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Hyperoxia-Induced Cardiac Pathophysiology in Guinea Pig Hearts

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by

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of the requirements for the degree of
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ABSTRACT

Hyperoxia, is regularly introduced to critically ill patients in ICU. Nevertheless, recent studies have shown the negative effects of this treatment on patients in critical care, including increased rates of lung and cardiac injury and thereby high in-hospital mortality. Large part of the literature related to hyperoxia was majorly focused on lung injury, with no or minimal investigations on cardiac injury. Our lab is the first to investigate the effect of hyperoxia on cardiac pathophysiology in mice and showed that mice exposed to hyperoxia for 3 days demonstrated brady-arrhythmia, cardiac hypertrophy, QTc prolongation along with other electrical remodeling and functional abnormalities due to dysregulation of important ion channels and other genes. Although, rodents are widely used animal model for research studies, guinea pigs are well known for its close similarity with human physiology. Therefore, to bring more clinical relevance to this study, we have utilized guinea pigs and investigated effect of hyperoxia on cardiac remodeling. For this study we exposed male guinea pigs (n=12) of one-year old age to hyperoxia or normoxia for three days and investigated physical and electrical parameters. Similar to our previous reports in mice, hyperoxia treated guinea pigs also showed significant reduction in body weight compared to normoxia controls. We also observed lung edema in hyperoxia treated guinea pigs, as evident by lung wet weight to dry weight ratio. Surprisingly in guinea pigs there are no significant changes in heart weight after hyperoxia treatment which is different from our previous studies in mice. Whereas we observed significant differences in left ventricle (LV), right ventricle (RV) and septum cardiomyocytes size in hyperoxia treated hearts compared to normoxia control hearts. ECG analysis revealed significant increase in RR intervals as a result of bradycardia, similar to what we reported in mice. Whereas significant decrease in P duration, QTc interval, R amplitude and T

amplitude, which is distinct from what we observed in mice. From qRT-PCR data results we found marginal difference in key ion channel Kv 1.4 and Kv 4.2 whereas in Kv 4.3 and Kv 2.1 showed significant difference between normoxia and hyperoxia group which may be the reason of arrythmias in hyperoxia guinea pigs. Overall, our data indicated that hyperoxia-induced cardiac pathophysiology in guinea pigs are distinct from mice, despite of we similarities in physical parameters.

The data obtained from this study will not only improve our understanding on hyperoxia related in-hospital mortality, but also help us to understand possible mechanisms through which hyperoxia induce cardiac pathophysiology. This will also open new avenues for targeted therapy

CHAPTER ONE

INTRODUCTION

1.1 Hyperoxia Treatment on ICU Patients:

High oxygen O_2 concentrations is mostly administered in the ICU patients to provide them with physiological levels of oxygen and carbon dioxide. In different conditions like cardiac arrest, stroke, respiratory failure, sepsis, Traumatic brain injuries (TBI) and after critical surgeries, oxygen is provided with mechanical ventilation to the patients [1]. Hyperoxia can be described as administering a higher-than-normal concentration of O_2 to lungs with mechanical ventilation. Hyperoxemia indicates a high level of O_2 in the bloodstream. In terms of pressure, the partial pressure of O_2 (PaO_2) greater than 100 mm hg/13.3 kPa refers to a hyperoxic condition [2].

Despite its extensive practice of administering O_2, a cohort study that evaluated arterial hyperoxia in critically ill patients confirmed that there is a direct association between hyperoxia and hospital mortality [3]. Also, another study showed that hyperoxia tends to significantly change cardiovascular and hemodynamic conditions which includes low cardiac output and stroke volume [4]. Adults who are treated with hyperoxic O_2 reduces the cerebral blood flow by 11 to 33%. Furthermore, we previously observed that the coronary artery blood flow decreases by 8-20% when treated with normobaric hyperoxia in subjects with chronic heart failure and coronary artery disease as well as in normal subjects [5]. We demonstrated that there are significant marked ion channel fluctuations and cardiac hypertrophy in mice when treated with extended hyperoxia, which causes significant pathophysiological remodeling of the heart [6]. Even though hyperoxia is used as the primary treatment for patients with respiratory disorder, with this treatment there comes a disadvantage that exposure to hyperoxia with longer duration (FIO_2>0.8) can ultimately cause

hyperoxia-induced acute lung injury (HALI), which is a pathological condition characterized by severe alveolar injury [7].

1.2 Hyperoxia and Lungs Pathophysiology:

It is estimated that in USA around 10000 to 15000 babies are born each year with bronchopulmonary dysplasia (BPD) alone [8]. Babies who are born prematurely with respiratory distress syndrome recurrently go through bronchopulmonary dysplasia (BPD) which is characterized by decreased coronary and alveolar development. Hyperoxia has a major role in back regulation in the growth of vascular endothelial cells in the lungs. It is demonstrated that interruptions in the regular growth and angiogenesis of capillaries with alveolar septa are a main contributory feature in the exhibition and occurrence of BPD. Bhaskaran et al[9] indicated that about 7 miRNAs are found to be altered in neonates when exposed to hyperoxia and the results indicated that these miRNAs can be the key factors in controlling the pathogenesis of BPD. They demonstrated that with hyperoxia the developing lungs induce p53 to produce miR-34a all over the lungs which in turn attacks a wide variety of genes. These attacks tend to cause significant changes to genes which alone or altogether trigger BPD pathway in neonates [9]. Schematic diagram of hyperoxia-induced ALI and BPD was illustrated in **Figure 1**.

