Effects of Hemorrhagic Shock and Fraction of Inspired Oxygen on Hydrogen Peroxide and Apoptosis in Rat Lung and Diaphragm

By

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Abstract

Hemorrhagic shock (HS) is the single most common cause of death in civilian and military personnel experiencing trauma (Alam & Rhee, 2007). Immediate resuscitation for HS can involve the administration of supplemental oxygen (O_2) above ambient levels (0.21) using a non-rebreather face mask and high flow O_2 of 10-15 L/min (Trauma, 2008). With supplementary O_2, reactive oxygen species (ROS) may be increased leading to oxidative damage to deoxyribonucleic acid (DNA), proteins and lipid membranes (Rushing & Britt, 2008). During hyperoxia, ROS can cause a secondary oxidative injury to cells and tissues especially in the lungs and diaphragm.

The optimal amount of O_2 to be administered following HS is not clearly defined. The purpose of this dissertation was to investigate the optimal fraction of inspired oxygen (FIO_2) to be administered following HS by determining the amount of hydrogen peroxide (H_2O_2) and apoptosis in the lungs and diaphragm. Previous animal studies have demonstrated that dopamine (DA) can scavenge free radicals or decrease ROS by increasing blood flow and can decrease apoptosis by activating β-2 adrenoreceptors (Communal, Singh, Sawyer, & Colucci, 1999; Patterson et al., 2004; J. D. Pierce, Goodyear-Bruch, Hall, & Clancy, 2006; J. D. Pierce, Goodyear-Bruch, Hall, Reed, & Clancy, 2008) Therefore, we conducted additional experiments to determine if DA with various FIO_2s following HS reduces apoptosis in these tissues.

Adult male Sprague-Dawley rats (n = 112) were anesthetized; a tracheostomy was performed and catheters were inserted in the carotid and femoral arteries. HS was elicited by withdrawing 40% of the rat's blood volume over 30 minutes. This was

followed by the rat breathing one of the following FIO_2 concentrations (0.21, 0.40, 0.60, and 1.00) without and with concurrent DA (10 mcg/kg/min) for 60 minutes. The animal was euthanized and the lungs and diaphragm excised and prepared for measurement of H_2O_2 and nuclear DNA damage (apoptosis). Hydrogen peroxide was quantified using dihydrofluorescein diacetate (Hfluor-DA) and laser scanning cytometry. Percent apoptosis was determined using differential dye up-take and fluorescent microscopy.

The amount of lung and diaphragm H_2O_2 and percent apoptosis were greatest in the 0.21 and 1.00 FIO_2 concentrations and the least amounts were observed in rats when using 0.40. Infusing DA significantly decreased H_2O_2 and apoptosis in both tissues at all FIO_2's except 0.40. The lack of difference in rats receiving 0.40 with DA was because of the already reduced H_2O_2 and apoptosis values. In conclusion, an FIO_2 of 0.40 was optimal for attenuation of lung and diaphragm H_2O_2 and apoptosis following HS. When greater FIO_2s are necessary, adding DA to the resuscitation regimen may diminish ROS-induced cellular injury.

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Common Abbreviations

ABG	Arterial blood gas
AO	Acridine orange
ATLS	Advanced Trauma Life Support
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Beecf	Base excess in extracellular fluid
DA	Dopamine
DBP	Diastolic blood pressure
DMSO	Dimethyl sulfoxide
DO_2	Delivery of O_2
EB	Ethidium bromide
FIO_2	Fraction of inspired oxygen
FAD	Flavin adenine dinucleotide
FCM	Flow cytometry
FLUOR	Fluorescein, the reduced form of Dihydrofluorescein diacetate
GSH	Glutathione peroxidase
HCO_3	Bicarbonate
HFLUOR-DA	Dihydrofluorescein diacetate
H_2O	Water
H_2O_2	Hydrogen peroxide
$HO^{\cdot}$	Hydroxyl radical

HR	Heart rate
HS	Hemorrhagic shock
ITP	Intrathoracic pressure
LSCM	Laser scanning cytometry
MAP	Mean arterial pressure
mcg/kg/min	Micrograms per kilogram per minute
mg/mL	Milligrams per milliliter
mL/min	Milliliter per minute
mm Hg	Millimeters of mercury
NADH	Nicotinamide dehydrogenase
O_2	Oxygen
$O_2^{-\cdot}$	Superoxide anion
PaO_2	Partial pressure of oxygen at sea level, arterial blood
$PaCO_2$	Partial Pressure of carbon dioxide at sea level, arterial blood
pH	Concentration of hydrogen ions in a solution Negative log of $[H^+]$
Redox	Reduction-oxidation
ROS	Reactive oxygen species
RR	Respiratory rate
SaO_2	Percentage of hemoglobin saturation with O_2
SBP	Systolic blood pressure
SOD	Superoxide dismutase
VO_2	Utilization of O_2

Chapter 1.

General Introduction

All cells require O_2 for aerobic metabolism and maintenance of normal cellular function. Cells cannot store O_2, thus a constant supply of O_2 is required to sustain the metabolic needs of each cell. Inspired O_2 moves across the alveolar-capillary membrane into the blood. The blood then transports O_2 to the tissues, sustaining normal cellular function and metabolism. Normal cellular function and metabolism are dependent upon appropriate proportions of molecular O_2 and various antioxidants. Sustained perturbation of this imbalance may result in either apoptosis or necrosis (Chandra, Samali, & Orrenius, 2000; Huang, 2005; M. E. Kerr, Bender, & Monti, 1996).

Hemorrhagic shock, a condition produced by rapid and significant loss of intravascular volume, is the leading cause of death in civilian and military personnel (Alam, Koustova, & Rhee, 2005; Alam & Rhee, 2007; Rhee, Koustova, & Alam, 2003). Hemorrhagic shock is a physiologic state in which decreased perfusion of vital organs leads to inadequate delivery of O_2 rich substrate necessary for normal tissue and cellular function (Brod, Krausz, Hirsh, Adir, & Bitterman, 2006; Gutierrez, Reines, & Wulf-Gutierrez, 2004; Guyton, 2006). Failure of compensatory mechanisms in the body results in further hemodynamic instability, decreased tissue perfusion, decreased O_2 delivery (DO_2) and O_2 utilization (VO_2), cellular hypoxia and hypoxic injury (Mauriz, Martin Renedo, Barrio, Culebras, & Gonzalez, 2007). Therefore, the treatment goals for HS patients are: 1) control of bleeding, 2) hemodynamic stability and 3) maintenance of tissue oxygenation (Angele, Schneider, & Chaudry, 2008).

Oxygen at a concentration of 100% is recommended to be given to patients experiencing HS based on the Advanced Trauma Life Support (ATLS) guidelines of the

American College of Surgeons. Administering O_2 for HS is accomplished using either a non re-breather mask or nasal cannula. Both methods of O_2 administration can deliver supraphysiologic FIO_2. The non-rebreather mask appropriately fitted can deliver 100% at high flow rates of 11 to 15 L/min. If the victim is unable to maintain an open airway, immediate resuscitation involves the use of supraphysiologic levels of O_2 that can be as high as 100% with an endotracheal tube (Trauma, 2008).

Supraphysiologic concentrations ($FIO_2 > 0.21$) of O_2 are beneficial for reduced VO_2. Reduced VO_2 is the precipitating pathophysiologic event in HS indicating onset of supply dependant metabolism (Kvarstein, Mirtaheri, & Tonnessen, 2003; Meier et al., 2004; Shoemaker, 1987). During immediate life support when clinicians can access the vascular system, supraphysiologic concentrations of O_2 augment blood pressure and perfusion of vital organs (Adir, Bitterman, Katz, Melamed, & Bitterman, 1995; Al-Waili et al., 2006; Bitterman, 2009; Bitterman, Brod, Weisz, Kushnir, & Bitterman, 1996; Brod, et al., 2006; Sukhotnik et al., 2002).

During HS, supplying O_2 rich substrate to the brain, heart and kidneys takes precedence over skin and splanchnic organs; thus, the skin and gastrointestinal mucosa are the first to experience a decrease in O_2 and the last to achieve reoxygenation during resuscitation (D. F. Wilson et al., 2003; M. Wilson, Davis, & Coimbra, 2003). Reoxygenation does not uniformly restore cellular oxygenation to tissues (Dyson, Stidwill, Taylor, & Singer, 2007; Geer, Behnke, McDonough, & Poole, 2002). Reoxygenation with FIO_2 of 1.00 may not be superior to FIO_2 of 0.21 in restoration of tissue metabolism after critical hypoxia (Klaus et al., 2003).

The reintroduction of O_2 rich substrate can be a source of free radical injury by ROS (Callahan, She, & Nosek, 2001; T. Clanton, 2005; T. L. Clanton, 2007; Makazan, Saini, & Dhalla, 2007; Murphy & Steenbergen, 2008; Zuo & Clanton, 2005). From a mechanistic point of view, hyperoxic environments increase superoxide ($O_2^{-\cdot}$) and the free radical precursor hydrogen peroxide (H_2O_2) which is converted to the hydroxyl radical ($HO^{\cdot}$). The $HO^{\cdot}$ radical is a trigger for intrinsic pathway apoptosis which is responsible for most of the deoxyribonucleic acid (DNA), protein and lipid damage in cells of living systems (Droge, 2002; Jamieson, Chance, Cadenas, & Boveris, 1986; C. Li, Wright, & Jackson, 2002). Supraphysiologic O_2 administration may create hyperoxic environments in which free radical generation accelerates, causing oxidative injury from ROS (Ahn, Robertson, Vereczki, Hoffman, & Fiskum, 2008; Yis, Kurul, Kumral, Cilaker, et al., 2008; Yis, Kurul, Kumral, Tugyan, et al., 2008). A pathological condition created by O_2 administration in HS treatment is often considered a secondary injury and the free radicals produced by supplemental O_2 play an important role in damaging cells (Akioka, 2002).

In a volume-controlled HS model, the optimum level of O_2 that produces the least amount of H_2O_2 and apoptosis in the lung and diaphragm tissue has yet to be established. Determining the optimal FIO_2 that produces the least amount of DNA damage during HS will contribute to current understanding of HS and restoration of tissue oxygenation. In addition, no investigators have examined how dopamine (DA), a common intravenous drug administered sometimes in HS with supplemental O_2, affects lung and diaphragm free radical formation.

Aims and Research Questions

The overall aim of this research is to determine the optimal level of FIO_2 to be administered that minimizes lung and diaphragm H_2O_2 and apoptosis in a rat model of controlled HS. The research questions are as follows:

Aim one. To determine the optimal FIO_2 that minimizes nuclear DNA damage in the lung and diaphragm following 30 minutes of exsanguinations to attain HS.

Research question one. What is the amount of H_2O_2 in the lung and diaphragm after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60, 1.00) following 30 minutes of exsanguinations to attain HS?

Research question two. What is the percentage of lung and diaphragm apoptosis after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60, 1.00) following 30 minutes of exsanguinations to attain HS?

Aim two. To determine the effect of DA at different FIO_2's on lung and diaphragm nuclear DNA damage following 30 minutes of exsanguinations to attain HS.

Research question three. Does dopamine at a dosage of 10 mcg/kg/min administered with various FIO_2 (0.21, 0.40, 0.60, and 1.00) attenuate the amount of H_2O_2 and apoptosis in the lung and diaphragm following 30 minutes of exsanguinations to attain HS?

Background and Significance

Hemorrhagic Shock

Hemorrhagic shock occurring as a result of trauma is defined as an acute loss of blood from the intravascular space giving rise to systemic hypo perfusion caused by

reduction either in cardiac output or effective circulating blood volume resulting in inadequate tissue perfusion and oxygenation. The loss of blood volume leads to a decreased venous return to the heart and reduction of left ventricular end diastolic volume. Compensatory catecholamine stimulation eventually becomes ineffective and myocardial fiber length decreases, thus reducing contractility which further reduces cardiac output. The decline in cardiac output decreases tissue perfusion and O_2 substrate available for normal metabolism (Martel et al., 2002; Morton, 2005; Rushing & Britt, 2008).

To compensate for hypovolemia, blood is shunted from less vital organs such as the gut, and skin to organs that are vital for preservation of circulation such as heart, brain, kidneys and lungs. This shunting is triggered by a decreased cardiac output and pulse pressure signaling baroreceptors in the aorta, atrium and carotid sinuses. Neural reflexes cause sympathetic stimulation to the heart and other organs which increase heart rate and vasoconstriction. The renin angiotensin system is activated causing vasoconstriction and retention of sodium and water (H_2O), both of which support blood pressure. The anterior pituitary and adrenal medulla are stimulated to release adrenocorticotropic hormone (ACTH), epinephrine and norepinepherine to compensate for hypotension (Cottingham, 2006; Goodrich, 2006; Morton, 2005).

At the cellular level, the decreased perfusion from HS causes cells to change from normal aerobic metabolism to anaerobic metabolism. Lactic acid is formed leading to metabolic acidosis. Continued blood loss leads to further myocardial hypo- perfusion and worsening lactic acidosis. Cerebral hypo perfusion leads to cardiac and respiratory

depression and sympathetic nervous system decline. Without continuous sympathetic stimulation, venous pooling and increased capillary permeability occurs (Prasad, 2002; Spaniol, Knight, Zebley, Anderson, & Pierce, 2007).

At the cellular level, membrane integrity is altered causing irreversible damage to tissues. This occurs in part because of the effects of ROS and reactive nitrogen species (RNS). These types of free radicals possess an unpaired electron which reacts to cause oxidation of nuclear DNA membranes, oxidation of fatty acids and amino acid degradation. The electrical gradients are destroyed causing cell swelling, endoplasmic reticulum and mitochondrial damage (Prasad, 2002; Spaniol, et al., 2007).

The clinical significance of hemorrhage depends on the volume and rate of bleeding. The American College of Surgeons defines four classes of HS according to the percentage of blood lost. Class I (initial stage) is characterized by blood loss of up to 750 mL (15% of blood volume). Compensatory mechanisms maintain cardiac output in healthy individuals. No change in blood pressure, pulse pressure or respiratory rate (RR) occurs. Even if slow losses of larger amounts of blood occur, the hemorrhage may have limited physiologic impact. If blood volume is restored, fluid resuscitation is generally unnecessary (Trauma, 2008).

Class II (compensatory stage) is characterized by blood loss of 750 to 1500 mL (15% to 30% of blood volume). Decreased cardiac output from this much blood loss signals the body's compensatory mechanisms. Tachycardia of greater than 100 beats per minute (bpm) occurs. Pulse pressure narrows as diastolic pressure rises due to increased catecholamines causing an increased peripheral vascular resistance. The respiratory rate

(RR) also increases to improve oxygenation. The partial pressure of arterial carbon dioxide ($PaCO_2$) and partial pressure of arterial O_2 (PaO_2) indicate respiratory alkalosis and hypoxemia. Urine output is decreased because of reduced renal perfusion. The patient's skin becomes pale and cool and with slow capillary refill which occurs because of peripheral vasoconstriction. The patient may exhibit anxiety and confusion because of decreased cerebral perfusion. In Class II fluid resuscitation is required; however blood replacement is not (Trauma, 2008).

Class III (progressive stage) is characterized by blood loss of 1500 to 2000 mL (30% to 40% of blood volume). In this stage, compensatory mechanisms begin to fail and impaired tissue perfusion occurs. Heart rates of greater than 120 bpm lead to myocardial ischemia and dysrhythmias. Systolic blood pressure (SBP) falls and respiratory distress occurs. Respiratory acidosis, metabolic acidosis and hypoxemia occur as indicated by a high $PaCO_2$, low bicarbonate (HCO_3) and low PaO_2. Urinary output significantly decreases and renal failure occurs. Cessation of blood loss is a priority and transfusions of blood and other fluids are necessary (Trauma, 2008).

Class IV (refractory stage) is characterized by blood loss of greater than 2000 mL (40% of the patient's total blood volume). This amount of blood loss is not compatible with life. Compensatory mechanisms fail and organ failure occurs. In patients, tachycardia of greater than 140 bpm, severe hypotension, narrow or unobtainable pulse pressure, absent peripheral pulses and negligible urine output are observed. The patient becomes cold, cyanotic and clammy. The patient's level of consciousness declines to unconsciousness. Immediate life saving measures such as

rapid transfusion of blood and other fluids and surgical intervention are required (Trauma, 2008).

Patients experiencing HS require supraphysiologic (> 21%) concentrations of O_2 to maintain and restore normal tissue function. However, the use of O_2 at such a dose can be harmful to tissues and can contribute to ROS formation (Ahn, et al., 2008; Brueckl et al., 2006; Fabiano, Pezzolla, Filograna, & Ferrarese, 2008). At concentrations greater than 21%, O_2 can be toxic to normal tissues causing observable morphometric changes to airways and lung tissues (Ellman et al., 2005; Zhang & Foda, 2004; Zhang, Zhu, Liu, & Foda, 2008). Toxic concentrations of O_2 can alter the innate host defense mechanisms, alveolar macrophage functioning and inflammatory cytokine production (Baleeiro, Wilcoxen, Morris, Standiford, & Paine, 2003).

Lung and Hemorrhagic Shock

The lungs are the basic respiratory organs of humans and are responsible for inhalation of O_2 and exhalation of carbon dioxide (CO_2). The lungs occupy the entire bilateral aspects of the thorax with two left lobes and three right lobes. The lungs are encased in a thin membrane-like sac called the pleural sac. The lungs are connected by the heart and pulmonary arteries and an inverted tree like structure (trachea and bronchus) which occupy the mediastinum. The trachea and bronchus give rise to intricately branching bronchioles that communicate with the thin walled terminal alveoli after 23 divisions. The vast 70 m^2 diffusion surface area closely apposed to an alveolar capillary network separated by less than one micrometer is capable of accommodating

blood flow between 5 L/min at rest and 25 L/min at maximal O_2 consumption (Kumar, 2009).

Hemorrhagic shock up-regulates genes in the lung responsible for inflammation, protein activation, oxidation and apoptosis (N. L. Jernigan, Walker, & Resta, 2004; T. W. Jernigan, Croce, & Fabian, 2004; Q. Lu, Xu, Davidson, Hasko, & Deitch, 2004; Shih, Wei, & Lee, 2005). In the lung, these proteins activate alveolar macrophages and recruit neutrophils which release oxidants. These substances also damage capillary endothelium and alveolar epithelium, disrupting the barriers between capillaries and airspaces. Edema fluid, protein and cellular debris fill the airspaces and interstitium, causing disruption of surfactant, air space collapse, ventilation-perfusion mismatch, shunting and stiffening of the lungs with decreased compliance and pulmonary hypertension. Diffuse alveolar damage causes an increase in intra-alveolar neutrophils, red blood cells (RBCs), cellular debris and altered epithelial basement membranes, with formation of hyaline membranes. The effects of HS and additional O_2 may damage lung tissues by altering intrinsic antioxidant defenses and apoptosis (N. L. Jernigan, Resta, & Walker, 2004; Perl et al., 2007). For example, several studies demonstrated the presence or absence of injury to the lungs and airways by altering superoxide dismutase (SOD) levels in experimental models. The consequences of altering oxidant sources or SOD are either injury producing or protective to tissues (Gongora & Harrison, 2008; Gongora et al., 2008; Zheng, Yu, Zhang, Ye, & Yi, 2009).

Researchers examined hypoxic systemic and non-systemic mesenteric lymph factors as mechanisms activating macrophages and neutrophils leading to oxidant

damage (Diebel, Liberati, Ledgerwood, & Lucas, 2008; Feinman et al., 2007). Feinman et al., (2007) developed an in-vivo rat HS model that distinguished the non-injury systemic effects of hemorrhage from pulmonary tissue injury. By ligating the mesenteric lymph duct, the investigators were able to compare the pulmonary and molecular responses of rats subjected to HS. Hemorrhagic shock down regulates genes for catalase and SOD in the lung.

Researchers used fluorescent microspheres to measure blood flow in a study that examined the effects of hemorrhage on perfusion distribution among alveoli. At blood loss of 30% inter-alveolar perfusion distribution becomes markedly maldistributed and may explain why lung injury occurs with HS (Conhaim, Watson, Heisey, Leverson, & Harms, 2006).

Diaphragm and Hemorrhagic Shock

The primary muscle of respiration is the diaphragm which performs 75% to 85% of the work of breathing (Silverthorn, 2006). The diaphragm accounts for more than 50% of the tidal volume during quiet breathing (Boczkowski, Lanone, & Aubier, 2000). The diaphragm forms the floor of the thoracic cavity as a curved and flat muscle that separates the thoracic from the abdominal cavity. The convex upper surface faces the thoracic cavity and the concave inferior surface faces the abdominal cavity (Poole, Sexton, Farkas, Powers, & Reid, 1997; Reber, Nylund, & Hedenstierna, 1998; Standring, 2009). The muscles of the thoracic cage, the diaphragm and pleural fluid are a functional pump facilitating ventilation. With each breath, the diaphragm shortens and flattens, displacing abdominal viscera, expanding the lower rib cage and creating a

pressure gradient which allows the lungs to expand (DiMarco, Connors, & Kowalski, 2004; Farkas, Cerny, & Rochester, 1996).

The diaphragm is a skeletal muscle having similar fiber composition as the limb muscles. The diaphragm consists of type I, IIa and IIb skeletal muscle fibers (Rives & Baker, 1942; Silverthorn, 2006). The diaphragm's muscle fibers arise from the highly oblique circumference of the thoracic outlet, the attachments being low posterior and laterally but high anterior. Although the diaphragm is a continuous flat muscle, the muscle can be considered to form three parts; crural, sternal and right and left hemidiaphragms (Rives & Baker, 1942; Standring, 2009).

The crura are tendinous at their attachments and blend with the anterior longitudinal ligament of the vertebral column. The right crus is broader and longer than the left. The right crus arises from the anterior lateral surfaces of the bodies of the intervertebral discs of the upper three lumbar vertebrae. The left crus arises from the corresponding parts of the upper two lumbar vertebrae. The medial tendinous margins of the crura meet in the mid-line to form an arch across the front of the aorta at the level of the thoracolumbar disc (Rives & Baker, 1942; Standring, 2009).

