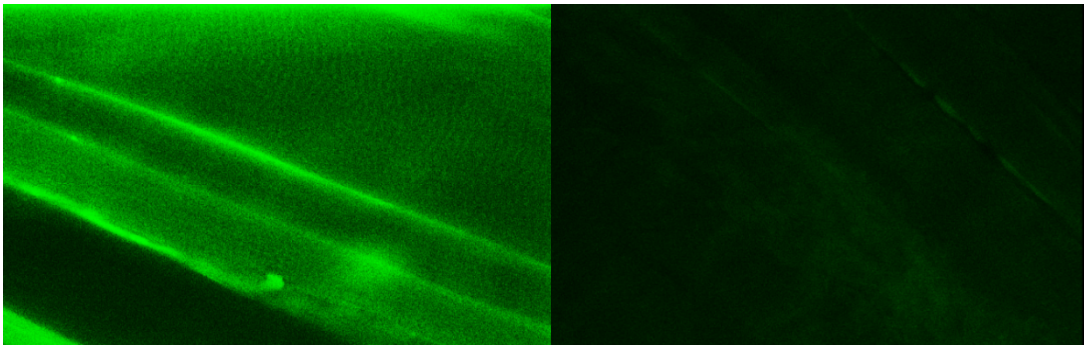
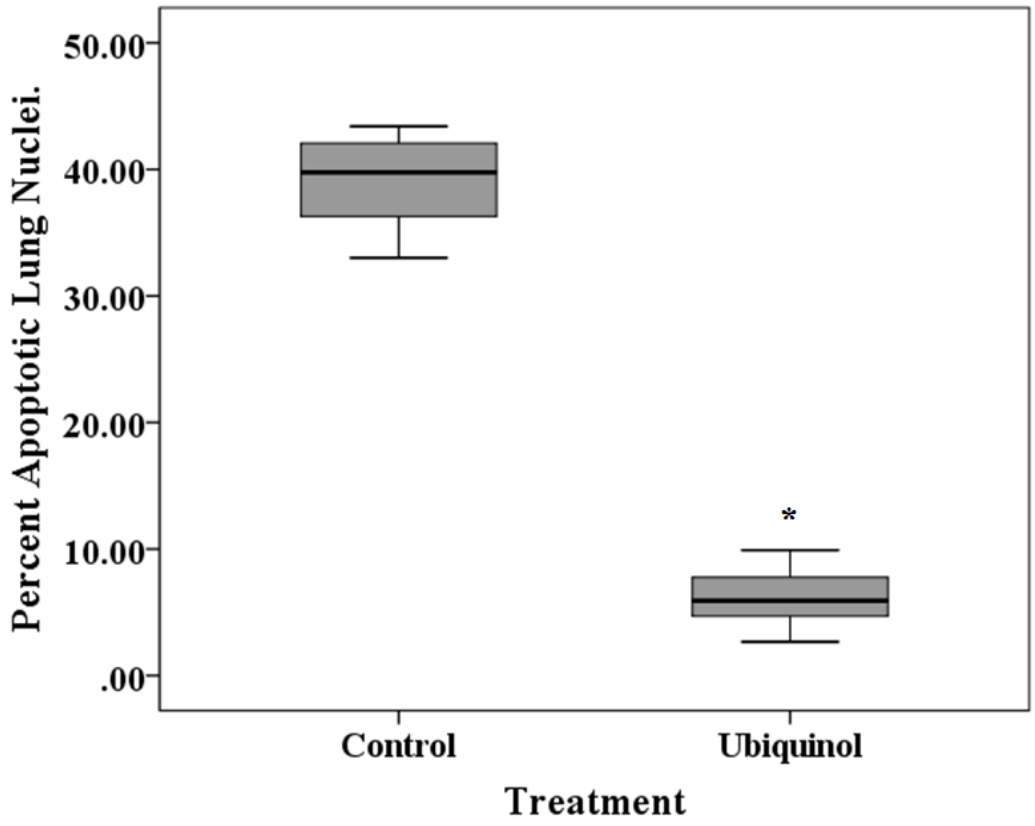
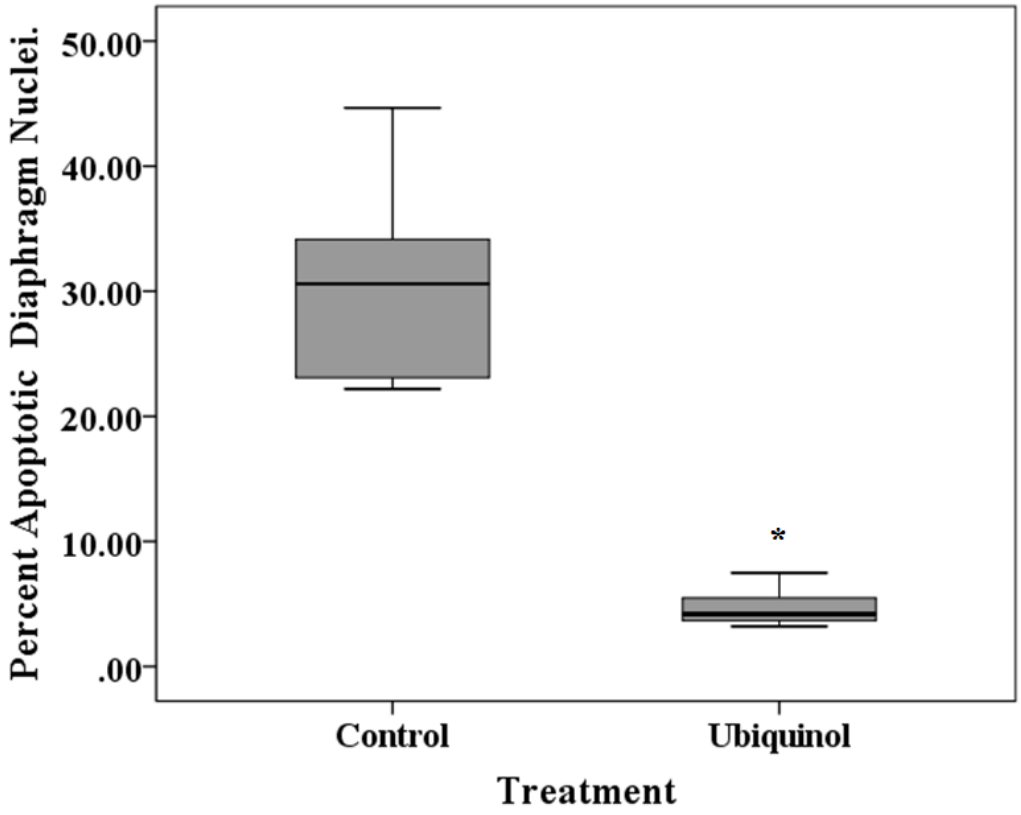