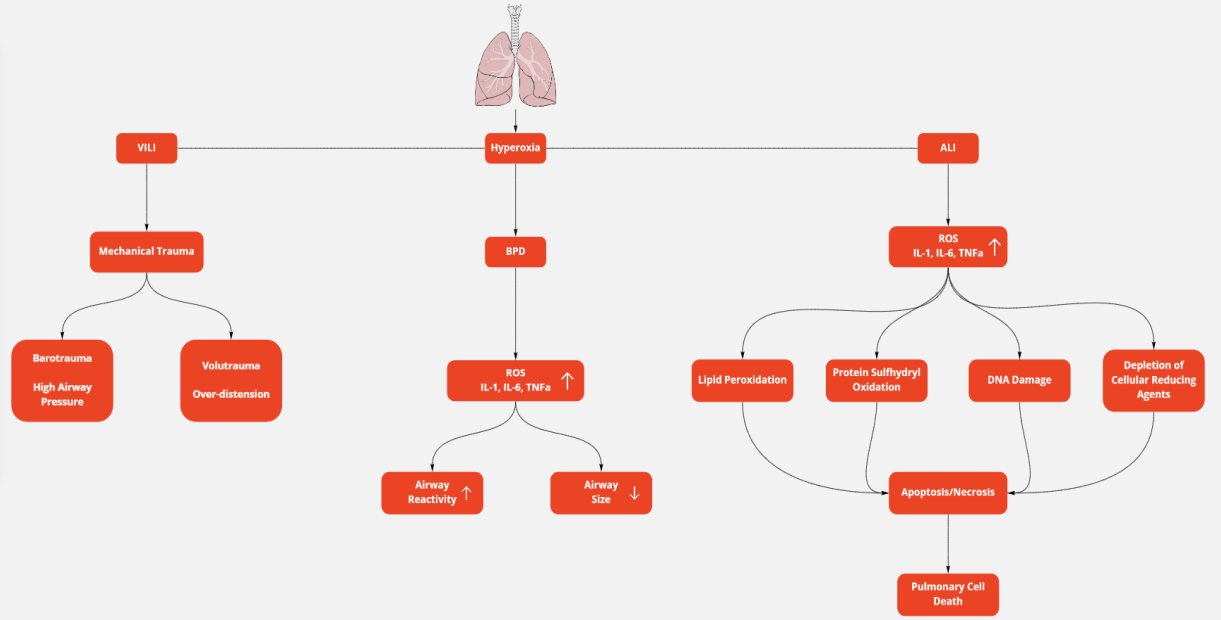

***Figure 1: Schematic chart of hyperoxia-induced lung injury*:** Hyperoxia induce major lung damage through mechanical injury, including biotrauma and volutrauma, which are collectively called **V**entilator-**I**nduced **L**ung **I**njury (VILI). Hyperoxia too actuates an incendiary response, primarily through the generation of **R**esponsive **O**xygen **S**pecies (ROS). ROS generation is the essential component of the improvement of **B**ronch**o**pulmonary **D**ysplasia (BPD) and **A**cute **L**ung **I**njury (ALI) [Color figure can be viewed at wileyonlinelibrary.com][10]

1.3 Hyperoxia and Oxidative Stress:

Cells produce reactive oxygen species (ROS), by reducing the molecular oxygen (O_2) to water (H_2O) [11]. These ROS facilitate normal lung pathophysiology within the body and are produced when there is exposure to hyperoxic conditions. Prolonged hyperoxic exposure causes an excess of ROS that disrupts the physiological balance between oxidants and antioxidants. This elevation of ROS directly interrupts the physiological homeostasis causing injury to cells and body tissues [12]. Epithelial cells of the alveoli and related pulmonary capillaries are a potential target of ROS. When targeted by ROS, the injury can lead to lung edema, fluid accumulation in alveoli, deposition of collagen, hyaline, and elastin fibers in membranes, and hemorrhage of capillary membranes [13]. At increased levels of oxygen, ROS devours all the endogenously produced

antioxidants enzymes henceforth causing cell death [14]. Edema and inflammation of the lungs lining are the most commonly reported harmful effects of hyperoxia, which is chiefly caused by the elevation in the levels of ROS, which is directly associated with O_2 levels in the lungs [15].

Various factors like atmospheric pressure, FIO_2, duration of exposure and cumulative oxygen dose determine and cause oxygen toxicity. When exposed to oxygen for a longer duration i.e. (≥24 hours and FIO2 greater than (>0.60) at normal atmospheric pressure 1ATA (atmosphere absolute), the oxygen becomes poisonous for the lungs. This kind of exposure is also called as Lorraine smith effect, pulmonary toxicity, or low-pressure O_2 toxicity [16]. This oxygen toxicity also occurs when exposure time to high FIO_2 is small, but the ATA is increased i.e., 1.6-4, often known as Paul Bert effect or high-pressure O_2 poisoning. Such toxicity of oxygen has a lethal effect on the central nervous system (CNS) [17]. Conditions like congestion of lungs passageway, atelectasis, and pulmonary edema are triggered by an injury to epithelial lining bronchi and alveoli which is a consequence of prolonged exposure i.e., 12 hours to oxygen. This edema and fluid accumulation of fluids in the lungs leads to symptoms like burning sensation in the chest and throat, shortness of breath (SOB) and eventually turning the process of breathing painful for the subject [16]. With Paul Bert's effect, CNS toxicity occurs which causes seizures in patients consequently leading to coma within an hour i.e. (30-60 minutes) and can be potentially fatal to patients without any warning sign. Other symptoms which are associated with CNS toxicity are disorientation, nausea, and vomiting, touchiness, vision impairment, jerking of muscles, and giddiness [18]. **Figure 2** illustrates schematic representation of summary of pathophysiological events induced during pulmonary toxicity under hyperoxia exposure.