The fibers of the diaphragm converge into a central tendon. The central tendon of the diaphragm is a thin but strong aponeurosis of closely interwoven fibers. This tendon is located near the center of the muscle, at the top of the dome, yet closer to the front of the thorax, so that the posterior muscle fibers are longer. The central tendon lies immediately below the pericardium and provides attachment for the moving end of the muscle fibers (Rives & Baker, 1942; Standring, 2009).

The diaphragm receives its blood supply through the following arteries: the phrenic, lumbar, the superior phrenic branches of the lower descending thoracic aorta and the lower three posterior intercostal arteries (Loukas et al., 2008). The diaphragm is innervated receiving its motor supply through the phrenic nerve which arises from branches of the cervical three, four and five nerve roots. Sensory fibers are distributed to the peripheral part of the muscle by the lower six or seven intercostal nerves (Rives & Baker, 1942; Standring, 2009).

Diaphragm functioning during HS has been studied by investigators. Early studies examined HS using physiological, ventilation and oxygenation variables during HS using awake and anesthetized dogs (Baue & Nara, 1972). Scharf, Bark, Einhorn and Tarasiuk (1986) examined changes in blood flow to the diaphragm and O_2 consumption of the diaphragm during HS. In normal controls, there was decreased O_2 consumption associated with decreased blood flow. This was not seen with inspiratory loading or isoproterenol; therefore, the diaphragm retains the ability to increase blood flow in response to metabolic or pharmacologic stimuli primarily by shifting the pressure flow relationship which may represent a change in the critical closing pressure of the phrenic artery (Scharf, Bark, Einhorn, & Tarasiuk, 1986).

Researchers compared critical DO_2 and critical and maximum O_2 extraction ratios of resting and contracting left hemidiaphragm with those of non-diaphragm tissues. The investigators concluded that oxygenation of the diaphragm is not preferentially preserved during generalized reductions in DO_2. In diseases associated with increased work of breathing and decreased DO_2, the diaphragm may become

metabolically impaired before limitation of VO_2 is observed systemically (Ward & Hussain, 1994).

Diaphragm functioning during hypoxia can become impaired by ROS mediated injury to diaphragm muscle fibers (Callahan, et al., 2001; Machiels, van der Heijden, Heunks, & Dekhuijzen, 2001; Stofan, Callahan, Di, Nethery, & Supinski, 2000; Zuo & Clanton, 2005). In these in-vitro studies, researchers examined hypoxic muscle fibers' responses to ROS ($O_2^{-\cdot}$ and H_2O_2) which decreased diaphragm functioning. Research also shows that glutathione (GSH) exposure can lead to improved force generation (Tupling, Bombardier, Stewart, Vigna, & Aqui, 2007; Wright, Klawitter, Iscru, Merola, & Clanton, 2005).

Hemorrhagic shock leads to a hypoxic cellular environment and ROS are generated. Additionally, reoxygenation in HS can be ROS generating to a greater extent than HS alone. Thus, one goal of this research is to determine the optimal level of FIO_2 in HS. The optimal O_2 level will be indicated by the lowest H_2O_2 concentration and apoptosis in the lung and diaphragm.

Other investigations have examined diaphragm contractile dysfunction and apoptosis attenuation by DA. Low-dose DA during inspiratory resistance loading reduced myonuclei DNA damage. Examining the effect of DA on rat diaphragm apoptosis and muscle performance without blood flow and constant O_2 availability demonstrated that DA decreased apoptosis. Improving diaphragm blood flow with DA increased diaphragm O_2 availability decreased free radical formation and reduced diaphragm dysfunction. Diaphragm apoptosis was reduced by DA to a greater extent

than the effect of DA on muscle performance in these experiments (C. A. Goodyear-Bruch, Jegathesan, Clancy, & Pierce, 2008; J. D. Pierce, Goodyear-Bruch, Hall, & Clancy, 2006; J. D. Pierce, Goodyear-Bruch, Hall, Reed, & Clancy, 2008; J. D. Pierce et al., 2007).

In a study recently completed by this research group, the effects of four resuscitation strategies on diaphragm shortening, blood flow, H_2O_2 concentration and apoptosis was investigated. The resuscitation strategies were lactated Ringer's (LR), LR plus DA, Hespan (HES) and HES plus DA. Diaphragm shortening was significantly greater in the HES group and the DA groups than the LR group. This pattern continued when observing diaphragm blood flow. The same three groups showed significantly greater blood flow improvement than the LR only group. Hemorrhagic shock increased H_2O_2 concentration. The increase in H_2O_2 was greatest in the LR only group. Lactated Ringer's plus DA and HES plus DA resulted in significantly less diaphragm apoptosis than LR alone (J. Pierce, Knight, A., Pierce, J.T., Clancy, R. & Slusser, J. , 2009; J. Pierce, Mach, W., Knight, A., Pierce, J.T., Slusser, J. & Clancy, R., 2009; J. Pierce, Mach, W., Knight, A., Spaniol, J. & Clancy, R., 2007).

Free Radicals

Reactive Oxygen Species

Free radicals are molecular species possessing one or more unpaired electrons. The most common of these species are ROS and reactive nitrogen species (RNS). Aerobic organisms which derive their energy from the reduction of O_2 are susceptible to damage from the actions of O_2 free radicals such as $O_2^{-\cdot}$, H_2O_2 and $HO^{\cdot}$. The energy

generation or the reduction of O_2 occurs in the mitochondrial electron transport chain when the organelle establishes an electron gradient of hydrogen atoms or protons across their inner membranes. In this process free radicals are generated.

Molecular O_2 is converted to an O_2 free radical such as $O_2^{-\cdot}$. An O_2 free radical precursor, the peroxide radical in the form of H_2O_2 is also converted. A single electron transfer which converts molecular O_2 to the superoxide radical $O_2^{-\cdot}$ creates an unstable molecule. During oxidative phosphorylation $O_2^{-\cdot}$ decays to H_2O_2 through step-wise electron addition. The decomposition of H_2O_2 can be a source of $HO^{\cdot}$ which is responsible for most biological damage. This reaction requires both $O_2^{-\cdot}$ and H_2O_2 as precursors for $HO^{\cdot}$. These steps reduce O_2 to H_2O by the addition of four electrons yielding three ROS: $O_2^{-\cdot}$, H_2O_2 and $HO^{\cdot}$ (Cadenas, 1989).

An intact membrane is required for the formation of large quantities of adenosine triphosphate (ATP) by oxidation of hydrogen that occurs in the mitochondrial process of oxidative phosphorylation (Galluzzi, Maiuri, et al., 2007; Galluzzi, Zamzami, et al., 2007). Oxidation of hydrogen is accomplished by a series of enzymatically catalyzed reactions in the mitochondria (Hoye, Davoren, Wipf, Fink, & Kagan, 2008; Reynafarje & Ferreira, 2008).

Within the mitochondrial electron transport chain hydrogen atoms are removed. One immediately becomes a hydrogen ion and the other combines with an oxidizing agent called nicotinamide adenine dehydrogenase (NAD) to form NADH. The initial effect is to release the other hydrogen atom from NADH to form another hydrogen ion (Guyton, 2006).

Electrons removed from the hydrogen atoms to cause hydrogen ionization immediately enter the electron transport chain of electron acceptors that are an integral part of the inner membrane of the mitochondria. The electron acceptors can be reversibly reduced or oxidized by accepting or giving up electrons. Each electron is shuttled from one of these acceptors to the next until the electron finally reaches cytochrome A3. This is called cytochrome oxidase because it is capable of giving up two electrons and thus reduces elemental O_2 to form ionic O_2, which then combines with hydrogen ions to form H_2O (Guyton, 2006).

An electron leak pathway mediated by cytochrome C, which scavenges $O_2^{-\cdot}$ and H_2O_2 is proposed giving mitochondria a dual role of ATP synthesis by means of transferring electrons inside the respiratory chain while at the same time working on ROS generation and elimination as electrons leak out of the chain (Fu et al., 2008; L. Xu, Voloboueva, Ouyang, Emery, & Giffard, 2009; Y. Xu, Liu, & Xia, 2008; Zhang, et al., 2008; Zhao & Xu, 2004). During the transport of these electrons through the mitochondrial electron transport chain, a large amount of energy is released. This energy is used to cause the synthesis of ATP. This energy is also used to pump hydrogen ions from the inner matrix of the mitochondria into the outer chamber between the inner and outer mitochondrial membranes. The pumping of hydrogen ions creates a high concentration of positively charged hydrogen ions in this outer membrane and creates a strong negative electrical potential in the inner matrix (Guyton, 2006; Munns, Lui, & Arthur, 2005).

ROS Scavengers

Oxidant antioxidant imbalance can occur when over-produced O_2 radicals overwhelm depleted endogenous antioxidant defense enzymes (SOD, catalase and GSH), leading to oxidation of mitochondrial lipid membrane constituents and signaling apoptosis (Stoubannier, 2008). Superoxide dismutase is an endogenous free radical scavenger and was initially discovered in 1969 (McCord & Fridovich, 1969a, 1969b). This discovery launched other research related to the balance between oxidants and antioxidants (Salvemini & Cuzzocrea, 2002, 2003; Salvemini, Doyle, & Cuzzocrea, 2006).

Increased oxidant levels are due to either pathophysiological processes or the inability of antioxidant levels to offer balance. Lack of production of antioxidants or a destruction of scavengers results in the overproduction of free radicals thus oxidative stress (Cuzzocrea, Riley, Caputi, & Salvemini, 2001; Cuzzocrea, Thiemermann, & Salvemini, 2004). Glutathione peroxidase and catalase are two other important endogenous cellular antioxidant enzymes involved in H_2O_2 and lipid peroxide metabolism that increase with age and differ with respect to muscle. Glutathione peroxidase activity increases with age in the diaphragm more than in the intercostals. Catalase activity increases with age only in the diaphragm (Montecinos, Ramirez, Lisboa, & Borzone, 1999). The same antioxidants were measured in control and exercised lung, diaphragm, gastrocnemius and soleus muscles. Lung and diaphragm had higher antioxidant activities than locomotor muscles when compared to control thus; better antioxidant protection than locomotor muscles was observed (Caillaud et al., 1999). Superoxide scavengers were shown to protect muscle contractile function and

reduce contracture during 30 minutes of hypoxia in rat diaphragm muscle strips (Wright, et al., 2005).

Researchers examined the effect of blockade of two free radical generating pathways: cyclooxygenase pathway and the xanthine oxidase (XO) pathway on $O_2^{-\cdot}$ release by the contracting diaphragm. They also examined the effect of faltering muscle length, CO_2 levels and stimulation frequency on $O_2^{-\cdot}$ release during contraction. In isolated arterially perfused rat diaphragm preparations $O_2^{-\cdot}$ release was assessed by measuring arterio-venous cytochrome C reduction gradients across the muscle. Superoxide release during contraction was not altered by blocking the cyclooxygenase pathway. However $O_2^{-\cdot}$ was partially reduced by XO pathway blocking, by decreasing muscle length, increasing CO_2 concentrations, and decreasing stimulation frequency. Xanthine oxidase pathways contributed to free radical formation under these circumstances, cyclooxygenase did not. The last three findings suggest that these common physiological alterations have significant effects on free radical released by the contracting muscle (Stofan, et al., 2000).

Hemorrhagic shock can induce injury to multiple organs. The activities of endogenous antioxidants SOD, catalase and GSH on microvascular endothelial cells of the small intestine can vary in HS. Animals with hypovolemia and hypotension had edematous endothelial cells with injured cell membrane and mitochondria with the enhancement in SOD activity and a decrease in the activity of catalase and glutathione peroxidase (Korzonek-Szlacheta & Gwozdz, 2007).

In acute severe HS severe pathological microscopic injury occurs in major organs such as the heart, liver, spleen, lung, kidney and brain. Superoxide dismutase activities and malondialdehyde, a compound that results from lipid, amino acids and DNA degradation, were found to be increased continuously during HS (Izumi, McDonald, Sharpe, Chatterjee, & Thiemermann, 2002; Long, Liu, Sun, Gao, & Liu, 2009; Zheng, et al., 2009).

ROS and Hemorrhagic Shock

Hemorrhagic shock can initiate ROS-mediated injury to various cells, tissues and organs. Hemorrhagic shock results in vascular hypermeability and mitochondrial ROS formation triggering activation of the mitochondrial intrinsic apoptotic signaling pathway (Tharakan, Holder-Haynes, Hunter, & Childs, 2008; Tharakan, Hunter, Smythe, & Childs, 2008; Tharakan, Whaley, Hunter, Smythe, & Childs, 2010). During HS, activation of neutrophil NADPH oxidase occurs through release of ROS (Abdelrahman et al., 2005). This ROS source is an important signaling function in mediating alveolar macrophage priming and lung inflammation (Fan, Cai, & Tan, 2007). Endothelial cell ROS production is increased in HS resuscitation resulting in leukocyte-endothelial cell adherence representing injury to the microvasculature. Reactive O_2 species generation and leukocyte adherence were reduced with pre-shock administration of a platelet activating factor antagonist (Childs, Tharakan, Hunter, Tinsley, & Cao, 2007; Childs, Udobi, & Hunter, 2005).

Hemorrhagic shock creates a hypoxic extracellular environment and reoxygenation of hypoxic tissues results in the formation of ROS. During hypoxia,

cellular ATP is degraded to form hypoxanthine. Normally, hypoxanthine is oxidized by xanthine dehydrogenase to xanthine. However, during hypoxia xanthine dehydrogenase is converted to XO. Xanthine oxidase uses O_2, thus during hypoxia XO is unable to catalyze the conversion of hypoxanthine to xanthine. Oxygen is reintroduced leading to conversion of excess hypoxanthine by xanthine oxidase which results in the formation of ROS leading to cellular injury (Mueller, Laude, McNally, & Harrison, 2005; Stofan, et al., 2000; Viel, Benkirane, Javeshghani, Touyz, & Schiffrin, 2008; D. Yang et al., 2007). This is referred to as the "O_2 paradox" where ROS levels are elevated in response to administration of high and low O_2 concentrations and where O_2 is given to remedy hypoxemia actually can become injury producing (T. Clanton, 2005; T. L. Clanton, 2007).

Hydrogen Peroxide Measurement

Hydrogen peroxide is formed in several biological processes depending on the enzymes capable of transferring electrons: 1) from dismutation of $O_2^{-\cdot}$ radicals catalyzed by the enzyme SOD, 2) from transition metal reactions with $O_2^{-\cdot}$ radicals and 3) from other enzymes (glycolate oxidase, urate oxidase) that produce peroxide directly without first producing $O_2^{-\cdot}$. Detection of H_2O_2 therefore becomes an important analytical task to perform and several techniques have been devised to accomplish this (S. Dikalov, Griendling, & Harrison, 2007; S. I. Dikalov, Li, Mehranpour, Wang, & Zafari, 2007).Hydrogen peroxide measurements have been conducted on lung samples that involve techniques measuring H_2O_2 in exhaled breath condensate (Brooks, Lash, Kettle, & Epton, 2006; Gerritsen, Zanen, Bauwens, van den Bosch, & Haas, 2005; Hitka, 2003; Hitka,

Vizek, & Wilhelm, 2003). Lung macrophage cells obtained by bronchoalveolar lavage have been assayed for H_2O_2 production using the horseradish peroxidase-mediated red phenol method (Souza, Poggetti, Fontes, & Birolini, 2000).

Other techniques employing dihydrofluorescein-diacetate (Hfluor-DA) in diaphragm muscle are based on peroxide-dependant oxidation of the reduced chemical probe to fluorescein (Fluor) by intra-cellular H_2O_2 (Zuo & Clanton, 2005). Hypoxic diaphragm strips were exposed to the probe and examined by tissue fluorometry. Dihydrofluorescein diacetate has advantages in that this probe loads easily and lacks sensitivity to nitric oxide. Fluorescence intensity alterations were examined. The investigators used two concentrations of the probe: Hfluor-DA 50 µM and Fluor-DA 0.05 µM for 30 minutes followed by a buffer wash. The second concentration focused on the reversibility of the Fluor-DA signal compared to ebselen, a free radical scavenger glutathione mimic that is known to scavenge H_2O_2.

Oxygen

Oxygen is a colorless, odorless, tasteless gas that makes up 21% of the atmosphere of the earth by volume when the atmosphere is dry. As a highly reactive gas, O_2 combines with most other elements to form oxides and O_2 is a component of hundreds of thousands of organic compounds essential to animal and plant life. When O_2 is absorbed through the lungs into the blood, it is used in cellular respiration, the process in which organisms release stored chemical energy. The reduction of O_2 to H_2O by the mitochondrial electron transport chain helps supply the metabolic demands of human life (Maltepe & Saugstad, 2008). Normal cellular function and survival depend on a

continuous supply of O_2 for aerobic metabolism since O_2 cannot be stored in cells (Huang, 2005).

Oxygenation

Oxygenation is the process of O_2 diffusing passively from the alveolus to the pulmonary capillary where it binds to hemoglobin in red blood cells or dissolves into the plasma (Levy, 2005). The PaO_2 is a reflection of the number of dissolved O_2 molecules in the plasma not bound to hemoglobin that are free to exert pressure on the measuring O_2 electrode. The number of O_2 molecules dissolved in the plasma along with other factors determines how many molecules will be bound to hemoglobin. Bound O_2 can no longer exert pressure on the measuring O_2 electrode. The PaO_2 does not indicate how much is in the blood without considering the amount of bound O_2 (SaO_2) and the hemoglobin content. The normal PaO_2 at sea level is 75 -100 mm Hg. (Morton, 2005).

The O_2 saturation (SpO_2) reflects the percentage of hemoglobin saturation by O_2, not the PaO_2. While monitored continuously the SpO_2 provides immediate information about O_2 transport and O_2 hemoglobin saturation but does not provide information regarding ventilation, acid base balance, serum hemoglobin or how well the oxygenated hemoglobin is being delivered to the tissues (Grap, Blecha, & Munro, 2002; Keidan et al., 2008).

Oxygenation can be further understood in terms of DO_2 from the lungs to the peripheral tissues and utilization (VO_2), whereby total DO_2 is the product of cardiac index (L/min per m^2) and arterial O_2 content and hemoglobin (Gutierrez, Reines, et al., 2004; Gutierrez, Wulf-Gutierrez, & Reines, 2004; M. Wilson, et al., 2003). In HS, the

DO_2 is reduced as cardiac output, arterial saturation and hemoglobin are unable to adequately deliver O_2 to the tissues (Cottingham, 2006; Goodrich, 2006).

Systemic O_2 utilization (VO_2) is proportional to the metabolic rate and varies according to the body's energy needs. Oxygen utilization is the difference between O_2 saturation in arterial and mixed venous blood. This is the rate at which O_2 is removed from the blood for use by the respiring cells and tissues (Gutierrez et al., 2004; Wilson et al., 2003). In HS, the VO_2 is reduced as delivery becomes impaired. Normally the DO_2 and VO_2 are in balance to maintain homeostasis, but when the DO_2 falls, the cells work to extract more O_2 for metabolism to continue from the mixed venous blood returning to the right side of the heart. When tissues can no longer extract O_2 to compensate for the fall in DO_2, consumption dependency occurs (Cottingham, 2006; Goodrich, 2006).

An oxygenation index assessing tissue oxygenation is the fraction of O_2 consumed to O_2 delivered (VO_2/DO_2) to the tissues, termed the O_2 extraction ratio. The normal O_2 extraction ratio is 22% - 32% (Gutierrez, Reines, et al., 2004; Gutierrez, Wulf-Gutierrez, et al., 2004; M. Wilson, et al., 2003). An O_2 extraction ratio > 0.4 indicates insufficient O_2 and the accumulation of O_2 debt (Wilson et al., 2003). While the formulas exist to assess oxygenation status and bedside technology provides immediate assessments, prescribing and monitoring O_2 has been shown to be sub-optimal and cautionary supplemental O_2 use is recommended out of concern for over oxygenation (Brokalaki et al., 2008; Saugstad, 2006a, 2006b; Saugstad, Ramji, & Vento, 2006). The only known cause of hyperoxemia is the excess and unnecessary

administration of O_2 by healthcare providers (Downs, 2003; Sola, 2008a, 2008b; Sola, Saldeno, & Favareto, 2008; Tin & Gupta, 2007).

Oxygen Toxicity

Oxygen is essential to all forms of aerobic life and all living organisms are at risk of oxidation from molecular O_2. Aerobic life forms have evolved antioxidant defenses to assist with this potential problem (Chandel & Budinger, 2007; Kazzaz et al., 1996; Kulkarni, Kuppusamy, & Parinandi, 2007). These antioxidant defenses can be overwhelmed by oxidative insults, including supraphysiologic concentrations of O_2 (hyperoxia). However, at sea level O_2 can be toxic when breathed for more than 24 hours at a percentage greater than about 40% (Patel, Paluszynska, Harris, & Thach, 2003).

Oxygen toxicity occurs when the partial pressure of alveolar O_2 (P_AO_2) rises above a critical level and the amount of O_2 transported across the alveolar capillary membrane dissolved in the blood reaches the tissues at higher than normal (20 mm Hg – 60 mm Hg) tissue PaO_2. Extremely high tissue PaO_2 that occurs when O_2 is breathed at very high P_AO_2 is detrimental to many of the body's tissues (Jamieson, et al., 1986).

Symptoms of O_2 toxicity often observed in patients include nausea, muscle twitching, dizziness, and disturbances in vision, irritability, disorientation, seizures, coma and death. These symptoms occur when the molecular O_2, converted to O_2 free radicals, overwhelm the tissues ability to remove them. Tissues possess enzymatic defenses mechanisms to prevent free radical damage such as glutathione peroxidase

(GSH), catalase and SOD. Therefore, when the hemoglobin O_2 buffering system maintains the normal tissue PaO_2 the oxidizing free radicals are removed rapidly enough to prevent cellular injury (Guyton, 2006).

There are two forms of O_2 toxicity: 1) central nervous system O_2 toxicity and 2) pulmonary O_2 toxicity. In pulmonary O_2 toxicity, the lung is the primary organ affected by high FIO_2 (Buccellato, Tso, Akinci, Chandel, & Budinger, 2004; Fu, et al., 2008; Joseph, 2008; Pendyala et al., 2009; Zaher, Miller, Morrow, Javdan, & Mantell, 2007). Lungs act as a barrier to keep the rest of the body from receiving elevated O_2 concentrations. The vast surface area of the lungs makes the alveolar capillary membrane an efficient filtering mechanism. The alveolar capillary membrane absorbs the effects of elevated O_2 concentrations. The amount and type of damage that occurs to lungs is dependent on the FIO_2 and duration of exposure to high O_2 consumption. There are five phases of pulmonary O_2 toxicity: 1) initiation, 2) inflammation, 3) destruction, 4) proliferation and 5) fibrosis (Crapo, 1986; Crapo, Barry, Chang, & Mercer, 1984; Mensack, 1999).