Figure 4. Percent Apoptosis of Lung Nuclei for Control and Ubiquinol Groups



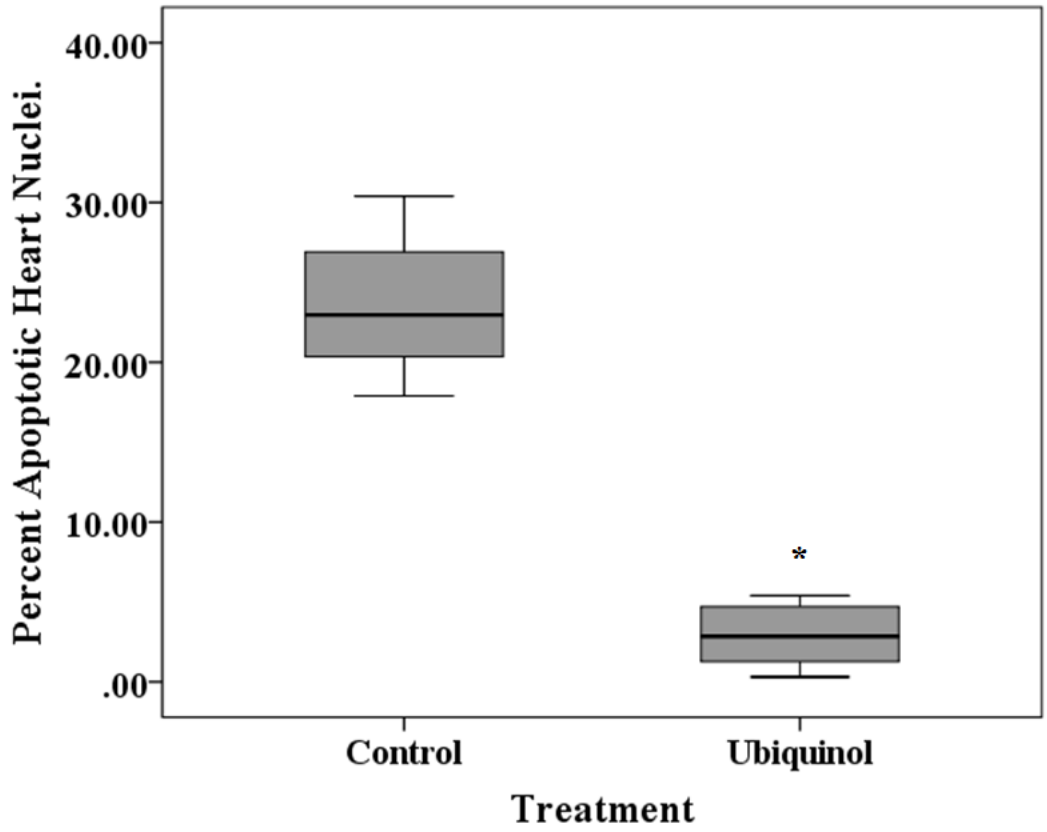
* = significantly different from control ($p < 0.05$).
n = 10 per group