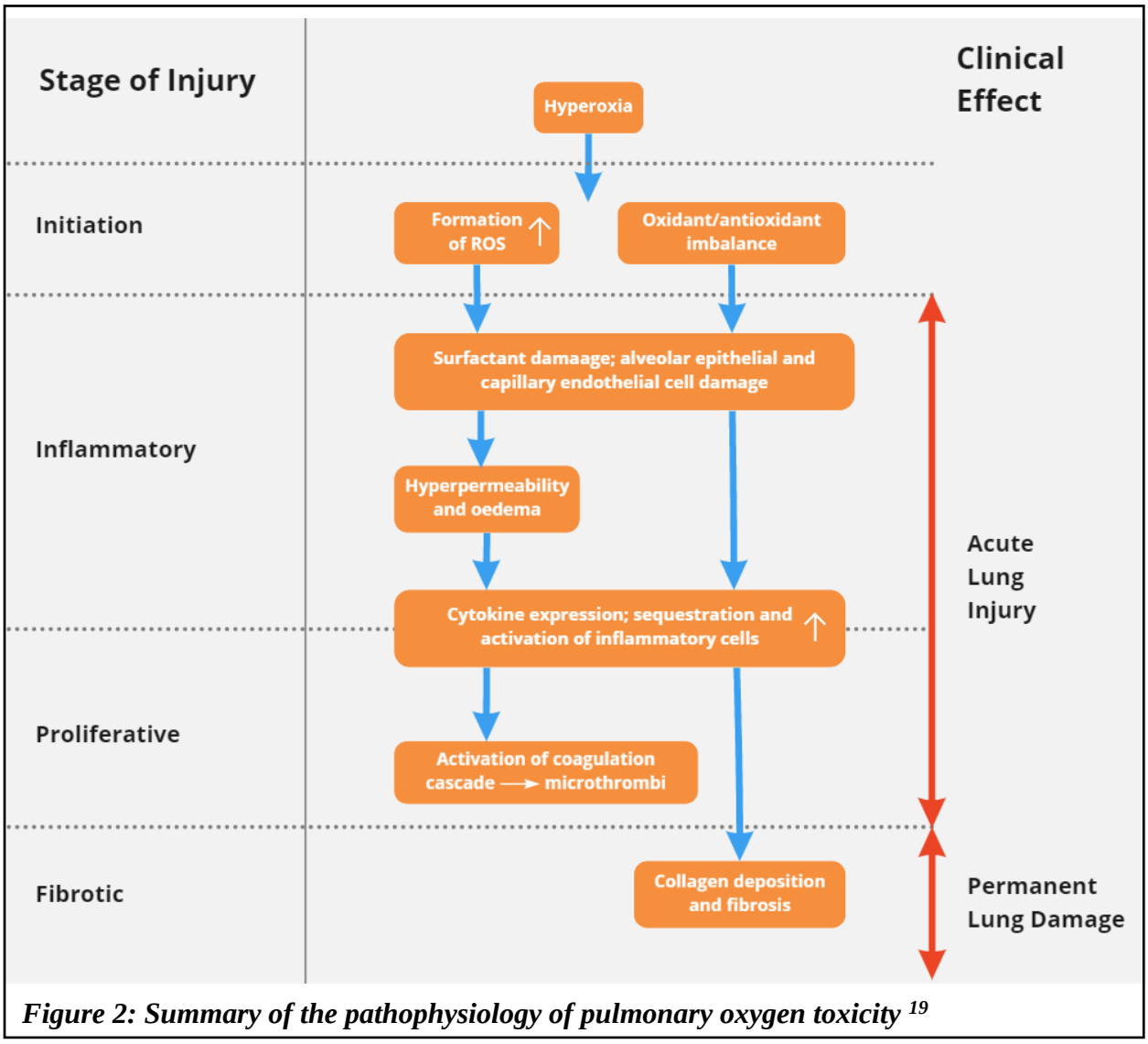

Figure 2: Summary of the pathophysiology of pulmonary oxygen toxicity [19]

The production of free radicals is also a potential pathway that contributes towards the pathophysiology of the lungs as the result of hyperoxia. Free radicals are known as reactive, very short-lived, and unstable chemical particles. These chemical entities are called free as they have possessed one or more unpaired electrons which are ready to react with chemical molecules in the environment [12, 20].

1.4 Hyperoxia and Heart Pathophysiology:

Hyperoxia has been associated with causing noteworthy changes in the hemodynamics which includes a decrease in cardiac output, heart rate, and stroke volume [21]. Study from our laboratory demonstrated that hyperoxia (>90% oxygen) contributes significantly towards various cardiac dysfunctions like hypertrophy and arrhythmias [6]. Furthermore, vasoconstriction and increase microcirculatory heterogeneity is caused by exposure to hyperoxia, henceforth compromising perfusion [22]. Vasoconstriction of coronary vessels is associated with a reduction in oxygen delivery heart muscles and amplifies the vulnerability towards ischemia, as well as decreases contractility of cardiac muscles [23].

Multiple research studies have demonstrated that hyperoxia is associated with cardiac complications and has a direct impact on blood vessels. The rise in ROS adversely affects the smooth muscle cells in the vessels by agonistic action of L-type Ca^{++} channels and closing the ATP-dependent potassium channels [23] [24]. ROS also affects the vascular tone in multiple ways i.e., decreasing prostacyclin, nitric oxide, and rising endothelin-1 levels [25]. Various complication like hypertension, cardiac parasympathetic induction and baroreflex function of Cardiovascular system arises simply because of vasoconstriction. This also contributes towards decreased stroke volume and heart rate [26].

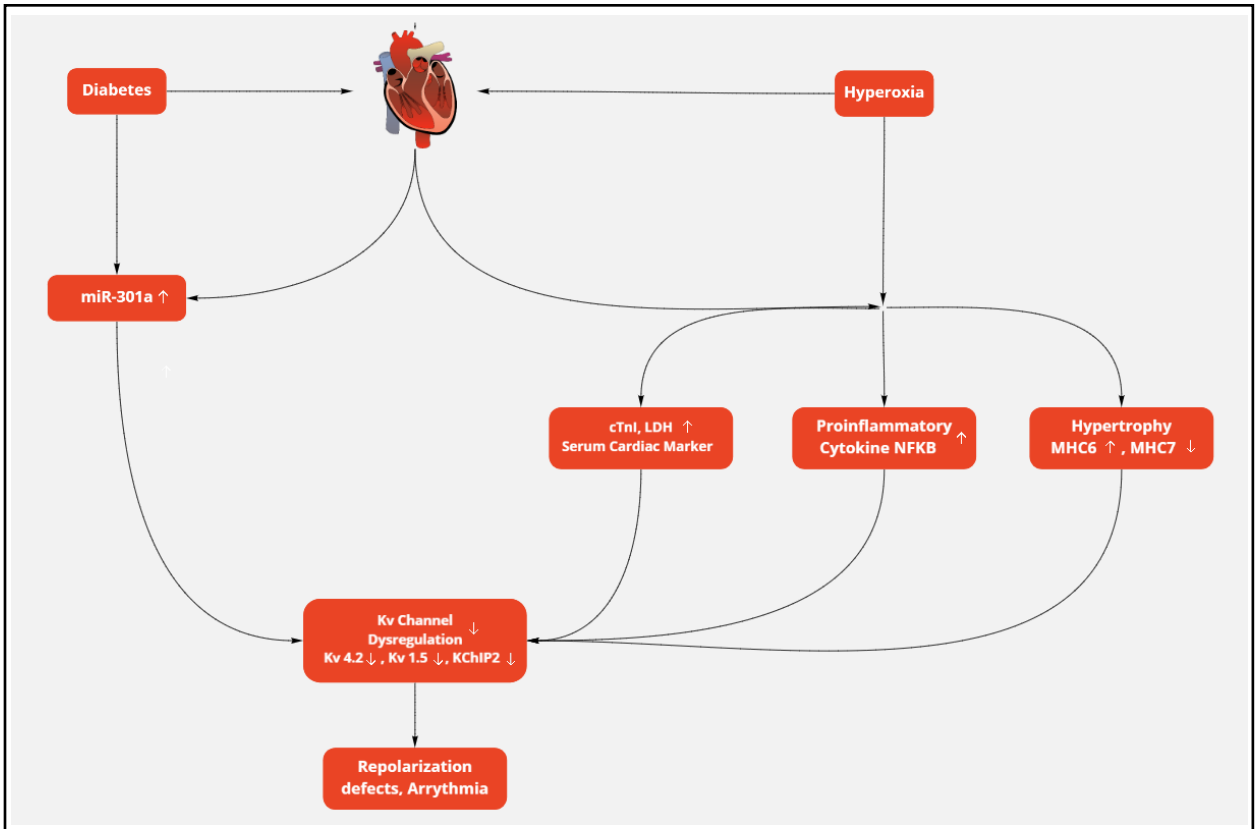

Figure 3: Schematic chart of hyperoxia-induced cardiac pathophysiology. Hyperoxia actuates cardiotoxicity by means of the production of serum cardiac markers **c**ardiac **t**roponin I (cTnI) and **l**actate **deh**ydrogenase (LDH). Hyperoxia too actuates inflammatory cytokines through NFkB and cardiac hypertrophy through acceptance of MHC6 and 7. Test models from our lab illustrate that hyperoxia induces critical Kv channel dysregulation through downregulation of Kv4.2, Kv1.5, and KChIP2, which comes about in repolarization absconds in mice, counting expanded activity potential lengths (APDs), QTc prolongation, arrhythmias, and diminished heart rates. Experimental results in diabetic mice moreover illustrate that Kv channel dysregulation and electrical remodeling may be a pre-existing condition in these mice, which has been connected to mir-301a upregulation. Thus, diabetes in mice increases electrical remodeling and Kv channel dysregulation due to hyperoxia presentation, as appeared by our experimental comes about. MHC6: myosin overwhelming chain 6 [Color figure can be viewed at wileyonlinelibrary.com] [10]

Multiple research studies have demonstrated that hyperoxia is associated with cardiac complications and has a direct impact on blood vessels. The rise in ROS adversely affects the smooth muscle cells in the vessels by agonistic action of L-type Ca^{++} channels and closing the ATP-dependent potassium channels [23] [24]. ROS also affects the vascular tone in multiple ways i.e.,

decreasing prostacyclin, nitric oxide, and rising endothelin-1 levels [25]. Various complication like hypertension, cardiac parasympathetic induction and baroreflex function of Cardiovascular system arises simply because of vasoconstriction. This also contributes towards decreased stroke volume and heart rate [26].