Hyperoxia

Hyperoxia is defined as an excess of O_2 in tissues and organs. Hyperoxia is also defined as a higher than normal O_2 tension such as that produced by breathing air or O_2 at greater than atmospheric pressure (C. Li & Jackson, 2002). Hyperoxia induces ROS formation in lung capillary endothelial cells, which initially originates from the mitochondrial electron transport chain. Subsequently there is activation of NADPH oxidase by endothelial calcium signaling and Rac1 activation associated with actin

remodeling. The researchers measured endothelial cell concentration of ROS and cytosolic calcium by in-situ imaging of fluorescence in isolated perfused rat lungs (Brueckl, et al., 2006).

Zhang and Foda (2004) investigated the pathways to cell death in hyperoxia-induced lung injury and the functional significance of apoptosis in-vivo in response to hyperoxia. Hyperoxia caused acute lung injury. Hyperoxic stress resulted in marked epithelium sloughing and increased apoptosis in both alveolar epithelial cells and bronchial epithelial cells. This was accompanied by increased expression of caspase-3 messenger ribonucleic acid (MRNA) in lung tissues after hyperoxia. Caspase-3 protein was located in the cytoplasm and nuclei of airway epithelial cells, alveolar epithelial cells and macrophages in hyperoxic lung injury (Zhang & Foda, 2004).

Exposure of O_2 (60% for 24 hours) to type II alveolar epithelial cells which are the source of pulmonary surfactant, produced ROS, malondialdehyde, apoptosis and a decline in total antioxidant capacity compared to rats in the 21% group (Fu, et al., 2008). In addition, in rat alveolar epithelial cells investigators have found that exposure to hyperoxia resulted in ROS generation that was completely prevented by the administration of the combined SOD/catalase mimetic EUK-134. Hyperoxic exposure resulted in the activation of Bax at the mitochondrial membrane, cytochrome c release and cell death (Buccellato, et al., 2004).

With hyperoxia, mitochondrial functioning of pulmonary epithelial and vascular endothelial cells is impaired (Pendyala, Gorshkova, et al., 2009; Pendyala, Usatyuk, Gorshkova, Garcia, & Natarajan, 2009; Zaher, et al., 2007). Hyperpermeability of

endothelial cells following HS and reoxygenation occurs by hyperoxic induced ROS (Ellman, et al., 2005; Zhang & Foda, 2004).

Hyperoxic reoxygenation during HS results in tissue injury and ROS is ubiquitous, therefore widespread cell injury occurs. Researchers demonstrated that 100% O_2 in progressive hemorrhage produced proportional declines in liver, muscle and bladder tissue PO_2. Renal cortical PO_2 was maintained until profound blood loss had occurred. There was variability in tissue O_2 extraction during HS thus variability in ROS generation was observed (Dyson, et al., 2007).

Apoptosis

Apoptosis (programmed cell death) is a non-inflammatory form of cell death. Apoptosis is necessary for eliminating unwanted damage to redundant cells and for maintaining homeostasis and growth in the tissues. Apoptosis represents the opposing function to cell division, cell death (J. F. Kerr, Wyllie, & Currie, 1972).

Regulation of apoptosis occurs by the interaction of three key genes; Ced-3, Ced-4, and Ced-9. Normally Ced-9 is associated functionally with Ced-4 and Ced-3, keeping Ced-3 inactive. Apoptosis stimuli cause Ced-9 dissociation, allowing Ced-3 activation, committing the cell to die by apoptosis (Ashkenazi & Dixit, 1998).

This process follows three progressive phases of initiation, execution and removal. The initiation phase is marked by a time when caspases become catalytically active. The execution phase is when these enzymes act to cause cell death. The removal phase occurs when phagocytes recognize marker molecules on the apoptotic cell surface and bind for uptake and engulfment (Kumar, 2009).

Caspase proteins are responsible for cell death and are involved in a wide variety of human disease. These proteins are activated through two distinct cell death signal transduction pathways. The extrinsic pathway may involve a death ligand receptor such as FasL/Fas. Fas are also known as Apol-1 and Ced-95. Fas are members of the tumor necrosis factor (TNF) receptor family. The cytoplasmic domain of Fas/FasL contains the death domain. Initiator Fas/FasL binding activates a trio of death domains, creating an intracellular binding site for FADD (Fas associated proteins with death domain). This Fas/FADD complex binds procaspase-8 with another death effector domain. This leads to activation of caspases 3, 6, and 7 which commit the cell to death (Gewies & Grimm, 2003).

The intrinsic mitochondrial cell death signal transduction pathway is activated by exogenous extra-cellular signals such as ultraviolet radiation, chemical toxins or cellular responses to metabolic or cell cycle disruptions. Transcytoplasmic protein signals trigger the mitochondria to initiate the cell death pathway and cytochrome c is then released into the cytosol. Cytochrome c binds Apaf-1 and caspase-9. This trio apoptosome cleaves procaspase-3 leading to cell death via its active form caspase-3 (Pollack & Leeuwenburgh, 2001).

Apoptosis and Hemorrhagic Shock

In HS apoptosis has been shown to occur in lung and visceral organs. Microvascular dysoxia initiates apoptotic signals. In animal models apoptosis has been demonstrated to be a major factor impacting survival (Davidson et al., 2004; J. Y. Lu et al., 2005; J. Y. Lu et al., 2004; Q. Lu, Harrington, & Rounds, 2005; Q. Lu, et al., 2004).

Additionally, certain treatment regimens have shown to decrease or accelerate programmed cell death (Deb et al., 1999; Deb et al., 2000).

Investigators studying HS have found that lung endothelial and epithelial apoptosis is stimulated by mesenteric lymph factors (Q. Lu, et al., 2004). Apoptosis in neutrophils, macrophages and alveolar cells was induced within the lung following HS (N. L. Jernigan, B. R. Walker et al., 2004). Acute lung injury occurred with HS. Activation of apoptotic and non-apoptotic/inflammatory Fas signaling on lung epithelial cells was found to be an early pathophysiological event in the development of indirect acute lung injury after HS and sepsis (Perl et al., 2007). Two studies have demonstrated that in rats following HS, lung microvascular endothelial cells were hyperpermeable by mitochondrial ROS and this initiate's intrinsic pathway Bak-induced apoptosis (Childs et al., 2008).

Visceral organ susceptibility to apoptosis occurs in HS (Childs, et al., 2007; J. Y. Lu, et al., 2005; Paxian et al., 2003; Sundar, Li, Rollwagen, & Maheshwari, 2005). In HS, visceral organ oxidative stress causes immediate up-regulation of SOD indicating existence of ROS, an intrinsic pathway trigger of apoptosis (Sundar, et al., 2005). Endothelial dysfunction resulting in mesenteric microvascular hyperpermeability is initiated by the apoptotic intrinsic pathway in HS (Childs, et al., 2008). The extent of liver apoptosis induced by trauma/HS can be prevented by administration of IL-6 at the start of resuscitation (Moran et al., 2008).

There is extensive research related to HS resuscitation and apoptosis (Murao, Hata et al., 2003; Murao, Loomis, Wolf, Hoyt, & Junger, 2003; Parreira et al., 2004;

Shires et al., 2005) and the data suggests that the type of fluid resuscitation therapy used may increase apoptosis. Several studies demonstrate increased apoptosis when LR was administered as a treatment for HS (Murao, Hata et al., 2003; Murao, Loomis et al., 2003; D. Subrato, Martin, B., Sun, L., Ruff, P., Burris, D., Rich, N., 1999; D. Subrato, Sun, L., Barry, M., Talens, E., Burris, D., & Kaufmann, C., 2000).

Though there are many studies related to HS and apoptosis, there are no studies specifically measuring lung and diaphragm H_2O_2 production and apoptosis in HS using various FIO_2 concentrations.

Apoptosis Measurement

Many cytochemical and immunohistochemical methods are available to determine if a cell is apoptotic. Two most frequently used methods are terminal deoxynucleotidyl nick end labeling (TUNEL) and in situ nick translation (ISNT). TUNEL and ISNT are based on the TdT-mediated dUTP-biotin nick end-labeling method which detects apoptotic cells by using the enzyme terminal deoxynucleotidyl transferase (TdT) to directly label the fragmented DNA ends (J. Y. Lu, et al., 2005; Save, Hall, & Coates, 2004; Sundar, et al., 2005; VandenBroeck, 2002).

Gel electrophoresis is another method which detects movement of charged DNA fragments associated with apoptotic changes (Cornelissen, Armstrong, & Holt, 2004; Guan, Jin, Jin, & Lu, 2002; Murao et al., 2003; Murao, Loomis, Wolf, Hoyt, & Junger, 2003). The single cell gel electrophoresis assay (comet assay), is a simple, visual and sensitive technique for measuring DNA breakage in individual cells (McKelvey-Martin et al., 1993). The sensitivity of the method has been compared to flow cytometry in

assessing apoptosis and found to be more sensitive than standard DNA flow cytometry to detect early DNA fragmentation events occurring during apoptosis (Godard et al., 1999).

Annexin-v staining is used to detect distribution changes of membrane phospholipids undergoing apoptosis. The presence of phosphotidylserine on the cell membranes external surface is a marker. Annexin-v is used with propidium iodide to distinguish apoptosis from necrosis as it also binds to the internal surface phosphotidylserine. When measured over time, Annexin-v and propidium iodide can be used to monitor the progression of apoptosis (Mari et al., 2004; VandenBroeck, 2002).

Flow cytometry (FCM) provides data on cell cycle distribution and cell death. This method uses light scatter analysis on cell particles passing single file through a saline sleeve intersecting its beam to indicate size and density. Flow cytometry uses a laser to excite DNA binding fluorescent dye such as PI (dark red) or Hfluor-DA (green). During and following laser excitation each cell or particle scatters the light or emits fluorescence characteristically allowing for analysis of fluctuations in brightness. Entire cell constituents are uniformly illuminated as the aim of the preparation is to prepare a suspension of single cell particles that have been stained in a specific way to identify a particular characteristic (Cram, 2002; Mittag et al., 2006; Mittag, Lenz, Gerstner, & Tarnok, 2006). The signal intensity indicates DNA content in each cell (Delogu, Moretti, Famularo, Antonucci, et al., 2001; Delogu, Moretti, Famularo, Marcellini, et al., 2001; Q. Lu, et al., 2005; McCarthy & Evan, 1998).

Laser scanning cytometry (LSCM) can be used to quantify apoptosis using whole tissue, thereby avoiding the steps required to get the cells into suspension. The data are generated by analysis of the light scatter emitted from fluorochromed individual cells on a microscope slide. The cells that have been treated with one or more fluorescent dyes in order to rapidly detail multiple cellular constituents and other features of the cells are scanned (Darzynkiewicz, Bedner, Li, Gorczyca, & Melamed, 1999; Darzynkiewicz, Huang, Okafuji, & King, 2004; Kamentsky, 2001; Luther, Kamentsky, Henriksen, & Holden, 2004). With this method there is less sample damage that comes from digestion and filtration of tissue components required in FCM. Prepared tissue is mounted on slides and confocal images plot cell coordinates and locate and analyze pixel fluorescence intensity of cellular constituents.

Apoptosis can be quantified by another frequently used method involving fluorescent microscopy and differential dye uptake and two fluorescent dyes: acridine orange and ethidium bromide (C. Goodyear-Bruch, Simon, Hall, Mayo, & Pierce, 2005). These dyes can be used to determine which cells in a population have undergone apoptosis and whether the cell is in early or late states of apoptosis, based on membrane integrity. Acridine orange intercalates into the DNA giving it a green appearance. This dye also binds to RNA giving it a red orange appearance. Hence, a viable cell will have a bright green nucleus and red cytoplasm. Ethidium bromide is only taken up by non-viable cells. Ethidium bromide binds weakly to RNA yielding a red color. Thus, dead cells have bright orange nuclei and red cytoplasm. Non-damaged nuclei in live cells will

fluoresce bright green. In contrast, damaged nuclei in dead cells fluoresce bright orange (McGahon, Martin, et al., 1995; McGahon, Nishioka, et al., 1995).

Dopamine

Dopamine (DA) is a hormone and a neurotransmitter occurring in a wide variety of animals. As a naturally occurring endogenous catecholamine, DA is an immediate precursor to norepinepherine and epinephrine found in both the central and peripheral nervous systems. Dopamine is a sympathomimetic agent that stimulates both alpha and beta adrenergic receptors. Used in the clinical setting, DA is an inotropic drug and is administered with caution to increase cardiac output and blood pressure in HS when fluids alone are ineffective as dopamine in HS can contribute to further injury (Beale, Hollenberg, Vincent, & Parrillo, 2004). Advanced Cardiac Life Support guidelines list use of DA for hemodynamic support during cardiopulmonary resuscitation (Dager, 2006).

Dopamine is sometimes used at low doses to improve renal perfusion (Dellinger & Vincent, 2005; Friedrich, Adhikari, Herridge, & Beyene, 2005; Schenarts et al., 2006; Venkataraman & Kellum, 2007a, 2007b). Dopamine is also used in the treatment of Parkinson's disease (Barone, 2003), heart failure (Hewing & Stangl, 2007; Shin et al., 2007) and hypotension (Amenta, Ricci, Rossodivita, Avola, & Tayebati, 2001; Banday, Lau, & Lokhandwala, 2008).

Dopamine and ROS

Dopamine can either be a free radical scavenger or a free radical generator, cytoprotective or cytotoxic (Cosentino et al., 2004). Depending on the concentration of

DA, O_2 availability and the type of tissue, DA can be beneficial or harmful (J. D. Pierce, et al., 2006). For example, diaphragm studies have demonstrated that DA improves blood flow and O_2 availability, thus improving diaphragm performance by scavenging ROS (J. D. Pierce, Clancy, Smith-Blair, & Kraft, 2002; J. D. Pierce, et al., 2008). In these studies, DA attenuated apoptosis possibly by scavenging ROS. Various FIO_2 (95%, 21% or 10%) superfused to the in-vitro diaphragm strips along with DA, inhibited apoptotic pathways, stimulated antiapoptotic pathways and scavenged free radicals (J. D. Pierce, et al., 2006).

Dopamine receptors are involved in regulation of ROS production. The activation of DA receptors in the cardiovascular and renal systems (Amenta, et al., 2001; Jose, Eisner, & Felder, 2003a, 2003b) may contribute to ROS-dependent hypertension or have an antihypertensive effect (D. Yang, et al., 2007; Z. Yang et al., 2006) depending on ROS effects on DA receptors.

Dopamine Receptor Activation

As a neurotransmitter, DA stimulates five types of dopamine receptors: D1 through D5 (Missale, Nash, Robinson, Jaber, & Caron, 1998; Neve, Seamans, & Trantham-Davidson, 2004; Zawilska, 2003). The effects of DA are determined by the interaction between DA and receptors. Depending on receptor location and type, effects of DA are complex. In 2007, Pierce et al. determined the presence of a specific subtype of DA receptors (D1-like) in the rat diaphragm. Using reverse transcriptase polymerase chain reaction, they found D1 and D5 receptors and not D2, D3, or D4 receptors in the rat diaphragm model (J. D. Pierce, et al., 2007).

The actions of DA on different receptors are dose dependent. Lower doses (< 5 mcg/kg/min) stimulate α adrenergic and dopaminergic receptors, which cause renal, mesenteric, cerebral and coronary bed vasodilatation, thus increasing blood flow to these tissues. Moderate doses (5-10 mcg/kg/min) stimulate β-1 receptors which increase myocardial contractility and heart rate, improving cardiac output. Higher doses (> 10 mcg/kg per minute) stimulate β-2 receptors and results in an increase in systemic vascular resistance, which may counteract the actions of the dopaminergic and β-1 receptors (Irwin, 2000; Kluwer, 2005; Thelan, 2005). At a dose greater than 20 mcg/kg/min, DA stimulates α adrenoreceptors leading to peripheral vasoconstriction and decreased renal perfusion (Guy, 2010).

The stimulation of α adrenoreceptors is protective to cardiomyocytes. Researchers indicated that the role for α adrenoreceptors is to increase glucose transport by translocating (GLUT)-1 and (GLUT)-4 to the plasma membrane. There is also an increase in glycogenolysis by activating phosphorylase kinase with an increasing rate of glycolysis by activating phosphofructokinase. This reduces intracellular acidity by activating the Na+/H+ exchanger and inhibits apoptosis by increasing levels of the antiapoptotic protein Bcl-2 (Salvi, Carrupt, Tillement, & Testa, 2001).

The effects of α and ß adrenoreceptor stimulation on protein synthesis and apoptosis have been investigated. Alpha adrenoreceptors stimulate vascular smooth muscle cell DNA synthesis and may contribute to vascular remodeling that occurs in hypertension and atherosclerosis (deBlois et al., 1996). Researchers found a significant negative correlation between catecholamine-evoked apoptosis and catecholamine-

induced hypertrophy in rat ventricular cardiomyocytes (Ponicke, Heinroth-Hoffmann, & Brodde, 2003).

When examining vasopressor use in HS patients, investigators found that early vasopressor use (within 12 hours after injury) was associated with significantly greater mortality than those resuscitated with early crystalloids. Phenylephrine, norepinephrine, DA and vasopressin were examined. These patients are more severely injured and have greater physiologic derangements that require intervention and critical care. Vasopressor use is recommended with caution only after crystalloid resuscitation fails to maintain adequate tissue perfusion (Sperry et al., 2008).

Current American College of Surgeons Advanced Trauma Life Support (ATLS) guidelines caution that the use of exogenous vasoconstrictors in patients experiencing HS because their use can increase peripheral vascular resistance. However, the increase in resistance does not necessarily indicate an increasing cardiac output and may further reduce end organ perfusion and oxygenation, thus having a negative effect. ATLS guidelines recommend the use of DA to support hemodynamics when crystalloid solutions are ineffective (Trauma, 2008).

Dopamine and Apoptosis

Dopamine can decrease apoptosis by scavenging ROS, stimulating ß-2 adrenoreceptors, and by altering apoptotic proteins. Investigators found less apoptosis during inspiratory resistance loading when there was an infusion of low dose DA (2 mcg/kg/min) versus infusion of saline alone. There was less DNA damage as measured

by comet assay. Dopamine decreased ROS measured by apoptosis and comet analysis (J. D. Pierce, et al., 2008).

A study by Goodyear-Bruch et al. has determined that in the rat diaphragm during inspiratory resistance loading there was a significant increase in the expression of SOD (CuZn) and Proprioceptive Event Related Potential (PERP) with DA, not normal saline, suggesting that DA reduces apoptosis by increasing SOD. Out of 27 apoptotic-related proteins tested, 12 were found in the rat diaphragm (C. A. Goodyear-Bruch, et al., 2008).

Additionally, research has found that β-1 adrenoreceptor stimulation to be pro-apoptotic and β-2 adrenoreceptor stimulation can be antiapoptotic in rat cardiomyocytes. Neither ß-1 nor ß-2 stimulation had an effect on basal apoptosis; however, selective ß-*2* adrenoreceptor stimulation protected cells from apoptosis (Chesley et al., 2000; Singh, Communal, Sawyer, & Colucci, 2000) and can involve the ROS/JNK-dependant activation of the mitochondria death pathway (Remondino et al., 2003).

Other investigations have determined that the effects of DA on oxidative metabolism and apoptosis can be dose dependent. Higher doses of DA (100-500 µM), increased intracellular ROS and apoptotic cell death and lower doses (0.1-5 µM) decreased ROS levels and apoptosis. Dopamine at 1 µM and 500 µM partially counteracted the decrease in Cu/Zn SOD (Cosentino, et al., 2004).

Dopamine stimulates α and β adrenoreceptors affecting hypertrophy by α stimulation and apoptosis by β-2 stimulation in cardiomyocytes. Myocyte contractility increased the number of TUNEL positive nuclei significantly higher than in untreated

post ischemic hearts. Additionally, the investigators found PARP cleavage, activation of mitochondrial caspase 9, and the terminal effector caspase-3. Dopamine increased cellular content of proapoptotic Bax while decreasing antiapoptotic Bcl-2 (Stamm et al., 2002). Dopamine also induces apoptosis primarily by generating ROS, p38 kinase and the intrinsic apoptotic cascade (Eunsung, 2001).

Significance

Determination of optimal FIO_2 used to resuscitate HS patients is important to investigate to reduce organ and cell damage. Use of high FIO_2 concentrations may produce ROS which may cause lung and diaphragm injury. However, use of low FIO_2 concentrations may not provide sufficient O_2 supply and thus may not restore cellular functioning. By investigating the effects of administering various FIO_2 following HS, we can determine what is the optimal O_2 that reduces lung and diaphragm injury. When resuscitation from HS is based on cellular processes and not just hemodynamics, tissue injury may be significantly reduced.

With current wars in Iraq and Afghanistan, the highest rates of combat casualties since the Vietnam War have been seen by the U.S. military. The injuries have no common civilian equivalent. Many require greater than 10 units of packed red blood cells (PRBCs) in less than 24 hours (Beekley, 2008; Beekley et al., 2008; Trauma, 2008). Since HS is the leading cause of death in civilian and military populations (Alam & Rhee, 2007), determining an optimal concentration of O_2 for resuscitation could be effective in reducing lung and diaphragm injury and improving patient outcomes.

This research is significant because it is one way to demonstrate how to optimize cellular functioning in HS by optimizing oxygen concentrations. Hyperoxic-induced ROS generation in diaphragm tissue during reoxygenation in HS is not in the literature. Therefore this research would assist in understanding cellular processes occurring in the diaphragm in HS. If we can determine by LSCM a change in HFLUOR-DA as it is oxidized to FLUOR in the presence of cellular H_2O_2 in diaphragm tissue following HS, across various FIO_2s, maybe this would stimulate further research using other tissues in a similar experimental design.