Figure 5. Percent Apoptosis of Diaphragm Nuclei for Control and Ubiquinol Groups



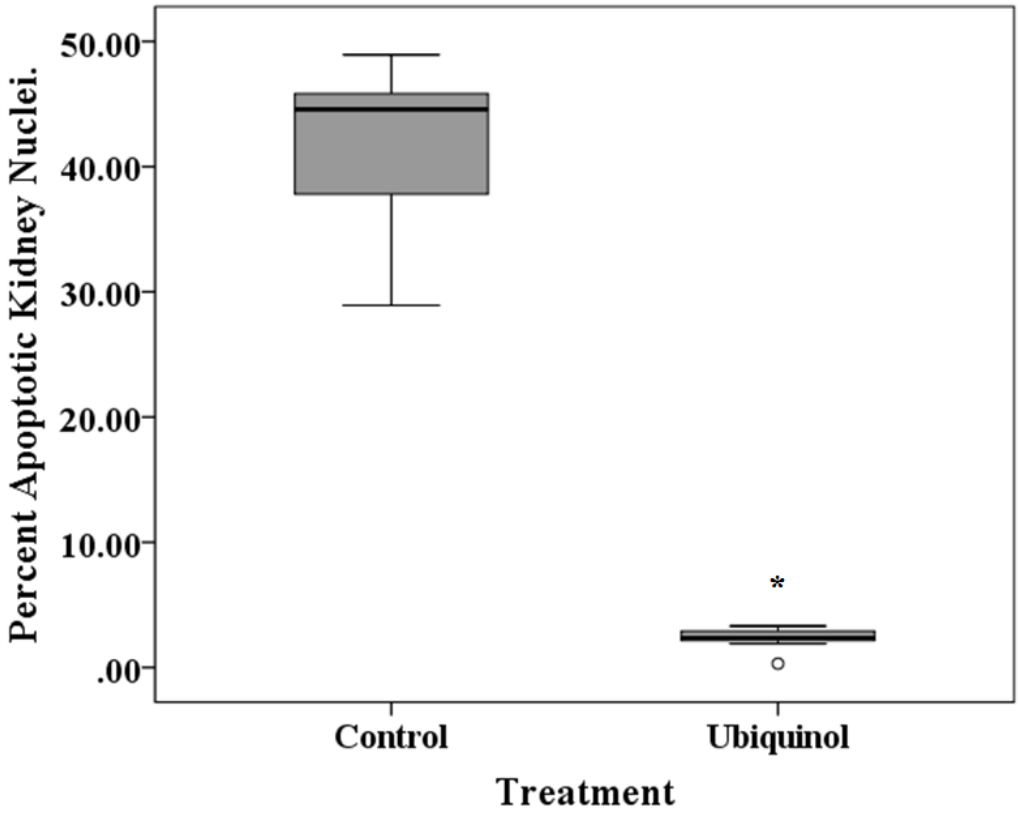
* = significantly different from control ($p < 0.05$).
n = 10 per group

Figure 6. Percent Apoptosis of Heart Nuclei for Control and Ubiquinol Groups



* = significantly different from control ($p < 0.05$).
n = 10 per group

Figure 7. Percent Apoptosis of Kidney Nuclei for Control and Ubiquinol Groups



* = significantly different from control ($p < 0.05$).
n = 10 per group

CHAPTER 5.