In a study conducted by Spoelstra et al., Hyperoxia may directly cause a negative inotropic effect on the heart. ROS can encourage the release of intracellular Ca^{++} by causing an alteration in the Sarcoplasmic reticular and sarcolemmal release mechanism of Ca^{++}. This overload in the Ca^{++} levels can lead to contractile dysfunction and ultimately cause cell death [27]. Injury due to ischemia and reperfusion to cardiac muscle and amplification in myocardial stunning is also associated with hyperoxia [28]. We previously demonstrated that hyperoxia activates the MHC6 and MHC7 which in turn releases the inflammatory cytokines inducing NFkB and metabolic anomalies. It was established that severe abnormalities occur in the Kv channels of rodents by Kv1.5, Kv4.5, and KChIP2 which cause conformations anomalies and alters the duration of action potentials as well [6]. Also, another study demonstrated hyperoxia-induced ion channel remodeling in which there are abnormalities in electrophysiological and mechanical function in heart failure. This dysfunction is related to a rise in the expression of Kv1.4 and downfall in the levels of Kv4.5 [6, 29]. We showed that critical decrease in Kv4.2 expression just as protein articulation happens concerning hyperoxic conditions [6]. An investigational trial of diabetic mice suggested that Kv channels abnormality and electromechanical renovation have become an issue in such mice that had been identified with mir-301a upregulation [30]. Some of the events of hyperoxia-induced cardiac remodeling were illustrated in **Figure 3**.

The poisoning of ROS mostly damages DNA, causes oxidation of proteins and lipids. The oxidation of lipids of the intra and extracellular membranes causes inhibition of mitochondrial

energy cascade, oxidation of thiols, and deactivation of enzymes [31]. Various defenses are prepared by the body to mitigate the impact of ROS by preventing, repairing the affected tissue. These defenses include enzymes (glutathione peroxidase, superoxide dismutase, and Catalase) and nonenzymatic entities like Glutathione, Vitamin A and E, and Albumin [32]. . ROS are proved to be closely associated with hyperoxia as Oxygen is the main key factor for the production of ROS and this cause upshift in the ROS levels, causing imbalance to oxidation and antioxidant balance which in turn leads to inflammatory conditions like cardiac arrest [33]. It is proved by various studies that hyperoxia directly increases vascular resistance and inversely affects the heart rate [34].

1.5 Impact of Hyperoxia on the Whole Body:

For the normal physiological function of the body and cell, an appropriate amount of oxygen is required. Also, low levels of oxygen saturation are life-threatening, especially in critical circumstances. For such hypoxic conditions, supplemental oxygen is provided so that physiological cell function and metabolism can be supported and avoid organ damage to the maximum extent. However, patients who are suffering from hypoxia if given supplemental oxygen will upsurge oxygen levels to the hyperoxemic range [18, 35]. Normally, humans are often subjected to hypoxic conditions like lung diseases or high altitude but administering supplemental oxygen or hyperoxic condition is something that can be termed as a non-physiological event [36].

When subjected to hyperoxia multiple organs are affected throughout the body. This hyperoxic state tends to increase the ROS which can directly injure different tissues when ROS levels exceed the antioxidants [37]. In a study, Orbegozo Cortés et al. stated that when evaluated by sublingual side-stream dark field (SDF) video-microscopy, there is a decrease in the capillary perfusion when a normal subjected to normobaric [38]. It is also suggested by a study that the

induction of vasoconstriction is a protective response to protect cells from the injurious effects of increased levels of PaO_2 [39, 40]

1.6 Why Hyperoxia is Important:

It is a common practice to administer supplementary oxygen to patients who are suffering from severe lungs or heart diseases so that the normal oxygen supply to peripheral tissue can be ensured. When exposed 9to a very high concentration of O_2 i.e., Z 50% for a longer duration, this can lead towards HALI [7]. There is an increase in the levels of free radicals of oxygen when a patient is exposed to high PaO_2 due to increase FIO_2, this upsurge in the levels of free radicals is associated with neuronal injury and cell death ultimately leading to apoptosis. Furthermore, various studies have proven by functional neurological testing that when administered to hyperoxia after ROSC, the extent of brain injury and mortality increases [41, 42]. Furthermore, in a clinical trial study, an ST elevation is observed in the patient with myocardial infarction and a greater risk of recurrence of MI, arrhythmias and MI size after six months when compared hyperoxia treated patients with non-hyperoxic, which indicated that increase PaO_2 might cause more reperfusion injury to heart muscles [43].

It has been observed in various studies that administration of a high level of O_2 harmfully affects the heart and lungs, which increases increase the inflammation and damage caused by inflammation. The damaged lungs caused a change in the structure of surfactant proteins as well [44]. When described anatomically, the lung's epithelial lining is prone to inflammation, research has indicated that the superficial layers suffer from oxidation and the number of antioxidants to protect the cell lining decreases. Consequently, ROS induces multiple signals transduction pathways to bring about the needed response which is an adaptation, repair of the damaged cell or oncosis, necrosis or apoptosis of the affected cell [45].

1.7 The Relation Between Hyperoxia and Delirium in Elderly Patients After Cardiac Surgery:

One of the occurred complications after heart surgery is delirium. According to a study it can occur in up to 52% of cases [46]. Lopez et al., demonstrated that after cardiac surgery when the cerebral oxygen levels were measured, it is observed that when exposed to a longer duration of hyperoxia during cardiopulmonary bypass, the occurrence of delirium in the patients in up to 30% after surgery. Additionally, modification in local and systemic perfusions, plasma pH and cellular metabolism also occurs, which tends to increase the oxidative stress henceforth enhancing the chances of delirium after surgery in patients [47]. Similarly, hydrogen peroxide another chemical specie that is produced by ischemic tissue also decrease the neuronal function and increase the susceptibility of delirium in mice with high oxidative stress [48]. Furthermore, some of the oxidative damage is also caused during the surgery which is caused by more or less removal of ROS, free radicals, and nitrogen species. These are the compounds that are produced intraoperatively and also add to the oxygenation stress produced due to hyperoxia administered during ischemia and reperfusion and hemolysis [49]. It was also stated in a study that the oxidative injury that occurs during the common surgeries is closely related to injuries of the kidney, heart and also associated brain injury and dysfunction after surgery [50].