Oxidant antioxidant imbalance impairs normal cellular functioning and is implicated in many pathological conditions. Reoxygenation during HS can inadvertently extend the pathological state in HS. Accurately describing this mechanism for such injury in diaphragm and lung is significant and could lead to further understanding of the effects of various FIO_2 on the mitochondria. With hyperoxia, increasing ROS are generated and this is damaging to the mitochondria as ROS consume antioxidant enzymes such as SOD, catalase and glutathione peroxidase. Mitochondrial release of cytochrome c follows. Cytochrome c activates caspase-9 and caspase-9 binds apoptosis protease activating factor 1 (Apaf-1) with ATP. This complex activates caspase-3 which initiates apoptosis. Thus, ROS and apoptosis are associated key factors in the pathological mechanisms associated with many conditions.

We believe that using two different types of measures (physiologic and molecular) is an innovative approach. Since no one has examined which FIO_2 is optimal to decrease lung and diaphragm damage following HS, these data will provide

information to assist scientists and health care professionals in understanding and treating patients who experience HS.

Chapter 2.

Consequences of Hyperoxia and the Toxicity of Oxygen in the Lung

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Abstract

Oxygen is life-essential but like a drug has a maximum positive biological benefit and accompanying side effects. Oxygen is therapeutic for treatment of hypoxemia and hypoxia associated with many pathological processes, one of which is HS. In HS, injury can occur secondarily during O_2 resuscitation as supraphysiologic levels of O_2 (> 21%) can be damaging to lung tissues. This pathophysiological process is associated with increased levels of hyperoxia-induced ROS such as $O_2^{-\cdot}$, H_2O_2 and $HO^{\cdot}$. Reactive oxygen species readily react with surrounding biological tissues exerting effects on lipids, proteins and nucleic acids. Endogenous cellular antioxidant defenses can become inundated with ROS leading to oxidative stress. Reactive oxygen species affect the pulmonary microcirculation. Activated alveolar capillary endothelium possesses increased adhesiveness causing accumulation of cell populations such as neutrophils, which are also a source of H_2O_2. Increased levels of ROS cause hyperpermeability, coagulopathy and collagen deposition among other changes occurring within the alveolar space. In hypoxia and hyperoxia, multiple signaling pathways determine the pulmonary cellular response; apoptosis, necrosis or repair. Understanding the effects of O_2 administration is important to prevent inadvertent alveolar damage in patients requiring supplemental oxygenation.

Key Words: oxygen toxicity, reactive oxygen species, antioxidants, acute lung injury

Introduction

Oxygen is vital to life, a basic element and a drug. Significant risk for inadvertent toxicity may exist when treating hypoxemia in emergent trauma patients as well as in patients with chronic disease. Annually, the need for supplemental O_2 is projected to be around 800,000 individuals at a cost of 1.8 billion dollars (Kim, Benditt, Wise, & Sharafkhaneh, 2008) and in excess of three billion dollars for long-term O_2 therapy for individuals who need it continuously (Casaburi, 2009; Cullen & Stiffler, 2009). Oxygen is a costly drug for patients and per day in-patient costs climb when O_2 is required (C. Wong et al., 2000; H. Wong et al., 2009). Suboptimal use of O_2 is reflected in prescription and treatment errors that exceed those related to antibiotics (Boyle, O'Connell, Platt, & Albert, 2006; Brokalaki et al., 2004; Hickey, 2007).

The lungs are anatomically an open, large epithelial, surfactant surface organized such that the alveolar epithelial and alveolar capillary endothelial cells become vulnerable targets for O_2 free radical-induced injury caused by hyperoxia. In acute lung injury, hyperpermeability of the pulmonary microvasculature causes flooding of the alveolus with plasma extravasations leading to pulmonary edema (Kumar, 2009). Abnormalities in the coagulation and fibrinolysis pathways promote fibrin deposition in the acutely injured lung (Idell, 2003; Shetty, Padijnayayveetil, Tucker, Stankowska, & Idell, 2008). Gap junctional proteins called connexins of type II alveolar epithelial cells are injured by O_2 free radicals leading to impairment of surfactant production (Johnson & Koval, 2009). Injury to type II alveolar cells causes collagen deposition and fibrosis of alveolar architecture leading to atelectasis (Kumar, 2009). Thus, the maximum

positive biological benefit for this life-essential but toxic element exists along a dose-response, deficiency toxicity continuum accompanied by an inverted bathtub curve.

The Formation of Free Radicals

Oxygen is a requirement for cellular respiration in the metabolism of glucose and ATP generation (Kulkarni, et al., 2007). However, toxic levels of O_2 lead to the formation of free radicals, especially ROS, which can impose cellular damage on lipid membranes, proteins and nucleic acids. Toxic levels of O_2 cause observable shape change to the alveolus. When O_2 is absorbed by the lungs into the blood and used in cellular respiration, organisms release stored chemical energy (O'Driscoll, Howard, & Davison, 2008). All eukaryotic aerobic organisms consume O_2 from the atmosphere and use the Krebs cycle and oxidative phosphorylation in turn to generate ATP from glucose (Taylor & Pouyssegur, 2007). Oxygen is required for the slow controlled metabolism of food materials by oxidation for ATP generation (Newmeyer & Ferguson-Miller, 2003). The form of energy directly involved in the process of ATP synthesis is not the chemical but the electrical component of the proton motive force (a proton voltage gradient across a membrane) as electrons move from an area of high concentration to an area of low concentration (Reynafarje & Ferreira, 2008).

Free radicals are a type of unstable, reactive, short-lived chemical species that have one or more unpaired electrons. The species is termed free because the unpaired electron in the outer orbit is free to interact with surrounding molecules (Tandon, Sinha, Garg, Khanna, & Khanna, 2005). Free radicals may possess a net charge or be neutral, and all are reactive. Two types of free radicals of biological importance are ROS and

RNS which are active in physiological and pathophysiological roles within the body (Valko et al., 2007). Cells generate free radicals, specifically ROS, by the reduction of molecular O_2 to H_2O (see Fig. 1) (Apel & Hirt, 2004; Sakac & Sakac, 2000). Reactive O_2 species are produced as a consequence of mitochondrial respiration (Halliwell, 1984a, 1984b; Voeikov, 2001). Superoxide and the $HO^{\cdot}$ are two examples of highly unstable molecules that have electrons available to react with lipids, proteins and nucleic acids (Pham-Huy, 2008; Raha & Robinson, 2001). Hydrogen peroxide, a non-radical O_2 derivative, is formed by the reduction of $O_2^{-\cdot}$ and further reduction to $HO^{\cdot}$ (Kazzaz, et al., 1996; Kohen & Nyska, 2002). Endogenous sources of ROS include enzymes, cells such as neutrophils, organelles such as mitochondria and disease. Other sources of ROS are exogenous such as radiation, pollutants, xenobiotics and toxins.

As aerobic organisms bathed in approximately 21% O_2, cellular survival and adaptation in an oxidative atmosphere are dependent upon adequate antioxidant defense mechanisms to counteract the effects of free radicals, especially ROS, on cells and tissues (Comhair, Bhathena, Farver, Thunnissen, & Erzurum, 2001). When an oxidant/antioxidant imbalance occurs as a consequence of hyperoxia, damage to tissues occurs (Misra, Sarwat, Bhakuni, Tuteja, & Tuteja, 2009; Tkaczyk & Vizek, 2007). The damaging effects of hyperoxia can lead to O_2 toxicity, cell death and can be a triggering factor in acute lung injury (Pagano & Barazzone-Argiroffo, 2003; Pagano, Donati, Metrailler, & Barazzone Argiroffo, 2004).

Functions and Classifications of Antioxidants

An antioxidant (a reductant or reducing agent) is anything that can prevent or inhibit oxidation (Halliwell, 1990; Halliwell, Aeschbach, Loliger, & Aruoma, 1995; Hernandez-Saavedra & McCord, 2007). Delay of oxidation can be achieved by preventing the generation of ROS or by inactivating ROS (Buccellato, et al., 2004). Prevention, diversion, dismutation (decay), scavenging and quenching are specialized antioxidant properties (Table 1). Superoxide dismutase, catalase and GSH divert or dismutate ROS into harmless products. Ascorbic acid, vitamin E, uric acid, bilirubin and glutathione scavenge ROS by expendable, replaceable or recyclable substrates. Vitamin E and beta-carotene quench by absorption of electrons and or energy. Antioxidants can be classified into four categories based on function: 1) Preventive antioxidants suppress formation of free radicals, 2) radical scavenging antioxidants suppress chain initiation and/or break chain propagation reactions, 3) the repair and de novo antioxidants such as proteolytic enzymes and repair enzymes of DNA function as the third line of defense and 4) a fourth defense level called adaptation occurs when the signal for the production and reactions of free radicals induces formation and transport of the appropriate oxidant to the right site (Noguchi, Watanabe, & Shi, 2000; Rahman, Biswas, & Kode, 2006).

Antioxidant defenses may be classified as non-enzymatic and enzymatic or endogenous and dietary. Examples of non-enzymatic antioxidants are glutathione, vitamin C, vitamin E, beta-carotene and uric acid. Major enzymatic antioxidants are SOD, catalase and peroxidase. Antioxidants can also be classified as endogenous or dietary based on the ability of the antioxidant to be synthesized by humans. Endogenous

antioxidants are SOD, catalase, GSH, uric acid and bilirubin. Dietary antioxidants are vitamin C, vitamin E and beta-carotene (Bulger & Maier, 2001; Yamaoka et al., 2008).

Superoxide dismutase converts $O_2^{-\cdot}$ to H_2O_2. Superoxide dismutase has three isoforms widely distributed in mammalian organisms. 1) Cytoplasmic SOD (SOD1 or copper zinc SOD (CuZnSOD) is located in the cytoplasm, nucleus and peroxisomes, 2) mitochondrial SOD (SOD2 or MnSOD) is located in the mitochondrial matrix near the electron transport chain and 3) extracellular SOD (SOD3 or EcSOD) is found in the extracellular fluids and extracellular matrix of all human tissues especially the heart, placenta, pancreas and lung (Auten, O'Reilly, Oury, Nozik-Grayck, & Whorton, 2006; Van Raamsdonk & Hekimi, 2009). The protective effects of Ec SOD in the lungs are well established (Bowler et al., 2001; Gao, Kinnula, Myllarniemi, & Oury, 2008; Nozik-Grayck, Suliman, & Piantadosi, 2005). Over-expression of EcSOD in the airways reduces hyperoxic lung injury, is associated with decreased physical evidence of lung damage, reduced inflammatory cells and reduced lung wet to dry ratio (Folz, Abushamaa, & Suliman, 1999).

Catalase, one of the most potent catalysts found mostly in the peroxisome functions to change H_2O_2 to H_2O. Catalase defense from oxidant injury to lung epithelial cells exists in the cytosol or the mitochondria. One study found that mitochondrial catalase cells had significantly higher survival rates, best mitochondrial function and the lowest c-Jun NH_2-terminal kinase (JNK) activity. The protection with mitochondrial catalase was observed primarily in pulmonary type II epithelial cells (Arita et al., 2006).

Glutathione reductase is an important antioxidant enzyme for maintaining the intracellular reducing environment. This enzyme catalyzes the reduction of glutathione disulfide (GSSG) to glutathione (GSH) (O'Donovan & Fernandes, 2000). Glutathione disulfide is produced through the oxidation of glutathione by oxidants such as ROS that arise during conditions of oxidative stress. Due to the high concentrations of GSH, GSH/GSSG is considered to be the principal reduction oxidation buffer of the cell and the ratio of GSH/GSSG is viewed as a major indicator of the cellular redox status. The ratio of GSH/GSSG decreases under an oxidative stress condition and is increased under reduced conditions (Shi, Stevenson, Campopiano, & Greaney, 2006; Smith & Levander, 2002). Glutathione is increased in the lung when exposed to moderate ($FIO_2 = 0.40$) hyperoxia (Lee et al., 2005). The expression of antioxidant enzymes is induced by the transcription factor Nrf2, also known as nuclear factor erythroid derived 2 or like 2 (Cho et al., 2002; K. W. Kang, Lee, & Kim, 2005).

Oxidative Stress

Homeostasis between oxidants and antioxidants is highly regulated and extremely important for maintaining vital cellular and biochemical functions (Davies, 1995). Changing the balance toward an increase in the oxidant over the capacity of the antioxidant is defined as oxidative stress and can lead to oxidative damage. Altering the balance toward an increase in the reducing power of the antioxidant can also cause damage and is defined as reductive stress. Interference in the balance between oxidant and reductant allowing each to accumulate to levels greater than the other defines

oxidative or reductive stress conditions (Kohen & Nyska, 2002; Nishikawa & Inoue, 2008; Pham-Huy, 2008).

Disturbances in the reduction oxidation state of the cell causes toxicity (Lavrentiadou et al., 2001). Normally all cells maintain a reducing environment. More specifically, any compound that accepts electrons is an oxidant while any compound that donates electrons is a reductant. A chemical reaction in which a substance gains an electron is defined as reduction. In oxidation, not a gain but a loss of electrons occurs. When a reductant donates its electrons, it causes another substance to be reduced. In addition, when an oxidant accepts an electron, it causes another substance to be oxidized. An oxidation process is always accompanied by a reduction process in which there is usually a loss of O_2. In an oxidation process there is a gain in O_2. In chemistry such reactions are called reduction oxidation (redox) reactions (R. Chang, 2007). In biology these reactions are called antioxidant or pro-oxidant reactions (Droge, 2002; Kohen & Nyska, 2002).

Pathophysiology of Oxygen Toxicity

Hyperoxia is defined as an abnormally high O_2 tension in blood, or an excess supply of O_2 in tissues and organs. Oxygen toxicity occurs when the partial pressure of alveolar oxygen (P_AO_2) exceeds that which is breathed under normal conditions. Gravitational force holds the atmosphere to the earth, thus the air we breathe possesses weight. Standard atmospheric pressure at sea level is 760 mm Hg. Atmospheric pressure of 760 mm Hg multiplied by the percentage of O_2 in the atmosphere (0.21) yields a partial pressure of 159 mm Hg. The barometric pressure or atmospheric pressure is the

sum of the weight of atmospheric and hydrostatic pressure exerted by gravity and is 14.7 pounds per square inch at sea level. With O_2 exposure at an atmospheres absolute (ATA) of 1.4, the central nervous system (CNS) may demonstrate signs of toxicity (Guyton, 2006).

The lungs have the highest exposure to atmospheric O_2. The 70 to 100 square meters surface area of the human lung is closely apposed to the intravascular space, which can be as thin as 1 micron. Only about 10% of the total lung is solid tissue; the rest is composed of air and blood. The lung surface area of 480 million alveoli absorbs the air that is inspired (Ochs et al., 2004). The mass of epithelial surface areas of the airways and lungs are constantly exposed to O_2. The average adult inhales 10,000 to 20,000 liters of air per day, of which approximately 21% is O_2 and the rest is oxidants, particulates and infectious agents (Crapo et al., 2004). To accomplish the process of successful respiration from initial inhalation of O_2 through exhalation of CO_2 requires over 40 different cell types and their respiratory tract signals (Franks et al., 2008; Zaher, et al., 2007). Over 95% of all the O_2 we breathe undergoes a sequential four-electron reduction to produce H_2O in a reaction catalyzed by cytochrome c oxidase of complex IV in the mitochondrial electron transport chain (Cadenas & Davies, 2000).

The majority of O_2 consumed by the mitochondria is utilized for ATP generation (Min & Jian-xing, 2007). The mitochondrial electron transport chain reduces the elemental molecular O_2 to ionic O_2 by the relay of electrons. While making O_2 usable for ATP generation, oxidizing free radicals are generated (Czarna & Jarmuszkiewicz, 2006; Jezek & Hlavata, 2005). Other cellular sources of free radicals include

inflammatory cells, fibroblasts, endothelial cells, xanthine and NADPH oxidase (Sanders, Huecksteadt, Xu, Sturrock, & Hoidal, 1999). Extracellular sources of free radical generation are cigarette smoke, radiation, carcinogens, drugs, hyperoxia and ozone (Ciencewicki, Trivedi, & Kleeberger, 2008). One type of free radical generated from cellular sources is ROS which are produced continuously in many biological processes demonstrated in experimental and human models (Ames, Shigenaga, & Hagen, 1993).

Oxygen is a drug, and over exposure makes O_2 a mutagenic gas that can be toxic, causing significant damage to cells and tissues. This seemingly benign element our cells depend upon for ATP generation can actually do harm to our cells. Exposure to greater O_2 than normal atmospheric conditions is not always better (Altemeier & Sinclair, 2007). With continuous exposure to supraphysiologic concentrations of O_2, hyperoxia may develop. Under hyperoxic pathological conditions, a large in-flux of free radicals specifically ROS are produced. In biological systems (intracellular and extracellular), the mass effect of ROS elevation disrupts the balance between oxidants and antioxidants and this disruption of homeostasis can result in damage to cells and tissues (Ciencewicki, et al., 2008; T. L. Clanton, 2007; Rahman, et al., 2006; Yee et al., 2006).

Exposure time, atmospheric pressure and FIO_2 determine the cumulative O_2 dose leading to toxicity. Oxygen is toxic to the lungs when high FIO_2 (> 0.60) are administered over extended exposure time ($\geq$ 24 hours) at normal barometric pressure (1 ATA). This type of exposure is referred to as low pressure O_2 poisoning. Toxicity also occurs when the ATA is high (1.6 ATA) and the high FIO_2 exposure time is short. This

type of exposure is referred to as high pressure O_2 poisoning and is toxic to the CNS (Guyton, 2006; Joiner, 2001). Direct exposure can cause observable morphometric change in tissue structure, a direct result of hyperoxia and toxicity. The O_2 itself is directly damaging to cells and tissues in addition to the over-abundance of ROS that are generated from supraphysiologic levels of O_2 exposure (Crapo, 1986; Crapo, et al., 1984; Crapo & Tierney, 1974).

Exposure to a hyperoxic environment (> 0.9 ATA) leads to O_2 toxicity (Brueckl, et al., 2006; Cho, et al., 2002; Crapo, 1986; Pagano & Barazzone-Argiroffo, 2003). The severity of toxicity is based on exposure time and dose (both partial pressure and concentration) and defines the effects on the brain and lungs (Ay et al., 2007; Demchenko, Welty-Wolf, Allen, & Piantadosi, 2007). There are two types of O_2 toxicity: pulmonary toxicity and CNS toxicity (Allen, Demchenko, & Piantadosi, 2009; Halliwell, 2006; Hedley-Whyte, 2008). Pulmonary O_2 toxicity occurs with exposure to elevated levels of O_2 for several hours, causing damage to cell linings along the lung walls. Oxygen exposure of 1 ATA after approximately 12 hours leads to lung passageway congestion, pulmonary edema and atelectasis caused by damage to the linings of the bronchi and alveoli. The reason for this effect in the lungs but not in other tissues is that the air spaces of the lungs are directly exposed to the high O_2 pressure. Oxygen is delivered to the other body tissues at almost normal PaO_2 because of the hemoglobin-O_2 buffer system (Dash & Bassingthwaighte, 2006; C. I. Jones, 3rd et al., 2008; Zhou, Shi, & He, 2007). The formation of fluid in the lungs causes a feeling of shortness of breath combined with a burning of the throat and chest (Mason, 2005).

Oxygen toxicity occurs when the P_AO_2 is in excess of that breathed under normal atmospheric conditions. The above normal O_2 levels over extended time periods cause cell damage and death. For example, breathing O_2 at 4 ATA will cause CNS toxicity with seizures followed by coma in most people within 30 to 60 minutes. Seizures often occur without warning and are likely to be lethal. Other symptoms of CNS O_2 toxicity include nausea, muscle twitching, dizziness, and disturbances of vision, irritability and disorientation (Guyton, 2006). According to the National Oceanic Atmospheric Administration (NOAA) Diving Manual, O_2 exposure at 1.6 ATA for 45 minutes, 1.5 ATA for 120 minutes, 1.4 ATA for 150 minutes and 1.3 ATA for 180 minutes will cause toxicity of the CNS (Joiner, 2001).

Elevated levels of ROS following hyperoxic exposure can overwhelm antioxidant systems in many pulmonary cell types making these cells targets for ROS-induced damage. Pulmonary capillary endothelial and alveolar epithelial cells are targets for ROS with injury-induced lung edema, alveolar flooding, hemorrhage and collagen, elastin and hyaline membrane deposits (K. W. Kang, et al., 2005; Pagano, et al., 2004; Yee, et al., 2006).

Above a critical P_AO_2, the hemoglobin-O_2 buffering mechanism fails and the tissue PaO_2 can rise to hundreds or thousands of mmHg. At high levels of O_2, the amounts of oxidizing free radicals literally swamp the enzyme systems designed to remove them thus, do the serious destructive and even lethal effects on the cells (Guyton, 2006; Mantell, Horowitz, Davis, & Kazzaz, 1999). One of the principal effects is to oxidize the polyunsaturated fatty acids that are essential components of many of the

cell membranes (Demchenko, et al., 2007; Guyton, 2006; Mason, 2005). The pathogenesis of O_2 toxicity is described as similar to paraquat toxicity, and the paraquat-induced pulmonary lesions are similar to the effects of O_2 toxicity on type I and type II pneumocytes (Bus, Aust, & Gibson, 1976).

Oxygen toxicity progresses in overlapping phases based on degree of severity and of reversibility of injury. The phases are initiation, inflammation, proliferation and fibrosis. Initially there is increased ROS, depleted antioxidant levels and failure of the lung to clear itself of mucous. The inflammation phase or exudative phase is characterized by the destruction of the pulmonary lining and migration of inflammatory mediators to the sites of injury. The proliferative phase after toxic injury is sub-acute. In this phase, there is cellular hypertrophy and increased secretions from surfactant secreting alveolar type II cells. There are also increased monocytes in this phase. The final terminal phase is the fibrotic phase in which the changes to the lung are irreversible and permanent. There is collagen deposition and thickening of the pulmonary interstitial space and the lung becomes fibrotic (Crapo, 1986; Crapo, et al., 1984; Mensack, 1999).