Discussion and Conclusions

Chapters 1, 2 and 3 provided the scientific foundation for the hypotheses that were tested and presented in Chapter 4. In this chapter, there will be a summary of the information presented in Chapters 1 through 4. Each hypothesis will be restated followed by a discussion of the results as well as the implications of our findings for clinical practice. The limitations of this research will be examined and recommendations suggested for future investigations.

In Chapter 1, HS was introduced as a life-threatening problem that affects victims of trauma in both military and civilian settings. For our investigation, we employed an ischemia-reperfusion model of HS where an increased production of ROS occurred in both leukocytes and organ tissues. This was the result of hypotension and tissue hypoxia during the acute phases of HS as well as a response to reperfusion from fluid resuscitation.[17,316]

Based on this model, we explored the therapeutic use of an antioxidant, ubiquinol, following HS as a possible approach to reduce ROS production and oxidative stress. In the literature, we found that a variety of compounds with antioxidant properties had been evaluated in HS research. One antioxidant that has received only minimal evaluation for use in HS is ubiquinone. This is an endogenously-produced molecule that functions in mitochondrial oxygen metabolism in the production of ATP. We identified only three studies that have investigated the use of ubiquinone in HS. These investigators did not associate their findings with ROS or oxidative stress and administered the ubiquinone before HS was induced.[47-49] No investigators have reported the use of ubiquinol in the treatment of HS. Ubiquinol has significant antioxidant properties and has been evaluated as a therapeutic adjunct in cardiovascular,[76] pulmonary,[50] renal [103] and neurological

disorders[75] but not in the treatment of HS.

In Chapter 1 there is a review of ROS production in mitochondria and the role of ubiquinol in the electron transport chain (ETC) where ATP is produced. In this chapter, a discussion was presented of how ubiquinol facilitates electron transport in the ETC, how it is a potent free-radical scavenger and is involved in other cellular mechanisms that may be protective in ROS-related cell damage.[4,85] Based on this review, a problem statement was developed focusing on gaps in the literature concerning the use of ubiquinol as a treatment for HS within an oxidative stress/ischemia-reperfusion framework. The aim of the study, hypotheses and conceptual definitions for this dissertation were presented. At the end of Chapter 1, a study conceptual design was presented and assumptions and anticipated limitations of the study were addressed. In the conceptual design, the four experimental hypotheses were incorporated according to the pathway that linked HS, hypoxia and reperfusion injury to an increase in production of $O_2^{\bullet-}$, H_2O_2, oxidative stress and percent of apoptosis. The design also includes ubiquinol as an intervention to reduce both ROS production and apoptosis. In our experiments we demonstrated that ubiquinol was effective in lowering the production of $O_2^{\bullet-}$ in leukocyte mitochondria and H_2O_2 in the diaphragm as well as attenuating apoptosis in the lungs, heart, diaphragm and kidneys. These findings support the continued usage of this model for future investigations of ubiquinol in relation to HS and fluid resuscitation.

Chapter 2 was a manuscript that has been accepted for publication entitled *A Review of Reactive Oxygen Species and their Measurement in Nursing Research*. In this chapter there was a summary of the challenges involved when using biomarkers for ROS that may be used in clinical settings. This discussion begins with an historical review of

the discovery of free radicals and their roles in aging and disease. Early methods of detecting the effects of ROS damage resulted in the identification of biomarkers that were so-called “footprint” biological products of cell injury. Tests were reviewed that are capable of measuring damage to lipid membranes, proteins and DNA and which are useful as biomarkers for research and clinical applications. In this manuscript, methods to directly quantify levels of ROS, particularly $O_2^{\bullet -}$ and H_2O_2 were discussed and how those advancements led to the development of chemiluminescent and fluorescent probes that directly react with ROS in blood or tissues. In particular, there was a review of how the combination of ROS with a fluorescent probe results in the formation of a new molecule, a fluorophore, which is excited by a laser source at a specific wavelength causing fluorescence that can be measured and quantified. Several detection systems that have been used with fluorescent probes were discussed including flow cytometry, laser scanning cytometry, spectrofluorometry, confocal microscopy and high performance liquid chromatography (HPLC). A review was presented of the fluorescent probes MitoSOX Red (a mitochondrial variant of HE) and analogues of 2′,7′-dichlorodihydrofluorescein, including Hfluor (dihydrofluorescein-diacetate). MitoSOX Red and HFluor were used in our experiments. At the conclusion of Chapter 2 was a discussion of the recent developments in ROS detection, including the development of new-generation probes, encapsulation techniques and the use of microchip electrophoresis.