In a study conducted by Kupiec et al., it is demonstrated that hyperoxia increases the chances of delirium after cardiopulmonary bypass surgery, so limiting the hyperoxia below 6.6kPa during cardiopulmonary bypass can be a potential way of avoiding and decreasing the risk of postoperative delirium [51].

1.8 Cardiac Arrest and In-Hospital Mortality Due to Hyperoxia:

The negative impact of hyperoxia has been reported in various studies in patients with MI which includes, decrease coronary blood flow, high vascular resistance and mortality [52]. Hyperoxia is directly associated with the mortality rate in severely ill patients, Stroke, traumatic brain damage, cardiac failure resuscitated subjects and patients in ICU [53]. Also in some studies, it has been observed that hyperoxia have an injurious effect on the patients who are severely ill and also the hyperoxic conditions adversely affect the cardiac parameters like vascular resistance, output, cerebral blood flow and functions of lungs [54, 55]. In a study conducted by Bodetoft et al., it has been observed that there is low cardiac productivity, coronary oxygen distribution and perfusion of the left ventricle when subjected to hyperoxia as compared to healthy individuals [56]. It has been reported by Kim et al. that there is a high 28-day in-hospital mortality rate in MI patients when administered oxygen during the first day of admission as well as more chances of occurrence of complications like liver dysfunction and coagulation [57]. Also, the younger ICU patients who face fewer comorbidities demonstrated greater hyperoxic events especially when they have shorter FiO2. Even though there is no strong correlation found between mechanical hyperoxia and 7 days in-hospital mortality but it surely increases the hyperoxic burden over the ICU patients [58]. Lastly, in a meta-analysis conducted by You et al., it is stated by arterial hyperoxia is directly related to the mortality rate of severely ill patients. This relation was established between the patients specially admitted to critical care units due to intracerebral hemorrhage, cardiac arrest and ischemia stroke [59].

1.9 Current Objective:

To investigate effects of hyperoxia exposure on cardiac pathophysiology in male (aged or older) guinea pig hearts animal model.

1.10 Long Term Objective:

We will also investigate the influence of gender on hyperoxia-induced cardiac remodeling in these animal model.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials:

2.1.1 Animals:

Guinea pigs of species Hartley Guinea Pigs (n=6 per group) of one-year old age and weighed average 900-1000g with health status Bright Active and Responsive (BAR) purchased from Charles River. The sex of these guinea pigs was retired male breeders. They were randomly selected for either hyperoxia or normoxia study groups. All guinea pigs were allowed access to both food and water *ad libitum* for the duration of this study and were maintained on a 12 h light/dark cycle. Each experimental research protocol was approved by the Institutional Animal Care and Use Committee at the University of South Florida (Tampa, FL), in accordance with the US National Institutes of Health.

2.2 Methods:

2.2.1 Hyperoxia Exposure:

Guinea pigs were divided in two groups of six guinea pigs each randomly as normoxia and hyperoxia group. For hyperoxia group, guinea pigs were treated with >90% oxygen (hyperoxia) for 72h, in an airtight chamber (50*50*30 cm) as per our previous reports [60]. The guinea pigs were set in a fixed hermetically sealed chamber with *ad libitum* food and water in 12h light/dark cycle. Animals were inspected for any discomfort after every 6h. An oxygen analyzer (Vascular Technology, Chelmsford, MA) was used to monitor oxygen levels throughout the exposure period. For normoxia guinea pigs were placed under normal air for 72h with unrestricted water and food supply. Immediately after normoxia or hyperoxia treatment, body weights were recorded for all the animal.

2.2.2 Electrocardiography (ECG):

Guinea pigs were anesthetized with 1–2% isoflurane, followed by insertion of surface probes in lead-II configuration for the acquisition of electrocardiogram (ECG) as shown in **Figure 4.**

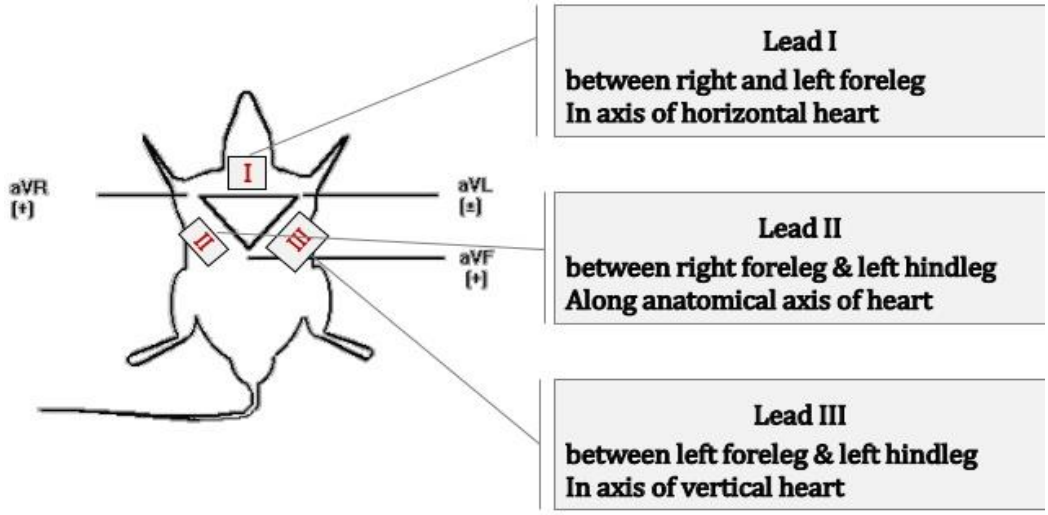

Figure 4: Difference between Lead I, Lead II and Lead III configuration of ECG traces recording.

ECG traces were recorded in a stretch of 30 s each and at three intervals from each animal. While collecting ECG, heart rate signals were analyzed using PowerLab system and LabChart 7.2 software (AD instruments, UK). As isoflurane and hyperoxia treatments significantly lower the heart rates, we tried to collect ECG traces from each group at a fixed heart rate.