Clinically, progressive hypoxemia requires increased FIO_2 and assisted ventilation, which further aggravate the pathophysiological changes associated with O_2 toxicity. Chest x-rays may show an alveolar interstitial pattern in an irregular distribution with evidence of a moderate loss of volume from atelectasis. There is no clinical way of diagnosing O_2 toxicity. Lung biopsy specimens may show changes consistent with O_2 toxicity but the primary value of the biopsy is to exclude other causes of lung injury. Air pressure changes within the enclosed lung cavity and ventilator

induced injury may accompany and be indistinguishable from O_2 toxicity. Oxygen toxicity can be minimized by keeping the P_AO_2 less than 80 mm Hg or the FIO_2 below 0.40 to 0.50 (Mason, 2005).

Research related to pulmonary cellular responses to hyperoxic exposure is well established. As the highly reduced cell becomes increasingly oxidized and levels of antioxidants fall, ROS-induced activation of signal transduction pathways determine the cellular response: either cell adaptation or repair or cell death by apoptosis and necrosis. This process includes the involvement of a cascade of pathways such as mitogen activated protein (MAP) kinase, toll-like receptor 4 (TLR4), signal transducers and activators of transcription (STAT) and nuclear factor kappa beta (NF kβ). The MAP kinase pathway includes extracellular signal regulated kinase (ERK1/2), a promoter of cell proliferation, C-Jun-terminal protein kinase (JNK1/2) and p38 kinase which induces cell death and inflammation. The TLR4, STAT and Nrf2 pathways are associated with survival gene expression. The NF kβ pathway is also associated with inflammation and cell death. These signaling pathways are regulators of the pulmonary epithelial cell response to hyperoxia (Halliwell, 2006; Zaher, et al., 2007). Cytokine and chemokine over expression in hyperoxic acute lung injury (HALI) is protective. Over-expression of tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), chemokine receptor 2 (CXCR2) and interleukin 11 (IL-11), as well as growth factor expression (insulin and keratinocyte growth factors) and the beta subunit of NaK-ATPase attenuate death signals (Barlos et al., 2009; Budinger & Sznajder, 2005; Waxman & Kolliputi, 2009).

Clinical Presentation of Hyperoxic Acute Lung Injury

Initial resuscitation strategies employed for a victim of HS are to secure an airway and ensure adequate breathing with appropriate FIO_2 levels to prevent hypoxemia and support cardiac output. The risk for hyperoxia and toxicity may be increased, triggering acute lung injury (ALI). Acute lung injury and acute respiratory distress syndrome (ARDS) are secondarily-occurring, inflammatory, syndromes caused by a wide variety of triggers or risk factors described as direct or indirect, pulmonary or extra pulmonary. The pathological changes associated with HALI mimic the ALI triggered by other conditions such as HS, reperfusion injury, pneumonia, sepsis or paraquat inhalation (Frutos-Vivar, Ferguson, & Esteban, 2006; Mantell, et al., 1999; Mantell & Lee, 2000; Ware, 2006). The risk of developing ALI or ARDS after inhalation injury is dependent on the toxicity and concentration of the inhaled substance (Joiner, 2001). For example, the cells and structure of the alveolar capillary membrane are highly susceptible to damage by toxic levels of O_2 (Nagata et al., 2007). Both ALI and ARDS are the same clinical disorder, differing only in severity of hypoxemia. The ratio between PaO_2 and the FIO_2 concentration delivered by ventilator support distinguishes the two syndromes. For ALI, the PaO_2/FiO_2 is $\leq$ 300 mmHg and for ARDS the PaO_2/FiO_2 is $\leq$ 200 mmHg (Frutos-Vivar, et al., 2006; Mantiega Riestra, 2006; Ware, 2006).

The injury to the alveolus is thought to develop when pulmonary or systemic inflammation leads to systemic release of cytokines and other pro-inflammatory molecules. Mast cells, which express mediators that exert effects on lung vasculature,

are also increased after hyperoxic exposure (Brock & Di Giulio, 2006). Cytokine release activates alveolar macrophages and recruits neutrophils to the lungs. Subsequent activation of leukotrienes, oxidants, platelet activating factor and protease occurs. These substances damage capillary endothelium and alveolar epithelium, disrupting the barriers between the capillaries and air spaces. Edema fluid, proteins and cellular debris flood the air spaces and interstitium, causing disruption of surfactant, airspace collapse, ventilation-perfusion mismatch, shunting and stiffening of the lungs with decreased compliance and pulmonary hypertension. There is no pattern to the injury; however dependant lung areas are most frequently affected (Wang, Song, & Shi, 2007; Ware, Camerer, Welty-Wolf, Schultz, & Matthay, 2006).

Tissue examination reveals that surfactant disruption, epithelial injury and sepsis initiate the increased expression of cytokines that sequester and activate inflammatory cells. Increased release of ROS alters normal endothelial function. Micro-array analysis has revealed increased expression of genes related to oxidative stress, anti-proteolytic function and extracellular matrix repair as well as decreased surfactant proteins (Leikauf, McDowell, Bachurski, et al., 2001; Leikauf, McDowell, Wesselkamper, et al., 2001). Diffuse alveolar damage results with intra alveolar neutrophils indicating the presence of an inflammatory response in the alveoli. Red blood cells, cellular fragments and eroded epithelial basement membranes are present with formation of hyaline membranes, indicating that serum proteins have entered and precipitated in the air spaces due to disruption of the alveolar capillary barrier. Formation of micro-thrombi

indicates the presence of endothelial injury and activation of the coagulation cascade (Matute-Bello, Frevert, & Martin, 2008).

The syndrome presents within 24 to 48 hours after the direct or indirect trigger. Initially, the patient may experience dyspnea, cough, chest pain, tachypnea, tachycardia, accessory muscle use, cyanosis, mottled skin and abnormal breath sounds (crackles, rhonchi and wheezing). Blood gas analysis reveals progressive worsening of hypoxemia, leading to respiratory failure. Bilateral infiltrates are seen on a chest x-ray and are consistent with pulmonary edema but without the cardiac component of elevated left atrial pressure. Treatment includes mechanical ventilation, supportive care and treatment of the underlying causes (Guyton, 2006).

Conclusion

Oxygen may be a triggering factor in HALI if the exposure is sufficiently concentrated and of adequate duration. The lung is a highly vulnerable target for oxidant-induced injury, initiating a cascade of protein signals that determine the cellular response, either death by apoptosis or necrosis. The alveolar epithelial and alveolar capillary endothelial surfaces are impacted whether the initial insult is direct or indirect. Hyperpermeability, micro-thrombi (resulting from altered coagulation and fibrinolysis), collagen deposition and fibrosis alter alveolar structure and function. Understanding the mechanisms of injury and pulmonary cellular responses to hyperoxia is essential for safe and efficient clinical practice.

Figure 1. Reduction of Oxygen

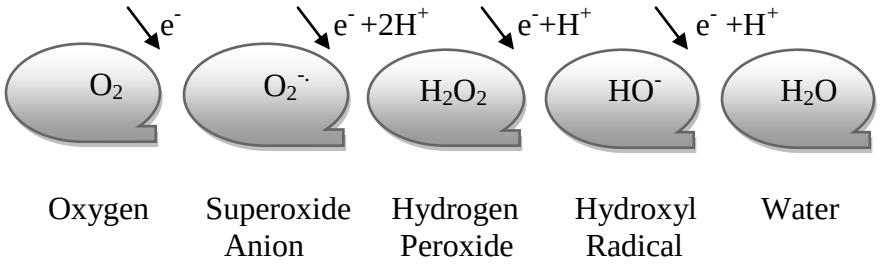

Figure 1. A single electron (e^-) transfer which converts molecular oxygen (O_2) to the superoxide anion ($O_2^{-\cdot}$), creating an unstable molecule. The decomposition of hydrogen peroxide (H_2O_2) can be a source of the hydroxyl radical ($HO^{\cdot}$), this reaction requires both $O_2^{-\cdot}$ and H_2O_2 as precursors. These steps reduce O_2 to water (H_2O) by the addition of four e^- yielding three reactive oxygen species $O_2^{-\cdot}$, H_2O_2 and $HO^{\cdot}$. H^+ hydrogen ion (Apel & Hirt, 2004).

Table 1. Antioxidants, Location and Properties

Enzymatic antioxidants located in mitochondria and cytosol	
Glutathione peroxidase	Removal of H_2O_2, hydro peroxides
Superoxide dismutase	Catalytic removal of O_2
Catalase	Catalytic reduction of H_2O_2 to H_2O
Non-enzymatic antioxidants located in cell membrane, exogenous dietary source	
Vitamin E (α tocopherol)	Chain-breaking antioxidant
β-carotine	Scavenger of ROS, singlet O_2 quencher
Co-enzyme Q	Regenerates Vitamin E
Compounds that reduce the availability of transition metals, Fenton reactions	
Transferrin	Sequesters iron and copper ions
Lactoferrin	Sequesters iron at lower pH
Albumin	Sequesters heme and copper
Ceruloplasmin (ferroxidase)	Scavenges superoxide radical, binds copper ions
Scavengers, products of metabolism, exogenous dietary source	
Bilirubin	Scavenges peroxyl radical
Uric acid	Scavenges hydroxyl radical
Vitamin C (ascorbic acid)	Scavenges hydroxyl radical, recycles Vitamin E
Thiol group donors	
Reduced glutathione (GSSH)	Binds free radicals, SH group oxidized to disulfide group (GSSG)
α-lipoic acid	Recycles vitamin C, glutathione substitute

Note. Multiple antioxidant properties counteract the damaging effects of oxygen free radicals in biological systems. H_2O_2: hydrogen peroxide, O_2: oxygen, H_2O: water, ROS: reactive oxygen species, pH: concentration of hydrogen ions in a solution, the negative log of $[H^+]$, SH: sulfhydryl group.

Chapter 3.

Flow Cytometry and Confocal Laser Scanning Cytometry: A Comparison of Techniques

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ABSTRACT

Objective. Flow and laser scanning cytometry are used extensively in research and clinical settings yet there is a lack of knowledge about the cytometers themselves and the techniques employed. These techniques provide clinicians and scientists needed information about cell and sub-cellular functioning in a variety of health and disease states. An in-depth knowledge and understanding of cytometry techniques can enhance interpretation of current research findings and may be useful in future research. Our goal with this review is to reacquaint clinicians and scientists with information concerning differences between flow and laser scanning cytometry by comparing their capabilities and applications.

Methods. A PubMed title and abstract search was conducted for articles on research and review articles relating to origins and use of flow and laser scanning cytometry. Attention was given to studies describing application of these techniques in the clinical setting.

Results. Both techniques exploit the interaction between the physical properties of light: fluorescence and light scatter and the cell properties. Data from these interactions are immediately and automatically acquired but they are distinctly different. Flow cytometry does provide researchers and clinicians with valuable rapid information about a wide variety of cellular or particle characteristics, yet this technique does not provide the scanned high resolution image analysis needed for investigators to localize areas of interest within the cell for quantification. Flow cytometry requires that the sample contain a large amount of cells and be disaggregated to a single cell suspension.

Laser scanning cytometry is slide-based and does not require as large of a sample. The tissue sample is not suspended into a liquid but affixed to a slide allowing repeated sample analyses. Flow and laser scanning cytometry are used in the clinical setting to understand pathophysiological derangements associated with many diseases such as cardiovascular disease, diabetes, acute lung injury, HS, surgery, cancer and Alzheimer's disease.

Conclusions. Understanding the differences between flow cytometry (FCM) and laser scanning cytometry (LSCM) can assist investigators in the planning and design of their research or clinical testing. Researchers and clinicians can optimize these technique capabilities with the cellular characteristics they wish to measure delineating molecular and cellular events. Discovery of new mechanisms in cells using FCM and LSCM may provide evidence needed to guide future treatment and interventions.

KEY WORDS. flow cytometry, laser scanning cytometry, fluorescence

Introduction

Cytometry is commonly used in the clinical setting for determining a variety of measurements such as cell adhesion, platelet activation and drug uptake. Cytometry refers to the measurement of physical and/or chemical characteristics of cells. Two types of cytometry techniques widely applied in the research laboratory and the clinical setting are: 1) FCM and 2) LSCM. With precision and accuracy these instruments can rapidly and simultaneously analyze, sort and quantify large samples of heterogeneous cells in suspension and determine protein functions in samples of tissue prepared on slides in very short time periods (Darzynkiewicz, Huang, et al., 2004; Pozarowski & Darzynkiewicz, 2004; Pozarowski, Holden, & Darzynkiewicz, 2006; Tarnok & Gerstner, 2002; Valet, 2003).

These cytometry techniques allow clinicians and researchers to discover cellular mechanisms related to free radical formation, inflammation and platelet activation in multiple diseases. This review article will provide investigators and clinicians the tools necessary to foster further understanding of cellular mechanisms in disease.

Background

Flow cytometry is a technique for counting, examining and sorting heterogeneous populations of cells and other biological microscopic particles suspended in a stream of fluid (Kamentsky, 2001; Nolan & Yang, 2007). In general, FCM allows for rapid and simultaneous multiparametric analysis of the physical and/or chemical characteristics of large numbers (20,000 - 40,000 cells/second) of single cells hydro-dynamically streaming through a fluid sheath sleeve. The laser within the instrument

intersects the stream of cells or particles. Parallel or perpendicularly placed light scatter detectors as well as fluorescence detectors acquire data on both scattered and fluorescent light (Cram, 2002; Rieseberg, Kasper, Reardon, & Scheper, 2001; Shapiro, 2003). Flow cytometry requires disaggregated cells and uses fluorescence to quantify a sample population. Laser scanning cytometry is similar in some ways such as the use of fluorescence but different in other respects.

Laser scanning cytometry is a technique that automatically measures laser-excited fluorescence at multiple wavelengths. The data are generated by analysis of the light scatter emitted from fluorochromed individual cells on a microscope slide. The scanned cells are treated with one or more fluorescent dyes in order to rapidly detail multiple cellular constituents (Darzynkiewicz, Li, & Bedner, 1999; Kamentsky, 2001; Luther, et al., 2004). Laser scanning cytometry combines into a single platform, fluorescence microscopy and automated sample scanning with the image analysis features of FCM to collect digital data on expression of biochemical or immunological features of cells or tissues in solid phase samples (Luther, et al., 2004; Wijsman et al., 2007).

The LSCM makes highly precise measurements on each cell at 0.1 micron or 0.25 micron spatial intervals per scan line. The line scan at the focal plane has a 10 micron diameter width and a 685 micron extent using a 10x objective, a 5 micron diameter width and a 342 micron extent using a 20x objective or a 2.5 micron diameter width and a 171 micron extent using a 40x objective. The nominal area covered each minute by movement of the scan beam and the stage is 4.25 mm^2 using a 20x objective.

Optimum cell rates are achieved with cell densities in the range of 100 to 1000 cells per mm^2 on the slide providing cell processing rates in excess of 1000 cells per minute. Cell acquisition rate depends on the objective used because the length of the scan is inversely proportional to the objective lens power cell (Kamentsky, 2001; Kamentsky, Burger, Gershman, Kamentsky, & Luther, 1997).

One common feature of both FCM and LSCM is the analysis of extrinsic fluorescence emission and light scatter. Thus, these techniques are often called fluorescence activated cell sorting (FACS) techniques. Dr. Mack Fulwyler, a physicist built the first prototype of a cytometer in 1965 (Cram & Arndt-Jovin, 2005). Dr. Leonard Herzenberg used this prototype to build an instrument that sorts cells using fluorescence, which was one of the first flow cytometers. Not until the 1990s did laser scanning cytometry become available (Kamentsky, Burger, et al., 1997; Kamentsky, Kamentsky, Fletcher, Kurose, & Sasaki, 1997; Shapiro, 2003). There are a large number of studies that have used both techniques (Bedner, Burfeind, Gorczyca, Melamed, & Darzynkiewicz, 1997; Mittag, Lenz, Gerstner, et al., 2006; Oswald et al., 2004). Cytometry techniques have added significantly to cell biology by offering researchers and clinicians information about cells and their subcomponents that was previously unavailable (Holden, 2001; Oswald, et al., 2004; Tarnok & Gerstner, 2002; Valet, 2003). Application of these techniques to the study of apoptosis and cell cycle mechanisms offers precise clarification of cell functions to researchers (Bedner, Li, Gorczyca, Melamed, & Darzynkiewicz, 1999; Pozarowski & Darzynkiewicz, 2004; Vermes, Haanen, & Reutelingsperger, 2000). The understanding of apoptosis has been advanced

significantly by the development of these cytometry techniques allowing researchers to further distinguish between types of cell death (Darzynkiewicz, Crissman, & Jacobberger, 2004; Darzynkiewicz, Huang, et al., 2004).

Fluorescence

Fluorescence is excitation light energy that is absorbed and emitted by a fluorescent molecule. The molecule transitions to an excited state and as it returns to an unexcited ground-state, a specific wavelength of light is emitted. The energy in the emitted light is dependent on the energy level to which the fluorophore or multiple fluorophores are excited. The light emitted has specific wavelengths, and consequently, specific colors, depending on the number of fluorophores used. Fluorescence intensities are quantified by flow and LSCM (Cram, 2002; Matyus, 1991; Shapiro, 2003).

Flow cytometry and LSCM applications are based on fluorescence and light scatter quantification. Intrinsic and extrinsic fluorescence are quantified and are directly proportional to the mass of the specific constituent fluoresced. Intrinsic fluorescence is the fluorescence of the cells and particles without the added fluorescent probe. Extrinsic fluorescence requires that the probe, a fluorochrome with known specificity, excitation and emission thresholds, be loaded prior to analysis. During and following laser excitation each cell or particle scatters the light or emits fluorescence characteristically allowing for analysis of fluctuations in brightness. Entire cell constituents are uniformly illuminated as the aim of the preparation is to prepare a suspension of single cell particles that have been stained in a specific way to identify a particular characteristic (Cram, 2002; Kamentsky, Burger, et al., 1997; Mittag, Lenz, Gerstner, et al., 2006).

When the cell or particle passes through the laser beam, it will refract or scatter light at multiple angles. The magnitude of the forward light scatter directly correlates with cell volume or size and is plotted from left to right on a histogram. Side light scatter correlates with the granularity and inner complexity of the cell or particle. A two dimensional scatter plot or dot plot of the data can distinguish size from density, further characterizing the cell or particle (Rieseberg, et al., 2001; Shapiro, 2003). The size range of particles can be from approximately 0.0001 microns to 10,000 microns (Nolan & Yang, 2007). Different sized particles containing different fluorochromes can be efficiently analyzed at rates up to 10,000 cells per second (Cram, 2002). In 2003, technology was capable of measuring 2 scatter and 12 fluorescent parameters simultaneously and individually for each cell. New cytometers can process up to 17 different fluorescent probes simultaneously during a single scan, and new cell sorters can sort up to 70,000 cells per second (Mittag, Lenz, Bocsi, et al., 2006).

Tissue Preparation

There are differences between FCM and LSCM with the greatest being related to the sample. Samples used in FCM must have a large number of single cells that are in a liquid form. Whereas in LSCM, the tissue used is whole tissue, a non-fixed preparation on a slide. The sample prepared for FCM is suspended as single cells or other biological particles that are forced hydro-dynamically through a sleeve. These cells or particles are placed into single-file prior to intersecting a laser beam. The light scatter and the fluorescence generated from the cell beam interaction are detected and analyzed by the

instrument. Re-analysis of this sample is not possible, as it is discarded (Kamentsky, 2001; Kamentsky, Burger, et al., 1997).

The sample prepared for LSCM is stationary and slide-based and there is no cell position change during analysis. Movement of the microscope stage is computer-assisted, guiding the fixed sample through the computer controlled laser beam. High resolution image analysis is generated from the data acquired and re-analysis of the sample is possible. Methods for sample preparation vary significantly. Flow cytometry requires disaggregation methods with digestion and filtration to get the fluorescent labeled microscopic particles into suspension, LSCM requires absolute precision in loading of fluorescence probes and expert skill in slide preparation.

Comparison of Techniques

Laser scanning cytometry offers similar information to researchers and clinicians as FCM without the limitations (Deptala, Bedner, & Darzynkiewicz, 2001). Specifically, cells or particles are non-fixed tissue, slide-based, not in suspension. With LSCM, there is no hazardous waste or no carryover of specimen or dyes. Laser scanning cytometry avoids the associated risk of cell damage during disaggregation and centrifuge steps are eliminated. The laser wavelength can be changed to image different constituents and the images can be combined if needed.

With LSCM, the slide and the laser beam are moved under computer control to excite the cells. The computer controls the positions of the slide and laser beam shutters, therefore cell position on the slide can be a measurement feature. This is not possible with FCM because cell position is not maintained (Kamentsky, 2001; Kamentsky,

Kamentsky, et al., 1997). The interactions of each cell and the laser are measured and recorded many times in a two dimensional pattern. Features computed from these multiple interactions are derived from pixel intensities, whereas in FCM, properties of a single analog pulse are recorded as each cell flows past the laser (Kamentsky, 2001; Kamentsky, Burger, et al., 1997). This is one reason LSCM is slower than FCM in terms of processing rates. Table 1 is a comparison of the main components of cytometers and definitions related to the two cytometers (Deptala, et al., 2001; Luther, et al., 2004; Matyus, 1991; Shapiro, 2003).

A LSCM is similar in function to that of a FCM with lasers, optics, detectors and computers. The components of the LSCM are: three lasers, (407 nm solid state, 488 nm argon ion, and 633 nm helium-neon). With a LSCM, to create a scan line at the specimen, several components are needed. Multiple photomultiplier fluorescence sensors, two dichrotic mirrors, a laser scanning computer with an oscillating computer controlled scanning mirror, a microscope with a computer controlled stepper motor stage equipped with absolute position sensors, a beam blocking bar assembly and a charge coupled device, scan the specimen (Luther, et al., 2004).

Data analyses of both techniques depend primarily upon what the researcher needs to know about the cells (Darzynkiewicz, Bedner, et al., 1999; Deptala, et al., 2001; Luther, et al., 2004). The most commonly used methods of displaying data for FCM are: multivariate frequency distributions, two-parameter dot plots, two-dimensional histograms, density and contour plots, three-dimensional cloud plots as well as multiparameter displays of emission spectra and forward and side light scatter

(Herzenberg, Tung, Moore, & Parks, 2006; Roederer, Brenchley, Betts, & De Rosa, 2004; Shapiro, 2003).