Chapter 3 was a manuscript that has been published entitled *Apoptosis: Understanding Programmed Cell Death for the CRNA*. This article was a review for nurse anesthetists of the essential pathways of apoptosis. Apoptosis was defined and a

brief history presented of the evolution of the scientific understanding of apoptotic mechanisms. Apoptosis, which is energy-dependent and characteristic of the normal cell life-cycle, was differentiated from necrosis, which is a process that is non-energy dependent and results from prolonged hypoxic tissue injury or disease. Morphologic characteristics of both processes were discussed. The four phases of apoptosis: 1) signaling, 2) regulation, 3) execution and 4) removal, were discussed. Two primary pathways, both involving caspase activation, were outlined and compared. The intrinsic (mitochondrial) pathway, which is initiated by the leakage of cytochrome c from mitochondria, was especially relevant to the conceptual model of our study and was used in the development of our experimental hypotheses.

Cardiac, pulmonary, neurological, endocrine disorders and trauma are common problems in surgical patients and it is important for nurse anesthetists to appreciate the causal relationships between apoptotic pathways and such disorders. To educate nurse anesthetists about these relationships, Chapter 3 provided a review of the current knowledge about apoptosis. A controversial area of concern in nurse anesthesia has been the possibility of apoptosis-mediated neurological injury in young pediatric patients as a result of commonly used anesthesia drugs. A discussion was presented of the neurotoxic effects of anesthesia drugs administered in experiments with young animals during the developmental phase of synaptogenesis. Some investigators have suggested that an increased production of ROS and activation of apoptotic pathways may result from the use of certain anesthetic agents, placing young surgical patients at increased risk of neurological injury. Drugs commonly administered by CRNAs that possess antioxidant characteristics were discussed and how those pharmaceutical agents could be beneficial

in limiting ROS production during anesthesia.[278]

Chapter 4 is a manuscript that will be submitted for our study entitled *Effects of Ubiquinol on Leukocyte Mitochondrial Superoxide, Diaphragm Hydrogen Peroxide and Tissue Apoptosis following Hemorrhagic Shock and Fluid Resuscitation*. The aim of this study was to examine the effect of ubiquinol on leukocyte mitochondria and in the lungs, diaphragm, heart and kidneys as a supplemental treatment for HS. In this chapter were the findings and discussion of the experiments we conducted in our laboratory. A review of the findings and a brief discussion for each hypothesis will follow. Hypotheses three and four will be discussed together because of their similarity.

Research Hypothesis 1: Administering ubiquinol will reduce leukocyte mitochondrial production of superoxide following hemorrhagic shock.

We used flow cytometry and the fluorescent probe MitoSOX Red to determine whether the intravascular administration of ubiquinol would reduce the production of the radical $O_2^{\bullet-}$ in leukocyte mitochondria. No significant difference in MFI was detected between treatment and control groups at either the baseline or following 60 minutes of HS. A significant difference in MitoSOX Red fluorescence did occur at the end of the treatment period 120 minutes after fluid resuscitation. Mean fluorescence intensity of MitoSOX Red was significantly lower in the ubiquinol group than in the control group.

It is well known that $O_2^{\bullet-}$ is produced in polymorphonuclear neutrophils (PMNs) via membrane-bound NADPH oxidase which catalyzes the production of $O_2^{\bullet-}$ in oxidative "bursts" from oxygen and NADPH.[190] The role of leukocyte mitochondria in $O_2^{\bullet-}$ production is not as well understood, however there is evidence that mitochondria in leukocytes do participate in the generation of $O_2^{\bullet-}$.[302] Only one other study was identified