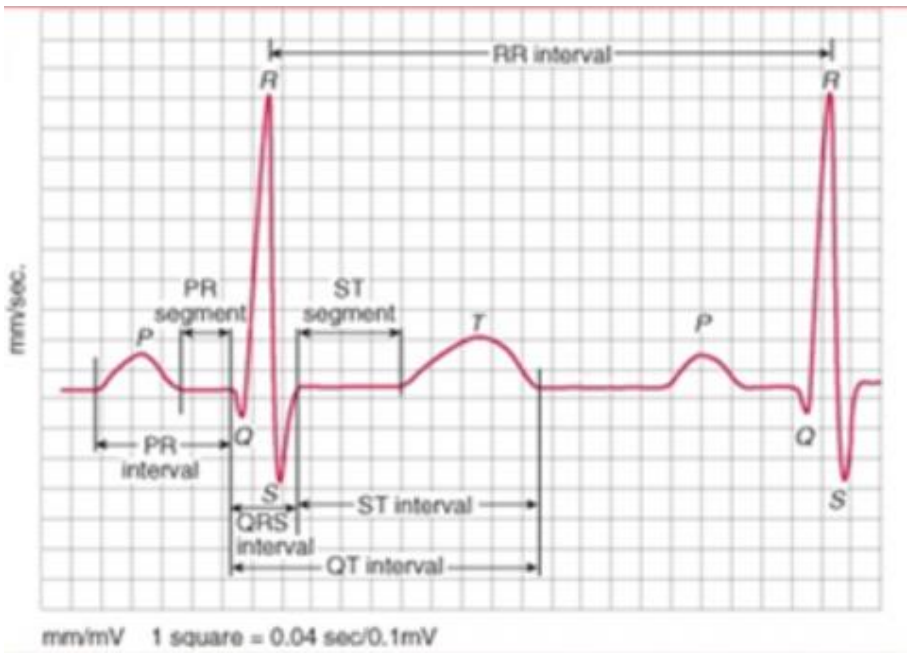

Figure 5: Example of starting and ending of Intervals for RR, PR, QRS and QT intervals.

As shown in the **Figure 5**, RR interval, representing the time elapsed between two successive R waves on the electrocardiogram and is a function of intrinsic properties of the sinus node as well as autonomic influences. The PR interval is the time from the onset of the P wave to the start of the QRS complex. It reflects conduction through the AV node. The QRS complex represents ventricular depolarization. Ventricular rate can be calculated by determining the time interval between QRS complexes. A corrected JT interval (JTc) was derived simply by subtracting the QRS duration from the QTc. All values were calculated in milliseconds, Intervals for RR, PR, QRS, and JT were each measured, with QT intervals measured at the start of Q peak up to TP baseline.

2.2.3 Euthanization and collection of organs:

Soon after acquiring ECG recordings, animals were euthanized for thoracotomy via intraperitoneal injection (IP) of 50 mg/kg euthasol. We also administered heparin (Sigma-Aldrich, MA) (500 IU/kg/10 ml) via intraperitoneal injection (IP). This was immediately followed by blood collection, heart and lungs excision. Blood was centrifuged at 5500 rpm for 5 min to collect plasma serum and frozen at -80 until further use.

2.2.4 Lung edema:

Lungs wet to dry ratio was measured. To measure the whole sum of lung edema, the guinea pigs were dissected and the lung weight was measured instantly after its extraction (wet weight) The lungs were at that point dried in an oven at 60°C for overnight and re-weighed as dry weight to evaluate lung edema in hyperoxia and normoxia treated lungs. Body weights and heart weights were compared in both hyperoxia and normoxia groups and normalized with corresponding tibia lengths.

2.2.5 Heart Weights and dissection:

Each heart was weighed and then dissected to separate right and left ventricles, apex, atrium, and septum and were stored at − 80 °C for subsequent analysis, including RNA and protein analysis.

2.2.6 RNA Isolation:

Total RNA was extracted from the left ventricles of all experimental groups, including in normoxia and hyperoxia guinea pig groups using miRCURY RNA isolation kit (Exiqon, Woburn, MA) as detailed below.

1. Approximately, 35mg of left ventricle tissue was homogenized in tissue lysis buffer.

2. 1/10 volume of miRNA Homogenate additive to the cell was added to the tissue lysate and mixed well by vortexing several times. Mixture was left on ice for 10 min.
3. A volume of Acid-Phenol: Chloroform that is equal to the lysate volume was added, before addition of the miRNA Homogenate additive. Vortex for 30-60 sec to make it mix properly.
4. After that it was centrifuged for 5 min at maximum speed (10,000 x g) at room temperature to separate the aqueous phases.
5. Then the interphase should be compact and to this 1.25 volumes of room temperature 100% ethanol were added (supernatant collected).
6. Filter Cartridge was placed into one of the Collection Tubes supplied for each sample. Mixture of lysate/ethanol was pipet onto the Filter Cartridge. For passing mixture through the filter it was centrifuged for 15 sec at 10,000 rpm. Flow-through was discarded and repeated until all the lysate/ethanol mixture was through the filter.
7. Collection tube was reused for washing steps. 700 µL miRNA wash solution 1 was applied to the filter cartridge and centrifuged for 5 to 10 sec. Flow-through from the Collection tube was discarded and filter cartridge was replaced into the same Collection Tube.
8. 500 µL wash solution 2/3 was applied and drawn through the Filter Cartridge as previously done.
9. Repeated with a second 500 µL aliquote of Wash Solution 2/3. After discarding the flow-through from the last wash, Filter Cartridge was replaced in the same Collection Tube followed by spinning the assembly for 1 min to remove residual fluid from the Filter.
10. The Filter Cartridge was transferred into a fresh Collection Tube (provided with the kit). 50 µL of pre-heated (95ºC) nuclease-free water was applied to the center of the filter, and cap was closed. For recovering of RNA, it was spined for 20 to 30 sec at maximum speed.

Elute was collected (which contains RNA) and stored at -20ºC. RNA elution was repeated in separate tubes.

11. The total RNA concentration for each test was measured by a NanoDrop spectrophotometer, and RNA quality was decided by 18S/28S ribosomal peak intensity.

2.2.7 cDNA Preparation:

Components of cDNA Supermix are qScript cDNA Supermix (5x) (qScript cDNA SuperMix 5X reaction buffer from Bio-Rad laboratories, containing optimized concentrations of MgCl2, dNTPs (dATP, dCTP, dGTP, dTTP), recombinant RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer and stabilizers) 4 µL to make final concentration 1x. RNA template was variable (1µg to 10 pg total RNA). RNase/DNase-free water was also variable to make total volume 20 µL. Procedures are as detailed below:

1. Components were placed on ice and mixed and then briefly centrifuged to collect contents to the bottom of the tube before using.
2. Reagents were combined in 0.2-mL micro-tubes.
3. After sealing each reaction, it was gently vortexed to mix contents. Then briefly centrifuged to collect components at the bottom of the room reaction tube.
4. Centrifuged tubes were incubated for 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC and hold at 4ºC.
5. After completion of cDNA synthesis, $1/5^{th}$ to $1/10^{th}$ of the first-strand reaction (2-4) was used for PCR amplification. cDNA samples were stored in -20 ºC until further use.

2.2.8 qRT-PCR:

Real-time RT-PCR reaction was set-up using iQ™ SYBER® Green supermix (2x concentrated) ready to use reaction master mix optimized for dye-based quantitative PCR (qPCR). It is consisting of antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBER® Green I dye, enhancers, stabilizers, and fluorescein.

1. iQ™ SYBER® supermix and other frozen reaction components were thawed to room temperature.
2. They were mixed thoroughly, centrifuged briefly to collect solutions at the bottom of tubes, and then stored on ice protected from light.
3. Enough assay master mix for all reactions were prepared by adding all required components, except the DNA template. iQ™ SYBER® Green supermix (2x) diluted to (1x) and 5 µL of it is taken.
4. Forward and reverse primer were used in 100 nM each (see **Table 1.** for sequence information) and were in 0.25 µL quantity each. In DNA template cDNA was taken 1:10 ratio was in total 4.5 µL quantity making total quantity of all the components to 10 µL.
5. Assay of the master mix was mixed thoroughly to ensure homogeneity and dispensed equal aliquots into each qPCR tube. Good pipetting practice was employed to ensure assay precision and accuracy.
6. DNA samples were added to the PCR tubes containing assay master mix, tubes were sealed to remove any air bubbles and the reaction mixture was collected in the vessel bottom.