The event or parameter data generated from the LSCM relate to maximum amounts of pixel fluorescence per event at specified coordinates on the slide. An initial contour line is drawn around all adjacent pixels above a preset threshold value defining an event. Contour lines can be based on forward light scatter or side scatter such as 90^0 right angle scatter. Additional contour lines are drawn to indicate areas of integration and background. These contours are used in computing the cells total scatter and total fluorescence which are the estimates of the amount of cell constituent resulting from scatter or fluorescence. Thus, the cell or particle can be "contoured" (Kamentsky, 2001).

Data values for both cytometers of all pixels include measurements such as the mean, count, standard deviation, coefficient of variation, minimum, maximum, sum and full width half maximum per well. Measurements are also taken on other gated subpopulations or scan areas depending on what was contoured (Deptala, et al., 2001). The precision of both techniques is based upon the efficiency of the instrument to distinguish between artifactual debris and background noise at times of weak fluorescence and light scatter (Wood & Hoffman, 1998).

Another method of data analysis with both cytometers is to allow the user to sum the pixel values between two contours after the initial scan. The initial scan performed at 10x magnification determines sample suitability. This initial scan is rapid and allows for placement of a number of 500 um^2 high resolution scan-areas so as to avoid sample edges and artifact. High resolution scans are then performed at 20x magnification using

0.1 um steps across the entire dimension of each prescribed scan area. Data are collected to reflect percent positive events per unit area or the percentage of cells that were extrinsically fluorochromed and are positive for this fluorochrome. The event data reflecting the distribution of fluorochromed pixel intensities and the mean pixel intensity of different populations of events in a given time can also be obtained. Table 2 provides examples of cytometer capabilities (Deptala, et al., 2001; Luther, et al., 2004; Shapiro, 2003).

Clinical Relevance

Flow cytometry and LSCM are used in the clinical setting for cell analyses associated with research and treatment of many pathological conditions (Tarnok & Gerstner, 2002). Flow cytometry and LSCM have been used to study the cardiovascular and pulmonary systems, the effects of surgery at the cellular level, diabetes, cancer and apoptosis. For example, the field of cardiovascular medicine has benefited greatly from advances in flow cytometry and LSCM for the characterization of disease etiologies (Bigalke et al., 2009; Fox, May, Shah, Neubert, & Heptinstall, 2009; Sibal et al., 2009; Taatjes et al., 2008). The vulnerable atheroma surface has fewer vascular smooth muscle cells (VSMCs). Normally VSMCs migrate to the neointima and this is modulated by the balance between plasminogen activator activity and plasminogen activator inhibitor type1 (PAI-1). Increased expression of protein PAI-1 by VSMCs reduces VSMC migration in-vitro, a process that was quantified by LSCM (Schneider et al., 2004).

Furthermore, in cardiac surgery patients, researchers were able to determine the effects of cardiac surgery on immune system functioning. This was accomplished in one

study by using FCM to quantify the density of expression of the receptors for the Fc part of IGg (CD64) and scavenger receptor CD163 on monocytes (Kolackova et al., 2008). Another study employed FCM to measure ROS and α integrin (CD11b) expression by polymorphonuclear (PMN) cells. Researchers conducted non-ischemic experiments to examine leukocyte function in cerebral ischemia-reperfusion in an aged rat model and concluded that increased intravascular PMN adhesion and vascular dysfunction may contribute to poor neurological outcome after cerebral ischemia-reperfusion (Ritter et al., 2008).

There has also been extensive pulmonary research utilizing FCM and LSCM. For example, activation of neutrophils in the lungs and CD11b expression were measured by FCM in a trauma HS study examining the optimal type of resuscitation fluid to be administered with shed blood. The trauma HS resuscitation regimens were associated with increased organ injury, gut permeability, pulmonary leukosequestration, and systemic neutrophil activation and decreased RBC deformability (Vega et al., 2008). In another study, researchers examined patients experiencing HALI. Using cytometry techniques, they characterized the morphology and phenotype of myeloid CD11b hematopoietic cells that co-express vascular endothelial growth factor receptor 2 ($VEGFR2^{+}$) and platelet derived growth factor receptor β ($PDGFR\beta^{+}$). Understanding the cellular response to hyperoxia by these two growth factors may offer a target for the treatment of the fibrotic changes occurring in HALI (M. C. Jones et al., 2009).

Other areas of pulmonary research utilizing FCM and/or LSCM include pulmonary vascular tissue and drug studies. For example, a study by Suzuki et al. (2000)

found that methylprednisolone down-regulates hyperoxia-induced intracellular adhesion molecule-1 expression by inhibiting NF-$_k$B activation via increased I_KB-α expression. Additionally, methylprednisolone inhibits neutrophil adhesion and reduces hyperoxia-induced H_2O_2 production in human pulmonary artery endothelial cells (Suzuki et al., 2000). Both of these conclusions would not have been possible without FCM techniques. Anti-inflammatory effects are being studied on two commonly prescribed blood pressure and cholesterol medications; losartan and simvastatin. Researchers were interested in the effects of these two medications on the C - reactive protein in human umbilical vein endothelial cells (HUVECs) and vascular cell adhesion molecule-1 (VCAM-1), this was determined by FCM (L. T. Chang et al., 2007). Researchers examined patterns of localization of phosphorylated extracellular signal-regulated kinases (ERKs) with respect to their role in cell cycle alterations by an epidermal growth factor, H_2O_2, or asbestos in pulmonary epithelial cells. Flow cytometry and LSCM were used to determine whether expression of phosphorylated ERK was related to the cell cycle and whether cell cycle alterations could be modified after the addition of the mitogen-activated protein kinase/ERK kinase (MEK) 1 inhibitor. Evidence supported that extra-cellular, signal-related kinase proteins mediate alterations in the cell cycle during development of apoptosis and during proliferation by diverse ERK stimuli (Buder-Hoffmann, Palmer, Vacek, Taatjes, & Mossman, 2001).

One new area of pulmonary research concerns the hemangiogenic propensity of lung cancer from the impact of surgery. Flow cytometry was used to assess levels of two biomarkers, CD133+ and VEGFR2+, circulating endothelial progenators (CEPs). Lung

cancer development involves a hemangiogenic switch towards increased CEPs. The study supported that surgical removal of primary lung cancer led to a normalization of hemangiogenic profile. Thus, collective assessment of hemangiogenic biomarkers may be a promising resource for predicting clinical outcomes, reoccurrence and for validating the potential impact of anti-angiogenic therapy on lung cancer (Jin, 2007).

Other studies have made use of FCM and LSCM techniques when examining the effects of surgery on the immune system. Flow cytometry was used to measure the proportion of CD4+ lymphocytes producing interferon γ, IL-2, IL-4 and IL-6 in abdominal cancer surgery patients compared to non-cancer abdominal surgery patients. Patients with malignancy showed an abnormal perioperative Th1/Th2 balance suggesting predominance of a type 2 immune response. Major abdominal surgeries induce a marked shift in Th1/Th2 balance toward Th2 in the early postoperative stage (Ishikawa et al., 2009). Another study by Park et al. (2007) investigated herniated nucleus pulposus (HNP) fragments, which are recognized as foreign and thus lead to an auto-immune reaction. Expression of human activation-inducible tumor necrosis factor receptor (AITR) and its ligand (AITRL) are important co stimulatory molecules in the pathogenesis of autoimmune diseases. Researchers investigated the possible over-expression of AITR and AITRL in HNP using FCM. Flow cytometry and LSCM analysis revealed significantly higher levels of AITR and AITRL in the HNP group compared to controls. Also, peripheral blood and disc tissue had increased expression of these molecules (Park et al., 2007).

Flow cytometry and LSCM have also been used in diabetes studies. For example, platelets have been studied with FCM in type 2 diabetes by measuring the expression of p-selectin and CD61 (Henry et al., 2009). In addition, the effects of clopidogril and aspirin on platelet p-selectin were studied using FCM, which showed that FCM can be used effectively to monitor effects of these commonly prescribed medications (Fox, et al., 2009). Circulating endothelial progenator cells (EPCs), which participate in endothelial repair, have been studied using FCM and LSCM in people with type I diabetes and hypercholesterolemia (J. Z. Chen, Zhang, Tao, Wang, & Zhu, 2004; Sibal, et al., 2009). During short-term hyperglycemia, in which specific recognition and clearance of foreign antigens is impaired, FCM determined a decreased monocyte expression of HLA-DR. Decreased levels of monocyte HLA-DR have previously been shown to correlate with infectious complications and mortality in critically ill patients (Turina, Miller, Tucker, & Polk, 2006).

The cellular uptake of compounds used in the treatment of cancer can be quantified by FCM. Temoporfin and hematoporphyrin both accumulate in the cytoplasm and in the mitochondria, the target organelle of these compounds in nasopharyngeal carcinoma cell lines (Yow, Chen, Mak, Cheung, & Leung, 2000). There also have been multiple studies using FCM to study multiple myeloma and with acute myeloid leukemia patients who have had autologous stem cell transplantation and minimal residual disease (MRD) (Al-Mawali, Gillis, & Lewis, 2009; Paiva et al., 2008). In addition, another study used FCM with antibodies to determine that monoclonal B-cell lymphcytosis occurs before the progression of chronic lymphocyte leukemia.

Conclusion

Both techniques employed in research and clinical laboratories provide researchers and clinicians with an abundance of information about specifically identified cell characteristics. There is a significant amount of FCM research which focuses on the study of cell size, cell cycle determinations, DNA analysis, immunophenotyping and detection of apoptosis (Darzynkiewicz, Huang, et al., 2004; Darzynkiewicz, Li, et al., 1999; Pozarowski & Darzynkiewicz, 2004; Pozarowski, et al., 2006). The LSCM techniques are capable of fluorescence differentiation imaging of many internally integrated translocation mechanisms of cell functioning. Other LSCM information may include specifics related to sub-cellular signaling, protein activity or pH within the cell or status of individual organelle components such as the mitochondrial membrane (Tomita et al., 1999; Ueno et al., 2005).

Flow cytometry and LSCM use extrinsic fluorescence with known excitation emission spectra and specifics about fluorochrome interactions with cell constituents. However, the fluorescent measurements are interpreted in different ways. In FCM, data acquisition about the fluorescence and light scatter occurs after one analysis using the laser beam. Data analysis on such cellular characteristics as cell size and density are provided in histogram, two-dimensional scatter gram displays and other formats. These data are available for re-analysis, but not the sample. In LSCM, data acquisition is a lengthier process using contouring techniques and high resolution image analysis to determine meaningful fluorescent pixel intensity values of a cell or sub-cellular constituent or particle in a specific position on a stationary slide. Both numerical and

image analysis files are available for initial and repeated investigator interpretations along with the slide containing the tissue. The preservation of the sample distinguishes the two techniques. With new high-quality digital imaging techniques, image files can be merged, post-scan with the sample itself for comprehensive morphometric analyses.

The use of FCM and LSCM in the clinical and research settings to characterize cellular derangements in disease is well established. The use of cytometry ranges from research on pathophysiological pathways in cardiovascular disease and diabetes, pulmonary disease, surgery/trauma, oncology and Alzheimer's disease to research examining the actions of various medications.

The future direction of cytometry and its capabilities and application is at least in part dependent upon further development of immunocytochemical probes that can detect protein function (Darzynkiewicz, Huang, et al., 2004; DeRosa, Bu, & Ford, 2003; Luther, et al., 2004; Nolan & Yang, 2007). Advanced flow cytometers already in use acquire integrated fluorescence signals and high quality fluorescence images and allow multiparameter analysis (Zuba-Surma, Kucia, Abdel-Latif, Lillard, & Ratajczak, 2007). The differences in the use of FCM and LSCM by researchers and clinicians are important to understand. Mechanisms can be discovered that may be fundamental in different cellular pathways and disease processes.

Table 1. Components and definitions of flow and laser scanning cytometers

Flow Cytometer	Laser Scanning Cytometer
The fluidic system: presents samples to the interrogation point and takes away the waste.	**Integral fluorescence (fluorescence and light scatter):** the sum of intensities of all pixels within the area.
The lasers: light source for scatter and fluorescence.	**Pixel:** the smallest single measurement component of an image, a measure of resolution.
The detectors: receive light.	**Area (um^2):** the number of pixels within the contour area.
The optics: gather and direct light.	**Background:** background fluorescence or signal nearby each event.
The electronics and the peripheral **computer system:** convert the analog signals.	**Event id:** a unique identifier for each event.
	Perimeter: the number of pixels that comprise the perimeter of the event.
	Maxpixel: brightest pixel value for each event for each saved channel
	Peripheral integral (peripheral contours only):the integral of the fluorescence in the area defined by the peripheral contour.
	Scan: pixel number (0-768) indicating the y position of the scanning mirror where the event was detected.
	X position and y positions: the x and y position in the well or slide for each event

(Deptala, et al., 2001; Luther, et al., 2004; Shapiro, 2003).

Table 2. Comparing capabilities of flow and laser scanning cytometers

Flow Cytometry	Laser Scanning Cytometry
Volume and morphological complexity of cells	Enzyme kinetics and other time resolved processes; drug uptake, efflux
Cell pigments (cell cycle analysis, proliferation, kinetics, etc.)	Data merge capability for each measured cell
Ribonucleic acid	Relocation of the measured cell for examination by fluorescence and image analysis
Chromosome analysis and sorting	Tissue section analysis
Proteins, phosphorylation, translocation via fluorescence resonance energy transfer	Ability to analyze hypo cellular specimens
Proteins, cell surface antigens (cell death markers)	Specimen stored for archival preservation for further studies using new probes
Intracellular antigens (various cytokines and secondary mediators)	Cell cycle
Nuclear antigens	Automated cell cycle
Apoptosis quantification, measurement of deoxyribonucleic acid degradation	Cell cycle combined with multiple fluorescence markers
Mitochondrial membrane potential and permeability changes	Live cell analysis
Cell viability	Cyan fluorescent protein, yellow fluorescent protein, fluorescence resonance energy transfer
Monitoring electropermeabilization of cells	Automated tissue analysis and tissue micro arrays
Oxidative burst	Immunophenotyping
Characterizing multi drug resistance in cancer cells	
Glutathione	

(Deptala, et al., 2001; Luther, et al., 2004; Shapiro, 2003).

Chapter 4.

Effects of increased inspired oxygen with and without dopamine on lung and diaphragm hydrogen peroxide and apoptosis following hemorrhagic shock

This manuscript has been submitted for publication to the journal

Injury, the International Journal of the Care of the Injured

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Abstract

Introduction*:* During resuscitation of HS victims, immediate-care providers typically employ high FIO_2 to restore and maintain maximal O_2 saturations. However, there are studies indicating that increased FIO_2 can be detrimental to cellular function. Optimal use of O_2 in HS resuscitation has not been explored in great detail. Our objective was to determine the FIO_2 that minimizes H_2O_2 production and apoptosis in lung and diaphragm following HS. In addition, we investigated the effects of DA at various FIO_2s.

Methods*:* Male Sprague-Dawley rats were randomized to FIO_2 groups: 0.21, 0.40, 0.60 and 1.00. Controlled HS was elicited by reducing the mean arterial pressure to approximately 40 mm Hg over 30 minutes. The rats were treated for 60 minutes with various FIO_2s or the same FIO_2s and DA infusion (10 mcg/kg/min). Hydrogen peroxide was measured using dihydrofluorescein diacetate (HFluor-DA). Apoptosis was determined based on nuclear differential dye up-take of ethidium bromide (EB) and acridine orange (AO).

Results*:* Compared to ambient air (0.21), lung and diaphragm H_2O_2 and apoptosis were significantly reduced in the 0.40 and 0.60 groups. However, at an FIO_2 of 1.00, H_2O_2 and apoptosis in these tissues were greater than at 0.21. With the exception of an FIO_2 of 0.40, infusing DA with various FIO_2s resulted in H_2O_2 and apoptosis being significantly decreased.

Conclusions*:* These results indicate that lung and diaphragm H_2O_2 and apoptosis (indicators of ROS damage) are affected by inspired O_2 and DA. These results indicate

using an FIO_2 of 0.40 with or without DA is most beneficial in attenuating tissue damage following HS.

Keywords*:* Reactive oxygen species, DNA damage, hemodynamics, acid-base balance, hyperoxia.

Introduction

Hemorrhagic shock is the result of an acute loss of blood from the intravascular space, often a consequence of traumatic injury and the leading cause of death in trauma patients (Alam, et al., 2005). During HS, there is decreased perfusion of vital organs which leads to inadequate DO_2 necessary for normal cell function (Gutierrez, Reines, et al., 2004). In patients who experience HS, failure of compensatory mechanisms leads to hemodynamic instability, decreased DO_2, and VO_2 resulting in hypoxic injury (Mauriz, et al., 2007). This alteration in cellular metabolism generates an increase in ROS formation (Cadenas, 1989).

In order to restore and maintain adequate tissue oxygenation during HS, clinicians apply increased FIO_2. This is an intervention based on the ATLS guidelines of the American College of Surgeons (Trauma, 2008). There are published data indicating that increased FIO_2 can be detrimental to tissues (Klekamp, Jarzecka, & Perkett, 1999; Pietarinen-Runtti, Raivio, Saksela, Asikainen, & Kinnula, 1998). Administering supplemental O_2 during HS increases tissue O_2, which may subsequently lead to increased amounts of ROS. This increased production of ROS can lead to lipid peroxidation, protein alterations and DNA damage (Yousuf et al., 2009).

Dopamine is a pharmacological agent that is sometimes used in patients experiencing HS when isotonic crystalloid administration fails to enhance tissue perfusion. Administering DA can augment tissue oxygenation by increasing cardiac output and thus, decreasing free radicals. In addition, DA has been shown to be a free radical scavenger (Gassen & Youdim, 1999; Miura, Muraoka, & Ogiso, 1998).

The objectives of this study were to determine the effects of different FIO_2s without and with the administration of DA (10 mcg/kg/min) following HS on lung and diaphragm damage. Tissue damage was assessed by measuring H_2O_2, a precursor of the $HO^{\cdot}$, and DNA damage, a component of apoptosis.

The Institutional Laboratory Animal Research Division at the University of Kansas Medical Center (KUMC) provided care of the animals. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animal care approval was obtained for this study (2008-1708).

Materials and Methods

Experimental design and protocol

Male Sprague-Dawley rats (n = 112) weighing between 350-450 grams were used in these volume-controlled HS experiments. The animals were ordered in lots of six and allowed to acclimate for 48 hours prior to experimentation. This study was an experimental design in which rats were randomized among eight treatment groups. Eight rats per treatment group totaling 64 rats were used. An additional 48 rats, (6 per treatment group) were used as sham, no shock animals for the determination of H_2O_2.

The independent variables for these experiments were FIO_2 and DA. The dependent variables were lung and diaphragm H_2O_2 and apoptosis. Mean arterial blood pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), arterial blood gases (ABGs), hemoglobin (Hgb) and body temperature were monitored and recorded during the experiment. The rat's core body temperature was maintained at 36-37 degrees Celsius.

In-vivo experiments

The rats were anesthetized with sodium pentobarbital (50 mg/kg body weight) administered intraperitoneally. Atropine (0.1 mg/100 g body weight) was administered intraperitoneally to reduce respiratory secretions. When a surgical plane of anesthesia was reached, the following procedures were performed. The trachea was exposed and cannulated using polyethylene (PE) 240 tubing. A PE 50 catheter was placed in the right carotid to monitor arterial pressures and heart rate (HR). Blood pressures were continuously monitored and measured with an accuracy of $\pm$ 2 mm Hg. A second PE 50 catheter was inserted in the femoral artery for blood withdrawal eliciting HS. A third PE 50 catheter was inserted in the right external jugular vein for administration of DA (10 mcg/kg/min) following HS. The ABGs were measured using an I-STAT instrument which measured pH, $PaCO_2$, HCO_3^-, Beecf, PaO_2, SaO_2 and Hgb.

Control arterial blood pressures (SBP, DBP and MAP), acid-base and Hgb measurements were obtained. Hemorrhagic shock was elicited by removing approximately 40% of the blood volume via the femoral artery over 30 minutes. During this time all rats breathed ambient air ($FIO_2 = 0.21$). At the end of the HS period, hemodynamic and acid-base parameters were recorded and one of the 8 treatments (various FIO_2 without and with DA) was initiated. Sixty minutes later, the treatment was completed and above parameters recorded.

The animal was euthanized with sodium pentobarbital (150 mg/kg body weight) and the lung and diaphragm were rapidly excised for H_2O_2 and apoptosis determination. The tissues were divided into two equal portions and immersed in a Krebs Ringer's

(KR) solution. Both of the lungs and the entire diaphragm were used for H_2O_2 and apoptosis measurements.

Hydrogen peroxide measurements

After rinsing the lung and diaphragm in KR solution, isolated lung and diaphragm strips were mounted in a paraffin dish and loaded with Hfluor-DA. This chemical is a probe that is oxidized to Fluor by H_2O_2. After 30 minutes of loading with Hfluor-DA, the lung and diaphragm strips were rinsed in phosphated buffer solution for 10 minutes. Lung and diaphragm strips were stretched and mounted on slides for measurement of fluorescent intensity (expressed as $x10^6$) which is directly proportional to the amount of H_2O_2 in the tissue. Changes in fluorescence due to Fluor were measured using LSCM with a detection limit of 1 μM H_2O_2.

In order to obtain a measure of H_2O_2 in both the lung and diaphragm, an additional rat was sacrificed for a sham sample. All tissues after the experiment were placed in the same Hfluor dye solution and processed identically. This method insured that the tissues were exposed to the same concentration of the dye. Following LSCM of the samples, the relative intensity of H_2O_2 of the sham was subtracted from the treatment sample which were the values used to express H_2O_2 concentration.

Apoptosis measurements

The lung and diaphragm were minced into small pieces and homogenized with a KR solution containing trypsin, collagenase and antioxidants. After 30 minutes homogenization at 37 degrees Celsius, the supernatant was removed and centrifuged for 30 minutes at 6,000 rpm. The pellet was resuspended in 2 mL of KR solution. A 250 μL

aliquot was added to a tube containing two µL of EB and two µL of AO. After vortexing, 20 µL of the sample was placed on a slide and fluorescent microscopy performed. Differential nuclear dye uptake microscopy was employed to measure DNA damage, an index of apoptosis. The images were analyzed with Boyce Scientific Analysis® software. The software eliminates human error by assessing exact hue values for each nucleus thus, allowing for determination of relative amounts of each dye (C. Goodyear-Bruch, et al., 2005). Approximately 300 lung and diaphragm nuclei were analyzed for apoptosis.