in which MitoSOX Red was used to evaluate the mitochondrial production of $O_2^{\bullet-}$ in leukocytes. [303] In that study, the effect of exercise on ROS production and apoptosis in neutrophils was measured and no treatments were administered. Study of the effects of ubiquinol or ubiquinone on the production of $O_2^{\bullet-}$ by leukocytes has been limited and has been focused primarily on the NADPH oxidase pathway. Suzuki evaluated the effects of ubiquinone on endotoxin-induced production of $O_2^{\bullet-}$ in PMNs collected from rats.[317] The treatment rats received ubiquinone which was followed by induction of endotoxin shock in both groups. Using the cytochrome-c method of $O_2^{\bullet-}$ measurement, he was able to demonstrate a significant decrease in $O_2^{\bullet-}$ production in the PMNs of the treated rats. Suzuki did not address the mitochondrial production of $O_2^{\bullet-}$. Consistent with his findings, in our study using MitoSOX Red to measure mitochondrial $O_2^{\bullet-}$ production in leukocytes following HS, we also found lower $O_2^{\bullet-}$ levels in ubiquinol treated rats compared to controls. Based on this, our data suggests that leukocyte mitochondria produce significantly higher levels of $O_2^{\bullet-}$ following HS and fluid resuscitation and that the use of MitoSOX Red with flow cytometry has potential for clinical usage in the measurement of ROS in blood. Continued development in this area is required to further clarify the use of leukocyte mitochondrial $O_2^{\bullet-}$ as a ROS biomarker following HS.

Research Hypothesis 2: Administering ubiquinol will attenuate hydrogen peroxide production in the diaphragm following hemorrhagic shock.

Hydrogen peroxide levels were measured in diaphragm tissue by confocal microscopy using the Hfluor probe. The MFI of the control group was significantly higher than for the group treated with ubiquinol, indicating significantly higher levels of H_2O_2 were present in the diaphragms of untreated rats. We concluded that ubiquinol significantly

reduced the production of H_2O_2 in the diaphragm because it scavenged ROS and may offer a protective treatment to reduce diaphragm dysfunction following HS.

Hydrogen peroxide is well established as a mediator of aging and disease[237] however, we found no other studies in which the effects of ubiquinol on the production of diaphragm H_2O_2 were examined. In one study of the effects of ubiquinone, Lakomkin and colleagues infused low and high concentrations of H_2O_2 into the coronary arteries of isolated rat hearts. Hearts that were obtained from rats that had received long-term dietary pre-treatment with oral ubiquinone had less negative inotropic effects, suggesting a mechanism of ubiquinone[304] involving protection from H_2O_2.

Increased levels of ROS are known to cause diaphragm dysfunction. In a previous study in our laboratory, we found that dopamine, a neurotransmitter with radical-scavenging capability, was effective in lowering the levels of H_2O_2 produced in the diaphragms of rats subjected to HS.[25] The rats treated with dopamine in this study were subjected to 60 minutes of HS followed by a 30 minute period of fluid resuscitation using LR or hetastarch with and without dopamine. The diaphragms were prepared similarly to our experiment however, measurement of H_2O_2 was made with laser scanning cytometry rather than confocal microscopy. In our present study, rats were also subjected to a 60 minute period of HS followed by fluid resuscitation, then rats were allowed to recover for 120 minutes prior to harvesting organs. In both studies, the administration of an antioxidant (dopamine or ubiquinol) was effective in decreasing diaphragm H_2O_2. Based on the results of the current study, we suggest that ubiquinol may be effective in reducing diaphragm dysfunction mediated by the increased generation of ROS following HS.

Research Hypothesis 3: There will be a decrease in the percentage of apoptotic

nuclei in the lungs and diaphragm following ubiquinol administered post hemorrhagic shock.

Research Hypothesis 4: There will be a decrease in the percentage of apoptotic nuclei in heart and kidneys following ubiquinol administered post hemorrhagic shock.

Experiments to determine the amount of apoptosis following HS were conducted in order to measure the effect of ubiquinol in decreasing HS-induced ischemia and reperfusion injury on tissues. Rats treated with ubiquinol had significantly fewer apoptotic cell nuclei in the lungs, diaphragm, heart and kidneys than the controls. One possible explanation for our findings is that ubiquinol was effective as a radical scavenger and protected against activation of the mitochondrial apoptosis pathway. Ubiquinol has also been shown to stabilize the mitochondrial membrane and prevent the opening of mitochondrial permeability transition pores (MPTP),[4,85] which also contributes to its effectiveness in suppressing the activation of the intrinsic apoptosis pathway. Our findings were consistent with the results of studies that suggest exogenously administered ubiquinol has antioxidant properties, inhibits the mitochondrial intrinsic cell death pathway and reduces apoptosis in other pathological states. Maroz et al demonstrated in-vitro that $O_2^{\bullet-}$ is quickly scavenged by ubiquinol to prevent lipid peroxidation.[87] Jing and colleagues observed that murine hippocampal cells exposed to ultraviolet B radiation and treated with ubiquinone were protected from apoptosis by reduction of $O_2^{\bullet-}$ and inhibition of the intrinsic mitochondrial pathway.[313] Both of these studies relate to our findings of reduced levels of leukocyte $O_2^{\bullet-}$ production following treatment, which supports the antioxidant function of ubiquinol. Papucci et al confirmed that the beneficial effect of ubiquinone in