7. Thermal cycling protocol on the real-time PCR instrument was programmed with initial step of 50ºC for 5 minutes followed by denaturation at 95º for 10 sec, primer annealing and extension at 60ºC for 30 sec for 39 cycles followed by melting curve cycle settings.
8. Afterwards, PCR tubes were loaded onto the real-time PCR instrument and run was started.
9. Data analysis was performed according to the instrument-specific instructions. Cq values for each sample from the iQ Cycler and fold values were collected. There were two main parts in collecting these values, first was through the 7300 system SDS software and secondly analysis through Excel. Steps are detailed below:

Table 1: List of Forward and Reverse Primer Sequence

	FORWARD	REVERSE
KV2.1	CTCCACCATTGCCCTGTC	TCCGCTTGATTGCTTTCTC
KV2.2	TCCCAGGAACAGATGAGC	GAGTGGTGAGCGGAAAGT
KV4.2	CTTCACTATCCCCGCCAT	GTTTCCACCACATTCGCG
Kv1.4	AACAGTCACATGCCTTATG	TAGTAAAACCTTCCCTCCTC
Kv4.3	GAAGAGGAGCACATGGGC	GTGATCTGGGATGTTTTGC
KvLQT1	GTTTGCCACATCAGCCATCA	GGGACCTTGTCGCCGTAA
minK	GGGGACAGTTCAACCCAGTA	TTCAATGACGCAACACGAT
ERG1	TGGCTCATCCTACTGCTGG	GGCTGACCACTTCCTCGTT
GAPDH (Housekeeping gene)	GCGCCGAGTATGTAGTGGAA	TGATTCACGCCCATCACGAA

ΔCt, and ΔΔCt calculations:

a. For normalization, the relative quantities, or absolute quantities of the housekeeping gene for all the samples were done once and used across all different primers for the same

samples. Sometimes, the PCR data will have a very bad standard curve. Even in this case, the data will have threshold cycle values (Ct values) for all samples. If there is a good standard curve obtained for the same cDNA in the previous run, the Ct values of each sample from the current run can be used to calculate the relative quantities from the previous standard graph using an equation given below the graph.

b. Generate average C_t values by calculating averages for the duplicates and triplicates of each normalizing gene and sample.

c. Calculate ΔC_t. ΔC_t = average $C_{t\ (sample)}$ – average $C_{t\ (normalizing\ gene)}$

   Note: Deltas are taken of the corresponding averages (average C_t of sample corresponding to average C_t of normalizing gene)

d. Take the average of all ΔC_ts to obtain final average.

e. $\Delta\Delta C_t = C_{t\ (sample\ or\ normalizing\ gene…can\ you\ make\ sure?\ I\ think\ its\ sample)} - \Delta C_{t\ (final\ average\ from\ part\ d)}$

f. To get the corresponding fold change for each sample calculate $2^{\Delta\Delta Ct\ (sample)}$

2.2.9 Immunohistochemistry:

Immunohistochemistry (IHC) is the foremost common application of immunostaining. It includes the method of specifically distinguishing antigens (proteins) in cells of a tissue segment by exploiting the rule of antibodies binding particularly to antigens in organic tissues.

2.2.9.1 Cryostat section:

1. Tissue was freeze up to 2.0 cm in diameter in OCT using a suitable tissue mold.
2. OCT was freeze with tissue onto the metal grids fitting the cryostat. At room temperature, OCT is viscous but freezes at -20ºC.
3. Sections were cut approximately 10 µm in the cryostat at the -20ºC.

4. To evaluate the preservation and orientation of the tissue, the first slide of each set can be stained using toluidine blue, eosin, hematoxylin, or various aqueous stain. Slide was immediately immersed into a fixative.

2.2.9.2 H&E Staining:

To evaluate total cross-sectional area of each heart, we performed H&E staining. Active ingredients for H&E staining include, 100% alcohol, 95% alcohol, Rapid-Fixx, Gill 3 Hematoxylin, Bluing reagent, Eosin-Y, Xylene and Shandon mounting medium.

The cryostat sections on the slides were kept on hot plates at 45ºC for 15 min. Sections were washed with 1xTPBS for 3 times to remove OCT from the slides. Staining procedures were performed as described below:

1. Rapid-Fixx was applied for 5-7 seconds, then distilled water was poured.
2. Hematoxylin was applied for 30 sec and again distilled water was poured on sample.
3. Sample was rinsed with few drops of Bluing reagent with the help of pipet and then rinsed a little by 95% alcohol.
4. Eosin-Y was applied to the sample for 10 sec.
5. Sample was rinsed a little by 95% alcohol followed by a small amount of 100% alcohol two times. In the end sample was rinsed twice by Xylene and frozen section was H&E stained.
6. Slides were let over-night to completely dry mounting the slides with coverslip using floromount medium (Fisher Scientific, USA).

2.2.9.3 WGA Staining:

To measure individual cardiomyocyte area, we performed wheat germ agglutinin staining using following protocol:

1. The 10-µm-thick frozen heart segments were settled with 4% PFA for 15 min. then washed with PBS three times.
2. The heart segments were then incubated with WGA (L4895; Sigma-Aldrich) solubilized in PBS for 20 min. at room temperature in darkness.
3. After washed three times with PBS, the segments were mounted with Prolong Gold containing DAPI (P36941; Invitrogen) and kept at 4°C in darkness.
4. The Keyence BZ-X800 fluorescent microscope was utilized to obtain cardiomyocyte measurements. A total of 20 fields per segment (80 LV, 40 RV, 20 Septum for each specimen) were observed at 40x amplification and analyzed with ImageJ computer program (National Organizing of Health). The cardiomyocyte area was measured by using the freehand selection tool to outline the areas of the delineated cardiomyocytes.

2.2.10 Statistical Analysis:

All the samples were labeled with unique numbers and codes to make sure we have done the analysis in blind fashion. For example hyperoxia and normoxia guinea pigs animal's heart and lungs samples numbering and codes were given to them randomly by mentor and student who is analyzing the data was not aware of these numbering and coding so that he don't know the treatment of each sample he is analyzing to avoid biased interpretation of treatment. After acquiring all the data then mentor revealed the identity and the analysis was done with the proper grouping.

All the experiments such as qRT-PCR and immunohistology were repeated at least three times with animal triplicates and internal duplicates. All the mean (± SEM) values were expressed in bar diagram and student t-tests was utilized to evaluate significance (p-values). The information analysis was utilized to compare the quantitative data populations of both typical dispersion and equal variance, where a value of $p \leq 0.05$ was considered statistically significant. The error represent means ± SEM for all information sets.