Statistical analysis

Data are presented as mean ± SEM. Differences within the FIO_2 only and FIO_2 + DA groups at control and shock were analyzed for identification of significant main and interaction effects by 2-way analysis of variance (ANOVA). Treatment differences within the FIO_2 only and FIO_2 + DA groups were analyzed by 1-way ANOVA followed by post hoc test (Fisher's LSD). Differences between FIO_2 only and FIO_2 + DA groups were analyzed similarly by 1-way ANOVA followed by post hoc test (Fisher's LSD). Hemodynamic and arterial blood gas analyses are based on data using six rats per group. For the analysis of the fluorescent intensity for H_2O_2 six rats per group were used and for the apoptosis analysis, eight rats per group were used. Significance was defined as $p < 0.05$. Statistical analyses were performed using SPSS software (version 17 for Windows®; Chicago, IL.).

Results

Hemodynamics

The hemodynamic data are summarized in Table 1 and Table 2. At control there were no significant differences in SBP, DBP, MAP and HR among the eight groups. While breathing room air, removal of 40% of the rat's blood volume resulted in significant decreases in all arterial blood pressures. The blood pressures at shock were not significantly different among all the groups. Consequently, the hemodynamic status of all rats was comparable at the onset of treatment. All statistical differences are with respect to FIO_2 at 0.21. In Table 1, increasing FIO_2 resulted in a significant increase in SBP at an FIO_2 of 0.60 ($p < 0.05$). Diastolic blood pressures and MAPs were significantly increased at all FIO_2s greater than 0.21. Varying FIO_2 had no significant effect on HR. In Table 2, administering DA and increasing FIO_2s resulted in a significant decrease in SBP at 0.60
($p < 0.05$). Increasing FIO_2 had no significant effect on DBP and MAP. Again HR was not significantly increased in any of the FIO_2 with DA groups.

Arterial Blood Gases and Hemoglobin

Tables 3 and 4 contain ABG data at control, HS and treatment at different FIO_2s without and with DA infusion. There were no significant differences among the variables at control and shock among all eight groups ($p > 0.05$). Plasma HCO_3 in rats breathing FIO_2 greater than 0.21 was significantly greater than that at ambient air (0.21) and correspondingly the Beecf was less in these rats (Table 3). As expected, increasing the FIO_2 resulted in significant increases in the PaO_2 ($p > 0.05$). In the treatment groups without and with DA, increasing FIO_2 had no significance effects on pH, PCO_2, O_2 saturation or Hgb.

Lung hydrogen peroxide

The effects of increasing inspired O_2 without and with DA infusion on lung H_2O_2 are illustrated in Figure 1. Increasing FIO_2 only (open bars) resulted in significant decreases in Fluor intensity (H_2O_2) at FIO_2 of 0.40 and 0.60 ($p < 0.05$). However, increasing FIO_2 to 1.00 resulted in H_2O_2 not being significantly different from FIO_2 equal to 0.21. Increasing FIO_2 plus DA (striped bars) was accompanied by significant decreases at all FIO_2s ($p < 0.05$). Dopamine significantly decreased ($p < 0.05$) H_2O_2 at FIO_2s of 0.21, 0.60 and 1.00 (striped bars versus open bars). However, DA did not significantly decrease H_2O_2 at FIO_2 of 0.40.

Lung apoptosis

Figure 2 summarizes percent apoptosis in lung tissue at various FIO_2s without and with the administration of DA. With the exception of FIO_2 equal to 1.00, increasing FIO_2 was accompanied by significant decreases (open bars) in lung apoptosis ($p < 0.05$), compared to lung apoptosis using an FIO_2 at 0.21. Increasing FIO_2 while infusing DA (striped bars) resulted in the percent of lung apoptosis being significantly increased only at FIO_2 of 1.00 ($p < 0.05$). When comparing the FIO_2 plus DA to FIO_2 only groups (striped bars versus open bars), a significant decrease in the percentage of lung apoptosis was found in all groups except FIO_2 equal to 0.40.

Diaphragm hydrogen peroxide

Figure 3 illustrates diaphragm H_2O_2 for FIO_2 only and FIO_2 plus DA groups. For the FIO_2 only groups (open bars), there were significant decreases in H_2O_2 at 0.40 and 0.60 with respect to 0.21($p < 0.05$). In contrast, when breathing 100% O_2 diaphragm

H_2O_2 was significantly greater than breathing room air. In the FIO_2 with DA groups (striped bars), there were significant decreases in H_2O_2 in 0.40 and 0.60 FIO_2 groups ($p < 0.05$). However at an FIO_2 of 1.00, diaphragm H_2O_2 was not significantly less than ambient air. Administering DA resulted in diaphragm H_2O_2 being significantly decreased at all FIO_2s except 0.40 (striped versus open bars) ($p < 0.05$).

Diaphragm apoptosis

Percent diaphragm apoptosis results are summarized in Figure 4. Increasing FIO_2 to 0.40 and 0.60 resulted in significant decreases in apoptosis (open bars). In contrast, administering FIO_2 equal to 1.00 resulted in the apoptosis being significantly greater than 0.21 ($p < 0.05$). Infusing DA (striped bars) at FIO_2 0.40 and 0.60 did not result in significant decreases in apoptosis. In contrast, at an FIO_2 of 1.00 apoptosis was significantly greater than at 0.21($p < 0.05$). With the exception of FIO_2 at 0.40, infusing DA significantly decreased apoptosis at all FIO_2s (striped bars versus open bars).

Discussion

In this study, we investigated the effects of FIO_2 (0.21, 0.40, 0.60, 1.00) and DA (10 mcg/kg/min) on lung and diaphragm H_2O_2 and apoptosis following 30 minutes of HS. We observed that H_2O_2 and apoptosis in lung and diaphragm were minimized when rats breathed 40% O_2. The extent of H_2O_2 and apoptosis were comparable and greatest in animals that were administered 21% or 100% O_2. With the addition of DA with supplemental O_2, significant decreases in lung and diaphragm H_2O_2 and apoptosis in all FIO_2 groups were observed except in the FIO_2 0.40 groups. At an FIO_2 of 0.21, arterial

blood pressures remained decreased throughout the treatment period. Arterial blood gases and Hgb results were similar to other investigations studying HS.

Arterial blood pressures increased with supplemental O_2 administration (0.40, 0.60, and 1.00) during the treatment period (Atkins, Johnson, & Pearce, 2007). Infusing DA at all FIO_2s resulted in arterial blood pressures not being significantly different at the end of the treatment period.

Lung H_2O_2

Hydrogen peroxide production contributes to lung injury during HS (Souza, et al., 2000). In HS, reduced DO_2 activates leukocytes resulting in increased free radical production (Kapoor & Prasad, 1996). In addition, cellular hypoxia results in increasing mitochondria free radical formation (Z. H. Chen, Saito, Yoshida, & Niki, 2008). It has been reported that increasing DO_2 by increasing inspired O_2 may result in hyperoxic induced lung damage. Turrens et al. (1982) observed increased lung mitochondrial H_2O_2 production at FIO_2 greater than 0.60. Other investigators reported lung injury accompanied by increased free radical formation during hyperoxia (Budzinska & Ilasz, 2008; Turrens, Freeman, & Crapo, 1982; Turrens, Freeman, Levitt, & Crapo, 1982; Valenca Sdos et al., 2007). Thus, our results related to lung H_2O_2 production being affected by the FIO_2 are consistent with these investigators. Our data indicate that an FIO_2 of 0.40 is most beneficial in minimizing lung H_2O_2 production following HS. In class IV HS when hemoglobin and cardiac output are decreased, negative effects on microcirculation leads to tissue and cellular hypoxia and acidosis impairing mitochondrial functioning. Oxygen administration of 0.40 improves DO_2 by supporting

HR, MAP and increasing vascular resistance and can enhance antioxidant functions. Vento et al. 2009 observed that an FIO_2 of 0.30 enhanced glutathione free radical scavenging in prenatal neonates (Vento et al., 2009). In addition, Lee et al. (2005) found that mice breathing 40% O_2 had increased tissue levels of vitamin E and C, known antioxidants (Lee, et al., 2005).

Administering DA with varying FIO_2s resulted in significant decreases in lung H_2O_2 at all FIO_2 except 0.40. Gero et al. (2007) found that activating DA receptors was cytoprotective against H_2O_2 induced lung injury (Gero et al., 2007). Our results suggest that DA scavenges ROS in lung tissue. Dopamine enhances tissue O_2 perfusion, increases cardiac contractility, systemic pressure and HR in HS by stimulating beta 1 adrenoreceptors (Holmes & Walley, 2003). We have observed that DA increases diaphragm blood flow in rats following HS (J. D. Pierce, et al., 2006). This was related to the need for the diaphragm to maintain respiration when systemic blood flow was reduced by HS. As a consequence, DO_2 to the tissues is increased with DA administration which could in part account for the reduction in H_2O_2 production (J. Pierce, Mach, W., Knight, A., Pierce, J.T., Slusser, J. & Clancy, R., 2009).

Lung Apoptosis

Programmed cell death is associated with HS (Moran, et al., 2008). Shih et al. (2005) studied lung differential gene expression and found HS induced up-regulation of genes responsible for apoptosis (Shih, et al., 2005). High concentrations of FIO_2 cause ROS (H_2O_2) mediated apoptosis (Buccellato, et al., 2004). Hydrogen peroxide production results in formation of $HO^{\cdot}$ leading to caspase activation that trigger

apoptotic events (Ryter et al., 2007). The increase in ROS causes a release of cytochrome c from the mitochondria, resulting in cell death (Pagano & Barazzone-Argiroffo, 2003). Administering FIO_2 of 0.21 and 1.00 after inducing HS resulted in the greatest percent lung apoptosis. Similarly, H_2O_2 was also greatest at these two FIO_2. Hypoxia and hyperoxia were associated with apoptosis. In HS, an FIO_2 of 0.40 produced the least amount of lung H_2O_2 and apoptosis. When using an FIO_2 of 0.60, there was a slight increase in lung apoptosis and H_2O_2 compared to 0.40.

In HS, activated leukocytes are a source of free radicals (Childs et al., 2002). DA has been shown to reduce polymorphonuclear leukocyte $O_2^{-\cdot}$ production (Yamazaki, Matsuoka, Yasui, Komiyama, & Akabane, 1989). Similar to other antioxidants, in this study DA decreased lung apoptosis at all FIO_2s (0.21, 0.40, 0.60, 1.00) presumably as of a result of decreasing H_2O_2. At FIO_2 of 0.40 lung apoptosis with DA was not significantly different than in animals not administered DA. The extent of lung apoptosis at FIO_2 of 0.40 was similar to sham lung apoptosis (2%). Our results are similar to the Teramoto et al. study in which they concluded that H_2O_2 induced lung apoptosis was in part attributable to ROS production (Teramoto et al., 1999).

Diaphragm H_2O_2

There is well established research of the effects of free radicals on diaphragm muscle function (X. Li et al., 2000; Supinski & Callahan, 2005). Free radicals attenuate calcium release from diaphragm muscle cells resulting in decreased force generation that leads to respiratory distress (Supinski & Callahan, 2005). Diaphragm ROS are generated during re-oxygenation following hypoxia (Zuo & Clanton, 2005). Oxygen radical

generation also occurs in the diaphragm during exposure to hyperoxia (Anzueto et al., 1994). In the present study, administering FIO_2 of 0.40 resulted in the least amount of diaphragm H_2O_2. In contrast, FIO_2s of 0.21 and 1.00 were accompanied by the highest amount of diaphragm H_2O_2. With an exception of FIO_2 of 0.40, adding DA decreased H_2O_2. We have reported that DA increases diaphragm blood flow (J. D. Pierce, et al., 2002). The resulting increase in DO_2 would reduce ROS formation.

Diaphragm Apoptosis

The percent of diaphragm apoptosis at various FIO_2s paralleled the changes in diaphragm H_2O_2 and lung apoptosis. The percent diaphragm apoptosis was greatest at FIO_2s at 0.21 and 1.00 and lowest at 0.40. The addition of DA with various FIO_2 resulted in a marked reduction in the percent of apoptosis. Thus, we believe DA is a free radical scavenger that attenuates apoptosis in the diaphragm by possibly stimulating beta 2 adrenoreceptors or by increasing antioxidant defenses (J. D. Pierce, et al., 2008).

Conclusions

Treatment of HS is directed at increasing DO_2 to cells. One hundred percent O_2 is routinely employed to achieve this objective. However, there are numerous studies indicating that 100% O_2 results in tissue injury which is attributed to increased free radical formation. We found that O_2 administered at an FIO_2 of 1.00 in HS increased H_2O_2 and apoptosis in both the lung and diaphragm. This study suggests that increased lung and diaphragm H_2O_2 and apoptosis resulting from the administration of 100% O_2 can be prevented by infusing DA (10 mcg/kg/min). Our results also indicate that while breathing 21% O_2 for 60 minutes following HS, increased lung and diaphragm H_2O_2 and

apoptosis can be markedly decreased by administering DA. This effect of DA appears to be attributable to its free radical scavenging capabilities. Administering 40% O_2 achieved the greatest reduction in H_2O_2-mediated apoptosis in lung and diaphragm accompanying HS.

Table 1. Hemodynamics for the different fraction of inspired oxygen concentrations.

	0.21	0.40	0.60	1.00
SBP (mm Hg)				
Control	148 ± 4	162 ± 9	157 ± 7	167 ± 9
Shock	97 ± 4	83 ± 3	93 ± 5	94 ± 7
Treatment	93 ± 9	121 ± 14	133 ± 10*	126 ± 14
DBP (mm Hg)				
Control	107 ± 5	122 ± 7	115 ± 7	124 ± 7
Shock	43 ± 1	39 ± 2	38 ± 3	46 ± 3
Treatment	38 ± 9	71 ± 13*	76 ± 6*	76 ± 15*
MAP (mm Hg)				
Control	127 ± 5	141 ± 7	135 ± 7	143 ± 7
Shock	61 ± 2	54 ± 3	57 ± 3	63 ± 4
Treatment	56 ± 9	94 ± 15*	98 ± 7*	95 ± 16*
HR (b/min)				
Control	357 ± 24	358 ± 10	355 ± 15	334 ± 26
Shock	366 ± 9	326 ± 17	380 ± 11	311 ± 24
Treatment	392 ± 23	379 ± 18	403 ± 19	405 ± 17

Data are presented as mean ± SEM, n = 6. SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure, HR: heart rate.
* $p < 0.05$ in comparison with $FIO_2 = 0.21$.

Table 2. Hemodynamics for the different fraction of inspired oxygen concentrations plus dopamine.

	0.21	0.40	0.60	1.00
SBP (mm Hg)				
Control	161 ± 3	153 ± 6	162 ± 9	155 ± 3
Shock	85 ± 4	86 ± 4	93 ± 6	78 ± 6
Treatment	157 ± 10	130 ± 12	122 ± 6*	133 ± 11
DBP (mm Hg)				
Control	118 ± 2	111 ± 4	108 ± 6	112 ± 3
Shock	38 ± 1	35 ± 2	36 ± 3	40 ± 2
Treatment	80 ± 9	65 ± 11	70 ± 9	90 ± 11
MAP (mm Hg)				
Control	138 ± 2	132 ± 4	135 ± 7	132 ± 3
Shock	55 ± 1	52 ± 2	55 ± 2	54 ± 2
Treatment	104 ± 8	89 ± 11	92 ± 9	109 ± 11
HR (b/min)				
Control	351 ± 11	357 ± 20	314 ± 24	372 ± 18
Shock	324 ± 20	332 ± 17	291 ± 29	311 ± 17
Treatment	402 ± 20	438 ± 31	361 ± 38	396 ± 31

Data are expressed as mean ± SEM, n = 6. SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure, HR: heart rate.
* $p < 0.05$ in comparison with $FIO_2 = 0.21$.

Table 3. Arterial blood gases for each fraction of inspired oxygen concentration.

	0.21	0.40	0.60	1.00
pH				
Control	7.42 ± 0.02	7.41 ± 0.02	7.40 ± 0.02	7.39 ± 0.02
Shock	7.46 ± 0.03	7.44 ± 0.03	7.47 ± 0.04	7.47 ± 0.04
Treatment	7.24 ± 0.07	7.39 ± 0.04	7.38 ± 0.05	7.31 ± 0.05
$PaCO_2$ (mm Hg)				
Control	38 ± 2	41 ± 3	41 ± 1	44 ± 2
Shock	24 ± 3	29 ± 3	24 ± 2	27 ± 3
Treatment	20 ± 2	25 ± 4	27 ± 3	27 ± 5
HCO_3 (mEq/L)				
Control	25 ± 1	26 ± 1	26 ± 1	27 ± 1
Shock	17 ± 1	19 ± 1	17 ± 1	19 ± 1
Treatment	8 ± 1	16 ± 3*	16 ± 2*	15 ± 3*
Beecf				
Control	0.3 ± 0.8	-1.3 ± 1.6	0.8 ± 0.9	1.2 ± 1.0
Shock	-7.3 ± 1.4	-5.0 ± 1.0	-6.3 ± 1.8	-4.3 ± 1.5
Treatment	-19.8 ± 1.5	-9.3 ± 3.1*	-8.8 ± 2.5*	-11.7 ± 4.0
PaO_2 (mm Hg)				
Control	80 ± 2	81 ± 4	78 ± 5	77 ± 3
Shock	89 ± 8	86 ± 6	112 ± 8	108 ± 9
Treatment	88 ± 5	196 ± 8*	244 ± 22*	519 ± 21*
O_2 Saturation (%)				
Control	94 ± 1	93 ± 1	95 ± 1	95 ± 1
Shock	97 ± 1	97 ± 1	99 ± 1	99 ± 1
Treatment	97 ± 1	99 ± 1	99 ± 1	99 ± 1
Hgb (g/100 mL)				
Control	12.6 ± 0.6	13.9 ± 0.3	12.5 ± 1.0	14.0 ± 0.9
Shock	7.7 ± 1.0	7.7 ± 0.5	5.8 ± 0.6	8.6 ± 1.0
Treatment	6.0 ± 0.7	6.8 ± 0.5	6.5 ± 0.8	7.0 ± 0.5

Data are expressed as mean ± SEM, n = 6. $PaCO_2$: partial pressure carbon dioxide, HCO_3: bicarbonate, Beecf: base excess, PaO_2: partial pressure of oxygen, O_2 Saturation: % of oxyhemoglobin, Hgb: hemoglobin.
* $p < 0.05$ in comparison with $FIO_2 = 0.21$.

Table 4. Arterial blood gases for each fraction of inspired oxygen concentration plus dopamine.

	0.21	0.40	0.60	1.00
pH				
Control	7.40 ± 0.09	7.43 ± 0.03	7.43 ± 0.04	7.41 ± 0.02
Shock	7.44 ± 0.07	7.43 ± 0.02	7.43 ± 0.05	7.39 ± 0.02
Treatment	7.43 ± 0.02	7.35 ± 0.06	7.39 ± 0.04	7.32 ± 0.05
$PaCO_2$ (mm Hg)				
Control	45 ± 1	35 ± 3	43 ± 5	41 ± 2
Shock	32 ± 5	27 ± 2	31 ± 5	34 ± 3
Treatment	25 ± 3	19 ± 2	28 ± 5	36 ± 7
HCO_3 (mEq/L)				
Control	28 ± 1	24 ± 1	27 ± 1	26 ± 1
Shock	21 ± 1	19 ± 1	19 ± 2	21 ± 1
Treatment	16 ± 2	11 ± 2	16 ± 3	19 ± 3
Beecf				
Control	2.0 ± 0.6	-0.3 ± 0.6	2.3 ± 0.6	1.0 ± 0.7
Shock	-3.5 ± 1.7	-5.0 ± 0.7	-5.8 ± 1.3	-4.5 ± 0.4
Treatment	-7.7 ± 1.5	-14.0 ± 3.3	-8.0 ± 3.0	-6.8 ± 3.0
PaO_2 (mm Hg)				
Control	72 ± 3	78 ± 5	75 ± 5	72 ± 4
Shock	95 ± 10	84 ± 6	92 ± 4	89 ± 5
Treatment	87 ± 6	181 ± 15*	282 ± 9*	506 ± 27*
O_2 Saturation (%)				
Control	94 ± 1	93 ± 1	95 ± 2	95 ± 1
Shock	97 ± 1	97 ± 1	99 ± 1	99 ± 1
Treatment	97 ± 1	99 ± 1	99 ± 1	99 ± 1
Hgb (g/100 mL)				
Control	13.1 ± 0.4	10.8 ± 0.5	13.1 ± 0.2	13.2 ± 0.5
Shock	8.1 ± 1.0	6.2 ± 0.3	7.3 ± 0.4	7.1 ± 0.7
Treatment	7.1 ± 0.6	5.9 ± 0.3	6.9 ± 0.4	7.1 ± 0.6

Data are expressed as mean ± SEM, n = 6. $PaCO_2$: partial pressure carbon dioxide, HCO_3: bicarbonate, Beecf: base excess, PaO_2: partial pressure of oxygen, O_2 Saturation: % of oxyhemoglobin, Hgb: hemoglobin.
* $p < 0.05$ in comparison with $FIO_2 = 0.21$.