reducing apoptosis was related to stabilization of the MPTP and maintenance of membrane potential.[4] In our study we did not specifically test the membrane stabilization activity of ubiquinol, however we believe it likely contributed to the reductions in $O_2^{\bullet-}$ and H_2O_2 as well as the attenuation of apoptosis. From a clinical standpoint, in the treatment of patients who have experienced HS, ubiquinol may be beneficial in reducing multiple organ failure and longer term mortality and morbidity following a hemorrhagic event.

Limitations

Access to flow cytometry and fluorescence probe technology provided us with an opportunity to evaluate the production of the radical $O_2^{\bullet-}$ in leukocyte mitochondria as a possible biomarker of oxidative stress. Our findings confirmed that ubiquinol did result in lower levels of $O_2^{\bullet-}$ as measured with MitoSOX Red. Two issues emerged with the use of this method that should be considered as limitations to our study. As we discussed in Chapter 2, the oxidation of hydroethidine in MitoSOX Red can occur as a result of reaction with cytochrome c and other artifact molecules as well as with $O_2^{\bullet-}$. This results in the production of two distinct molecules, only one of which actually provides a quantifiable marker of $O_2^{\bullet-}$. Laser scanning techniques with MitoSOX Red, including flow cytometry, do not distinguish between the two molecules. Using HPLC, investigators have accurately quantified the two individual products of MitoSOX Red. However, HPLC requires an extraction protocol for these products which is arduous and would be difficult to use for clinical application.[218] Thus, there is a continuing need for future investigation of new products that could rapidly and efficiently measure ROS biomarkers with limited volumes of blood in either laboratory or clinical settings. New

technology, including the development of highly specific fluorescent probes with less vulnerability to artifact may provide solutions to these challenges.

One other limitation related to the evaluation of $O_2^{\bullet-}$ in this study is that the optimum times to measure the production of $O_2^{\bullet-}$ by leukocytes following ischemia and reperfusion in HS has not been established. The response of leukocytes to systemic stress can result in sudden and dramatic increases in $O_2^{\bullet-}$ production known as "superoxide bursts" or "oxidative bursts" and it is unknown to what extent the production of mitochondrial $O_2^{\bullet-}$ contributes to these events. The appropriate times to capture these periods of increased $O_2^{\bullet-}$ production has not been explicated. This problem is compounded by the extremely short half-life of $O_2^{\bullet-}$ (10^{-6} s) which challenges investigators to identify the optimal times to obtain blood specimens. For our study, sample times corresponded with experimental stages (baseline, end of the 60 minute shock period and end of the 120 minute treatment period). Additional times to collect mitochondrial $O_2^{\bullet-}$ samples either during the experiment or after the experiment might have provided information concerning how quickly or how long the process occurs.

Future Research

Further laboratory research on the use of mitochondrial-specific fluorescent probes in the analysis of leukocyte $O_2^{\bullet-}$ is needed to understand the contribution of mitochondria to $O_2^{\bullet-}$ production. This is especially important in neutrophils which comprise the largest percent of ROS-producing leukocytes. Additional studies examining the timing of $O_2^{\bullet-}$ production in leukocytes to determine the appropriate times to remove blood specimens is needed. This study could be repeated with more frequent measures of leukocyte ROS through the various stages.

The antioxidant-specific function in decreasing apoptosis following HS is not assumed to be the sole mechanism of the beneficial activities of ubiquinol. Stabilization of the MPTP by ubiquinol is also a likely explanation and the contribution of each function should be determined by further research.