Figure 1. Fluor intensity of lung hydrogen peroxide for FIO_2 and FIO_2 plus DA

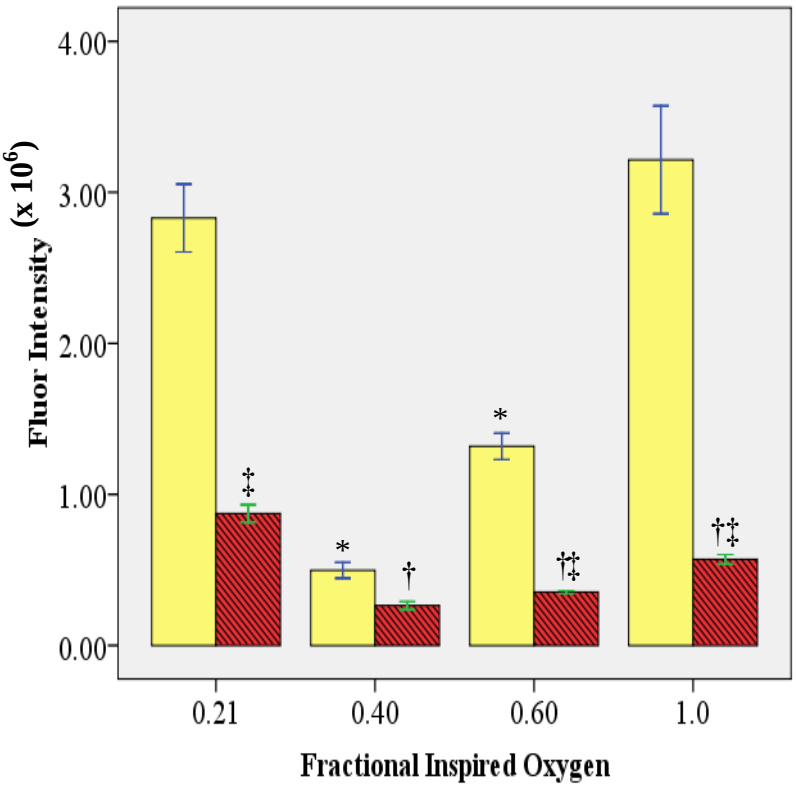

Figure Legend

Open bars = FIO_2 groups; Striped bars = FIO_2 plus DA groups.
* = significantly different from FIO_2 at 0.21 within the FIO_2 only group - □ ($p < 0.05$).
† = significantly different from FIO_2 at 0.21 within the FIO_2 plus DA group – ▧ ($p < 0.05$).
‡ = significantly different between the FIO_2 only and FIO_2 plus DA groups - □ vs. ▧ ($p < 0.05$).
Data are expressed as mean ± SEM, n = 6 (due to experimental design, see methods section).

Figure 2. Percent lung apoptosis for FIO_2 and FIO_2 plus DA

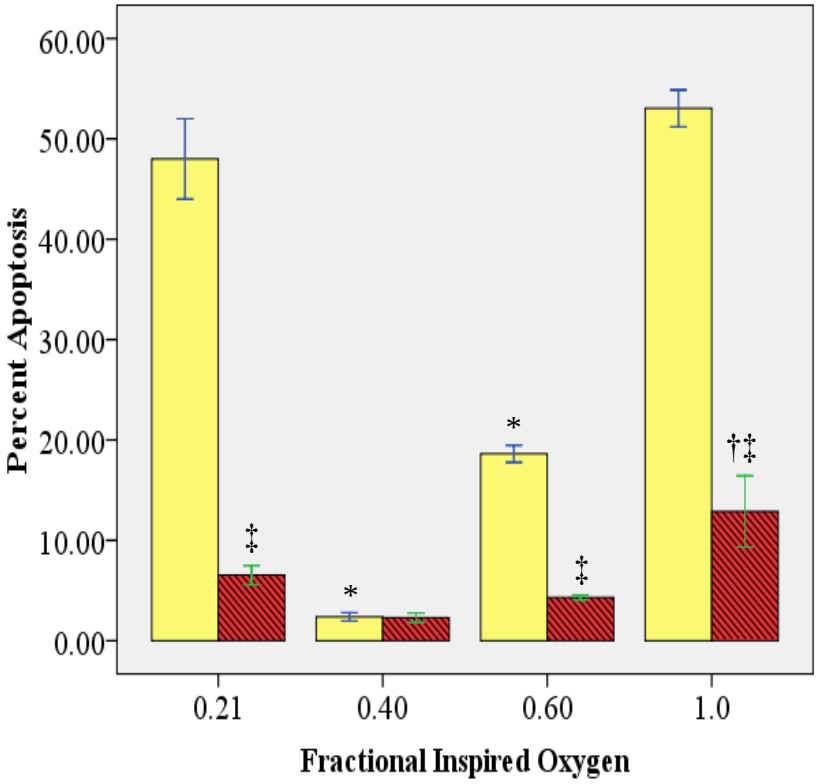

Figure Legend

Open bars = FIO_2 groups; Striped bars = FIO_2 plus DA groups.
* = significantly different from FIO_2 at 0.21 within the FIO_2 only group - □ ($p < 0.05$).
† = significantly different from FIO_2 at 0.21 within the FIO_2 plus DA group – ▨ ($p < 0.05$).
‡ = significantly different between the FIO_2 only and FIO_2 plus DA groups - □ vs. ▨ ($p < 0.05$).
Data are expressed as mean ± SEM, n = 8.

Figure 3. Fluor intensity of diaphragm hydrogen peroxide for FIO_2 and FIO_2 plus DA

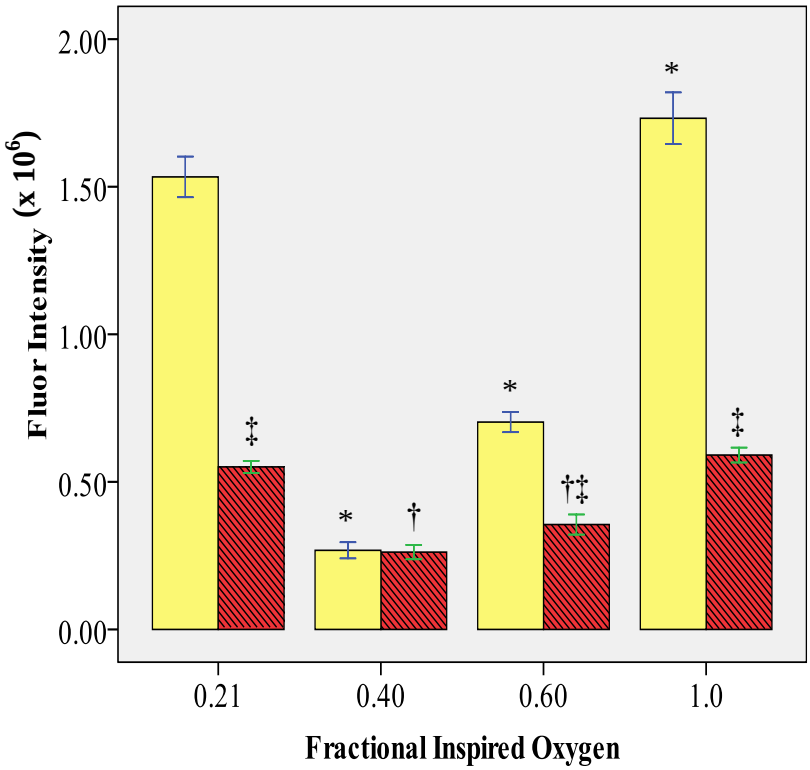

Figure Legend

Open bars = FIO_2 groups; Striped bars = FIO_2 plus DA groups.
* = significantly different from FIO_2 at 0.21 within the FIO_2 only group – □ ($p < 0.05$).
† = significantly different from FIO_2 at 0.21 within the FIO_2 plus DA group – ▨ ($p < 0.05$).
‡ = significantly different between the FIO_2 only and FIO_2 plus DA groups - □ vs. ▨ ($p < 0.05$).
Data are expressed as mean ± SEM, n = 6 (due to experimental design, see methods section).

Figure 4. Percent diaphragm apoptosis for FIO_2 and FIO_2 plus DA

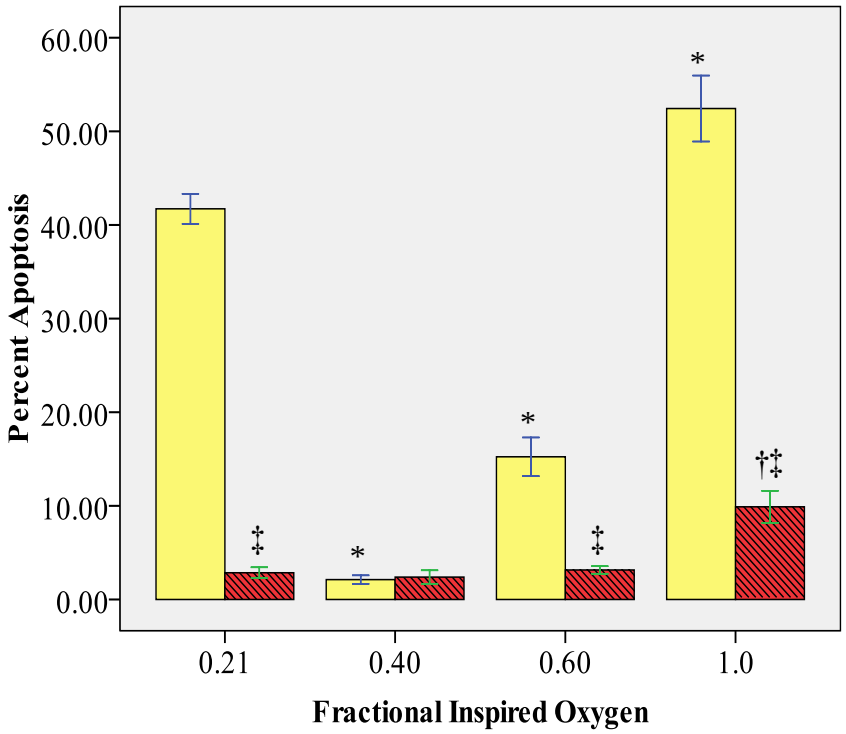

Figure Legend

Open bars = FIO_2 groups; Striped bars = FIO_2 plus DA groups.
* = significantly different from FIO_2 at 0.21 within the FIO_2 only group - □ ($p < 0.05$).
† = significantly different from FIO_2 at 0.21 within the FIO_2 plus DA group – ▨ ($p < 0.05$).
‡ = significantly different between the FIO_2 only and FIO_2 plus DA groups - □ vs. ▨ ($p < 0.05$).
Data are expressed as mean ± SEM, n = 8.

Chapter 5.

Discussion and Conclusion

The purpose of this chapter is to summarize and review the information in chapters one through four. A short review of chapter one, two and three is provided to offer a foundation on which the experimental studies were based. Next, each research question is restated and answered followed by a discussion of the experimental results. From our data, future studies are suggested and possible mechanisms to be investigated are addressed.

In chapter one, following the general introduction related to HS, the overall aims and research questions of this research were stated. The optimal FIO_2 to be administered following HS is unknown, thus one aim was to determine what the optimal FIO_2 that minimizes lung and diaphragm H_2O_2 and apoptosis in a rat model of controlled HS. Dopamine has been shown to be an antioxidant in other studies decreasing apoptosis. Hence, the second aim was to determine the effect of DA at different FIO_2s on lung and diaphragm apoptosis following 30 minutes of HS.

Background literature included in chapter one primarily related to the effects of HS on the lungs and the diaphragm. Since free radicals, specifically ROS are implicated in cell damage associated with HS, information about ROS was discussed. A brief review of O_2 and oxygenation, hyperoxia and O_2 toxicity was also included in this chapter since HS resuscitation involves the use of supplemental O_2. Hemorrhagic shock has been shown to cause apoptosis in many different cell types, for this reason, measurement of apoptosis during HS was examined. In HS, DA is frequently administered in addition to fluids to support blood pressure. Dopamine is a free radical

scavenger, thus there is a section related to DA and ROS, DA receptor activation and DA and apoptosis.

Chapter two was a review manuscript entitled *Consequences of Hyperoxia and the Toxicity of Oxygen in the Lung.* This review contained literature examining O_2 as a drug and O_2 toxicity. Three highly related topics 1) the formation of free radicals, 2) the functions and classification of antioxidants, and 3) oxidative stress were discussed. Since the effects of oxidative stress are associated with increased damage to lung structures, a review of lung physiology was included in this chapter. This was followed by a description of the similarities between the pathophysiology of hyperoxic acute lung injury (HALI) and the pathophysiological conditions which are associated with HS or reperfusion injury.

Chapter three was another review manuscript entitled *Flow Cytometry and Confocal Laser Scanning Cytometry: a Comparison of Techniques*. In this manuscript there was a comparison of both cytometry techniques. Cytometry refers to the measurement of physical and or chemical characteristics of cells. Flow cytometry is a cytometry technique which requires a sample of cells or cell constituents of interest to be disaggregated and suspended in solution. Laser scanning cytometry does not require that the sample be disaggregated. This technique utilizes slide-based whole tissue preparation which was more suitable for answering our research questions. In our studies we determined the relative intensity of H_2O_2 in the lungs and diaphragm by using LSCM. A fluorescent probe (HFLUOR-DA) was used that immediately oxidizes to

Fluor in the presence of cellular H_2O_2. The fluorescent intensity of this probe is directly proportional to the amount of H_2O_2 in the tissue sample.

Chapter four was a manuscript with our study data entitled *The Effects of Increased Inspired Oxygen with and without Dopamine on Lung and Diaphragm Hydrogen Peroxide and Apoptosis Following Haemorrhagic Shock*. The first research question in this study was:

Research question one. What is the amount of H_2O_2 in the lung and diaphragm after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60, 1.00) following 30 minutes of exsanguinations to attain HS?

The amount of H_2O_2 in the lung and diaphragm after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60, 1.00) following 30 minutes of HS was expressed as the mean fluorescent intensity (x 10^6). The figures in this chapter illustrate the amount of H_2O_2 in the lungs after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60 and 1.00) and the mean values were 2.80, 0.40, 1.30 and 3.20, respectively. The amount of H_2O_2 in the diaphragm after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60 and 1.00) was 1.50, 0.27, 0.70 and 1.70, respectively. Following 30 minutes of exsanguinations, increasing the FIO_2 from 0.21 (room air) to 0.40 significantly reduced the amount of H_2O_2 in lung and diaphragm tissue. Further increases in FIO_2 to 0.60 or 1.00 were associated with more H_2O_2 production in the lung and diaphragm, thus an FIO_2 of 0.40 was optimal at reducing tissue damage following HS.

Studies have shown that cellular H_2O_2 can be increased in both hypoxia and hyperoxia (Sigaud, Evelson, & Gonzalez-Flecha, 2005). Kutzsche et al. (2001) found that H_2O_2 production from neutrophils increases following hypoxia and re-oxygenation with an FIO_2 of 0.21 or 1.00 (Kutzsche, Ilves, Kirkeby, & Saugstad, 2001). Our results are similar in that there was an increase in H_2O_2 when using FIO_2s of 0.21 and 1.00. When using FIO_2s of 0.60 or 1.00, our results suggest that exposure to these concentrations of O_2 becomes less therapeutic then an FIO_2 of 0.40 and begins to contribute to H_2O_2 production and possible tissue damage in both the lung and diaphragm.

Research question two. What is the percentage of lung and diaphragm apoptosis after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60, 1.00) following 30 minutes of exsanguinations to attain HS?

The percentage of lung apoptosis after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60, 1.00) following 30 minutes of exsanguinations to attain HS was 48.0, 2.4, 18.6 and 53.0 respectively. The percentage of diaphragm apoptosis after 60 minutes of administration of various FIO_2 (0.21, 0.40, 0.60, 1.00) following 30 minutes of exsanguinations to attain HS was 41.7, 2.1, 15.2 and 52.4 respectively.

Cytochrome c oxidase within the electron transport chain of mitochondria is the primary site of ROS production which initiates apoptosis (Brunelle & Chandel, 2002; Cai & Jones, 1998; T. W. Jernigan, et al., 2004; Zhao & Xu, 2004). Other research suggests that during hypoxia, ROS production and paradoxical increases in ROS during hypoxia reflect the effect of O_2 within the mitochondrial membranes and complexes

(Chandel, Budinger, & Schumacker, 1996; Guzy & Schumacker, 2006). When mitochondrial membrane integrity is compromised cytochrome c enters the cytosol and apoptosis is initiated (Buccellato, et al., 2004; Budinger et al., 2002; Carbonell, Nussinov, & del Sol, 2009). Studies have shown that $O_2^{-\cdot}$ and H_2O_2 can initiate apoptosis in lung epithelial cells and the vascular endothelium (Kazzaz, et al., 1996; Yin, Stearns, & Gonzalez-Flecha, 2005). Reactive O_2 species can activate apoptotic pathways and our results indicated that in the FIO_2 groups when the amount of H_2O_2 was increased or decreased, so was the percent apoptosis.

Research question three. Does DA at a dosage of 10 mcg/kg/min administered with various FIO_2 (0.21, 0.40, 0.60, and 1.00) attenuate the amount of H_2O_2 and apoptosis in the lung and diaphragm following 30 minutes of exsanguinations to attain HS?

Our results indicated that DA at a dosage of 10 mcg/kg/min administered with various FIO_2 (0.21, 0.40, 0.60, and 1.00) does attenuate the amount of H_2O_2 in the lung. The amount of H_2O_2 in the lung was 0.87, 0.26, 0.35 and 0.57, respectively; this was much less H_2O_2 in this tissue than when using FIO_2s alone. The percent apoptosis in the lung was 6.5, 2.3, 4.3 and 12.9, respectively.

Our results show that DA at a dosage of 10 mcg/kg/min. administered with various FIO_2 (0.21, 0.40, 0.60, and 1.00) attenuated the amount of H_2O_2 in the diaphragm (0.55, 0.26, 0.36 and 0.59) and percent apoptosis in the diaphragm (2.9, 2.4, 3.1 and 9.9). Significant attenuation of both H_2O_2 and percent apoptosis occurred in all

FIO_2 groups except 0.40. An FIO_2 of 0.40 was already reduced; therefore, H_2O_2 did not significantly change with the addition of DA.

Studies have shown that DA functions as a free radical scavenger (Cosentino, et al., 2004; Gassen & Youdim, 1999; C. D. Kang et al., 1998). Several studies that have investigated the effect of DA on the diaphragm have demonstrated that DA improves diaphragm functioning in part by scavenging ROS (J. D. Pierce, et al., 2002; Yen & Hsieh, 1997), by improving blood flow (J. D. Pierce, et al., 2006), or by stimulation of β_2 adrenoreceptors (Communal, Singh, Sawyer, & Colucci, 1999; Patterson et al., 2004).

Strenuous diaphragm contractions can produce free radicals which can trigger apoptotic pathways. In HS, neuro-endocrine responses to decreased PaO_2 values increase RR and increase diaphragm contractions thus, increasing free radical production and apoptosis. The addition of DA with supplemental O_2 in our experiments attenuated H_2O_2 and apoptosis in the lungs and diaphragm.

Our results also demonstrated that in HS, adding DA improves MAP. Our data indicated that an FIO_2 of 0.21 with DA increased MAP more than when allowing the rat to breathe an FIO_2 of 0.21 following HS. However, the MAP was not significantly different between the groups that received O_2 plus DA and supplemental O_2 only following HS.

We also found that MAP increased when supplemental O_2 was administered after HS, which was similar to findings by other investigators (Bitterman, et al., 1996; Waisman et al., 2003). The increase in MAP was related to the vascular response to O_2 and to increased total peripheral resistance (Atkins, et al., 2007; Bitterman, et al., 1996)

which significantly enhanced blood flow. The reduction of H_2O_2 and apoptosis may be partially related to this increase in blood flow.

We demonstrated that a moderate increase in FIO_2 was beneficial physiologically but further increases to 0.60 and 1.00 were associated with increased H_2O_2. Administering O_2 may improve MAP following a hemorrhagic event and effectively treat tissue hypoxia. However, too much O_2 may be converted to damaging free radicals. Studies have shown that ROS generated from hyperoxic resuscitation increases leukocyte rolling and adhesiveness of PMNs and macrophages to alveolar endothelial cells leading to damage of cellular structures (Beck-Schimmer et al., 2001; Hierholzer & Billiar, 2001; Hierholzer, Harbrecht, Billiar, & Tweardy, 2001).

If an FIO_2 of 1.00 is necessary as a treatment following HS, adding DA to the resuscitation regimen to minimize inadvertent secondary injury to the lungs and diaphragm can be a supportive intervention. Our results suggest that the use of O_2 in HS reduces hypotension and hypoxia. However, our results also suggest that in HS, resuscitation with FIO_2's of 0.60 and 1.00 can increase ROS injury to the lungs and diaphragm if a scavenger such as DA is not utilized.

Future Research

Future research related to HS that would be important to investigate is specific cellular response pathways such as mitogen activated protein kinase and caspase pathways which are activated during HS. This research could assist in further distinguishing patterns of cell death either by apoptosis, necrosis or cell repair.

Additionally, research is needed in the area of caspase activation that has been linked to tissue damage in HS models.

Currently, the Pierce laboratory is beginning to identify activated cell types following HS which are ROS sources. The literature indicates that polymorphonuclear leukocytes (PMNs) and alveolar macrophages are the most likely cell types to generate or activate ROS. Future research endeavors of our laboratory group would focus on examining the cell source of ROS with the goal of discovering a biomarker to measure ROS in blood or urine in a HS model.

Other organs (heart, brain and visceral organs etc.) may also be affected by various FIO_2 and DA following HS. Studies could be conducted that determines the amount of H_2O_2 and apoptosis in these different organs and whether there is a similar pattern that we observed in the lungs and diaphragm. Additional research investigating the up-regulation of genes that stimulate superoxide, H_2O_2 and $HO^{\cdot}$ production would be important to study to assist in understanding pathways stimulated during HS.

Other ROS scavengers such as N-acetylcysteine (NAC) could be investigated to determine if there is a similar effect as DA on H_2O_2 and apoptosis. Researchers have demonstrated that this antioxidant can inhibit inflammatory gene expression thus reducing ROS-induced damage (Eberlein et al., 2008). Future research may assist in explaining the properties of NAC in relation to ROS formation during HS.

Conclusion

In conclusion, we demonstrated that HS induces H_2O_2 and apoptosis in the lungs and diaphragm. However, by administering FIO_2 greater than 0.40, an increase in H_2O_2

and apoptosis was found. With the addition of DA, H_2O_2 and apoptosis were reduced in all groups except 0.40. In HS resuscitation, infusing DA (10 mcg/kg/min) can be protective to cells when there are increased levels of ROS. Adding DA with supplemental O_2, lead to an overall reduction of H_2O_2 and apoptosis in the lung and diaphragm. These findings demonstrate the capability of DA to scavenge ROS, or stimulate β-2 adrenoreceptors, thus decreasing apoptosis. Continuing research related to optimal resuscitation strategies following HS will lead to further understanding of the cellular responses that will assist in translating data from the laboratory to the bedside.

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