Ideally, our findings should provide foundational data for clinical studies. Translational human studies will be important, especially related to the development of methods of ROS detection using small blood samples that may offer a method to assess oxidative stress in HS patients. Human clinical trials would provide the opportunity for furthering our understanding of the potential benefits of ubiquinol following HS. We are aware that conducting human research is difficult with traumatically injured individuals who have experienced HS, primarily due to the inability to obtain human subject consent to administer ubiquinol. Forms of ischemia-reperfusion other than HS exist in more controlled surgical settings and would provide potential subjects. Examples of alternative models of ischemia and reperfusion injury that could be investigated are the use of extremity tourniquets in orthopedic surgery and procedures involving the use of cardiopulmonary bypass and surgery involving the transplantation of tissues and organs. Cardiopulmonary bypass has well-documented detrimental effects on lung, heart and kidney tissues.[318-320] Transplanted organs are not perfused for periods of several hours until they are re-implanted into a recipient and are exposed to injury as a result of a reperfusion event. In these instances, randomized controlled studies could be employed in clinical settings where ubiquinol is administered before and after surgery and mitochondrial leukocyte $O_2^{\bullet -}$ production is measured as a biomarker of oxidative stress.

Investigators could examine additional tissues other than the diaphragm to

confirm the effect of ubiquinol administration on the generation of H_2O_2 following HS. We were limited to studying the diaphragm because the method used to measure H_2O_2 with confocal microscopy requires thin and flat tissue for examination. Similar studies of lungs, heart and kidneys could be conducted with a laser scanning microscope to confirm the H_2O_2 effect of ubiquinol in these organs. A laser scanning microscope is available at our institution however repair and activation of the equipment would be needed.

In this study we examined the extent of organ injury based on the percent of apoptosis present 120 minutes following fluid resuscitation for HS. As noted in Chapter 1, organ injury after HS follows a bimodal pattern of development with spikes at day three and during days five to seven. A longitudinal study could be conducted to evaluate the extent of organ damage at these later times to determine whether ubiquinol continues to attenuate organ injury through the period of recovery from HS.

Conclusions

The results of our study support the administration of ubiquinol as a treatment following HS. The primary benefits of ubiquinol based on our data appear to be the scavenging of ROS and decreased activation of the apoptotic pathway that leads to nuclear DNA damage. Thus, we concluded that ubiquinol attenuated apoptotic damage in rats subjected to HS followed by fluid resuscitation. Our observations of the control rats confirm that HS, treated only with fluid resuscitation, resulted in an ischemia-reperfusion event. This stimulated the production of leukocyte mitochondrial $O_2^{\bullet -}$ which continued to rise in the control animals during the treatment period. This effect was significantly reduced in ubiquinol-treated rats. Our measurements of H_2O_2 in diaphragm tissue also supported our conclusion that ubiquinol was an effective antioxidant. Any antioxidant

that effectively lowers $O_2^{\bullet-}$ will most likely lower levels of H_2O_2 because of the relationship of $O_2^{\bullet-}$ to H_2O_2 in the ROS pathway. Measurement of H_2O_2 is often used in studies as a corollary of the production of other ROS.[215] The ability of ubiquinol to scavenge $O_2^{\bullet-}$ would therefore be predicted to lower H_2O_2 in treated rats, which is consistent with our findings.

Finally, our examination of rat lungs, diaphragm, heart and kidneys for evidence of apoptosis confirmed that ubiquinol was effective in decreasing DNA damage. We suggest that protection of the cell mitochondria from ROS damage, whether by antioxidant effect or membrane stabilization, diminished activation of the intrinsic apoptosis pathway resulting in substantially fewer damaged nuclei. Previous investigators have suggested that using interventions specifically aimed at reducing ROS production would be beneficial in limiting organ damage following HS.[292,321] Our study with ubiquinol following HS and fluid resuscitation strongly supports that it is a potent antioxidant capable of reducing the activation of the apoptotic pathways.

Only three researchers have investigated the effects of administering ubiquinone prior to HS. These studies were conducted in the 1980s and the investigators observed beneficial effects of ubiquinone especially in heart and lung function following HS. They did not associate their findings with mechanisms involving ROS and apoptosis in their studies and ubiquinone was administered before the induction of HS. Our results in administering ubiquinol to reduce apoptosis in lungs, diaphragm, heart and kidneys following HS were especially encouraging as a potential supplemental treatment in HS and should be replicated with additional investigations to verify our findings.

The use of ubiquinol as an adjunct to the treatment of HS and reperfusion injury is

promising because of the antioxidant protective effects. Our future investigations of HS will be focused on examining ubiquinol on microcirculation measurements including leukocyte adherence, mast cell degranulation, vascular permeability and ROS production in the mesentery. We are also planning to expand our investigations of ubiquinol in different pathologic conditions such as heart failure, diabetes mellitus and high altitude pulmonary edema.

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