CHAPTER THREE

RESULTS

3.1 Physical Parameters:

Based on our previous experiments in mice[6], hyperoxia significantly changed the physical parameters such as body weight, heart weight and size, area of cross section of cardiomyocytes, and lung (wet weight/dry weight). Therefore, we investigated if some of these physical parameters are affected by the hyperoxia in the guinea pigs.

3.1.1 Body Weight:

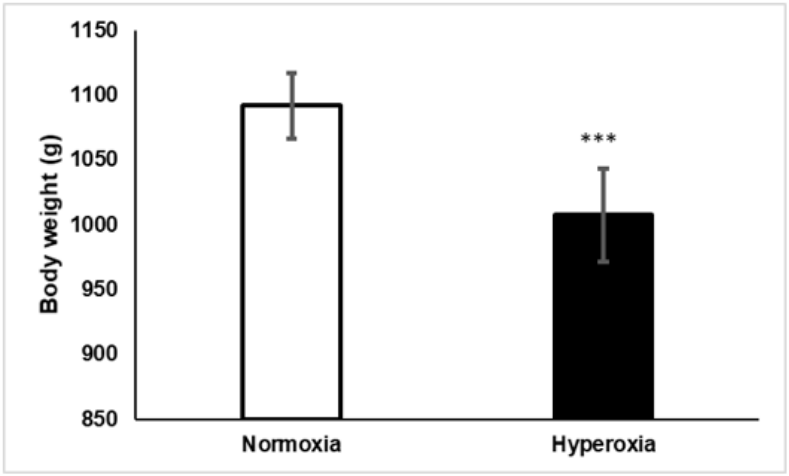

Figure 6: Body weight normalized to tibia length (g/cm), percentage weight loss. Figure demonstrates significant reduction in the body weight of Guinea Pigs who were exposed to hyperoxia for 72 hours. All the data presented here are mean of (±SEM) n = 6, ***$p \leq 0.0005$.

From our data, we found out that after 72h of hyperoxia treatment, the body weight of guinea pigs was significantly reduced by 8.135 %. (**Figure 6**) as compared to the normoxia treated guinea pigs. From this it is evident that hyperoxia treatment significantly diminished body weights

(normalized to tibia length). All the values presented in the bar are mean (±SE) of n=6. *** represents p-value ≤0.0005 compared to normoxia control.

3.1.2 Heart Size and Weight of Hyperoxia/Normoxia Treated Guinea Pigs:

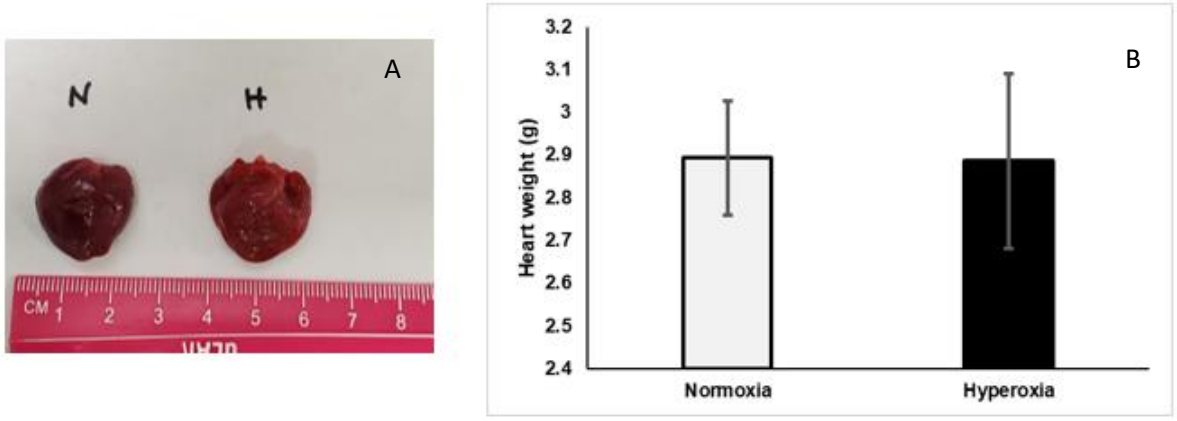

Figure 7: Physical parameters of heart. In (A) it is comparison of two physical hearts of normoxia and hyperoxia treated Guinea Pigs, to see the size difference between both of them. There was no size difference found. (B) shows the comparison between weight of normoxia treated group vs hyperoxia treated group, also there was no significant difference was found in both groups. All the values presented in the bar are mean (±SE) of n=6.

As we observed significant change in body weights in hyperoxia treated group, we also measured heart size and heart weight to see if there is any hypertrophy in heart after treatment with hyperoxia. Surprisingly, no significant changes in heart size (**Figure 7A**) and weight (**Figure 7B**) were observed after hyperoxia treatment. Even though there was no change in the heart size and shape, we measured the area of cross section of the entire hearts as well as each cardiomyocyte. H&E staining was used to measure the area of cross section of entire hearts (**Figure 8A**) and WGA staining was used to measure size of cardiomyocytes.

3.1.3 Wheat germ agglutinin (WGA) Staining of cardiomyocytes:

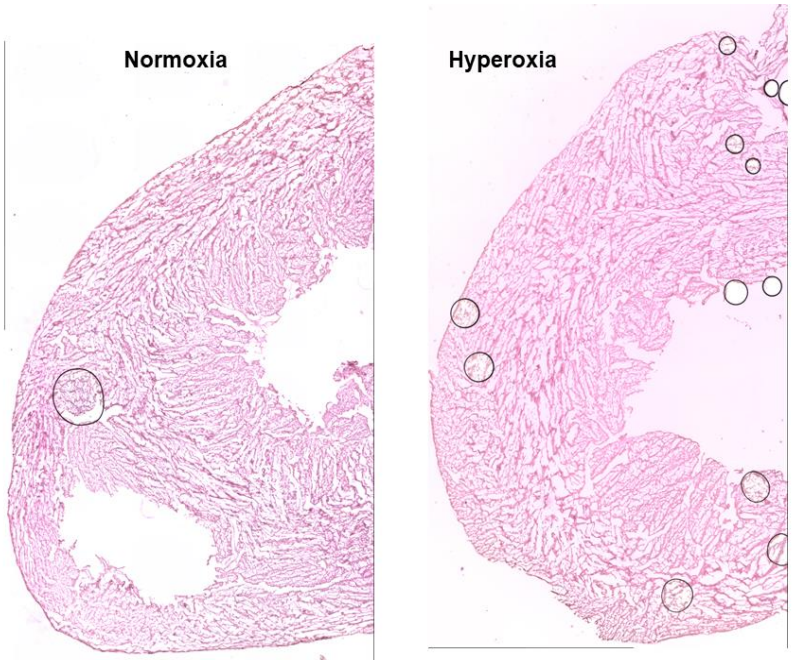

Figure 8 (A): **Comparative image of H&E staining of total cross sectional area of heart of both normoxia and hyperoxia guinea pig.** Total area of cross section is decreasing after hyperoxia treatment.

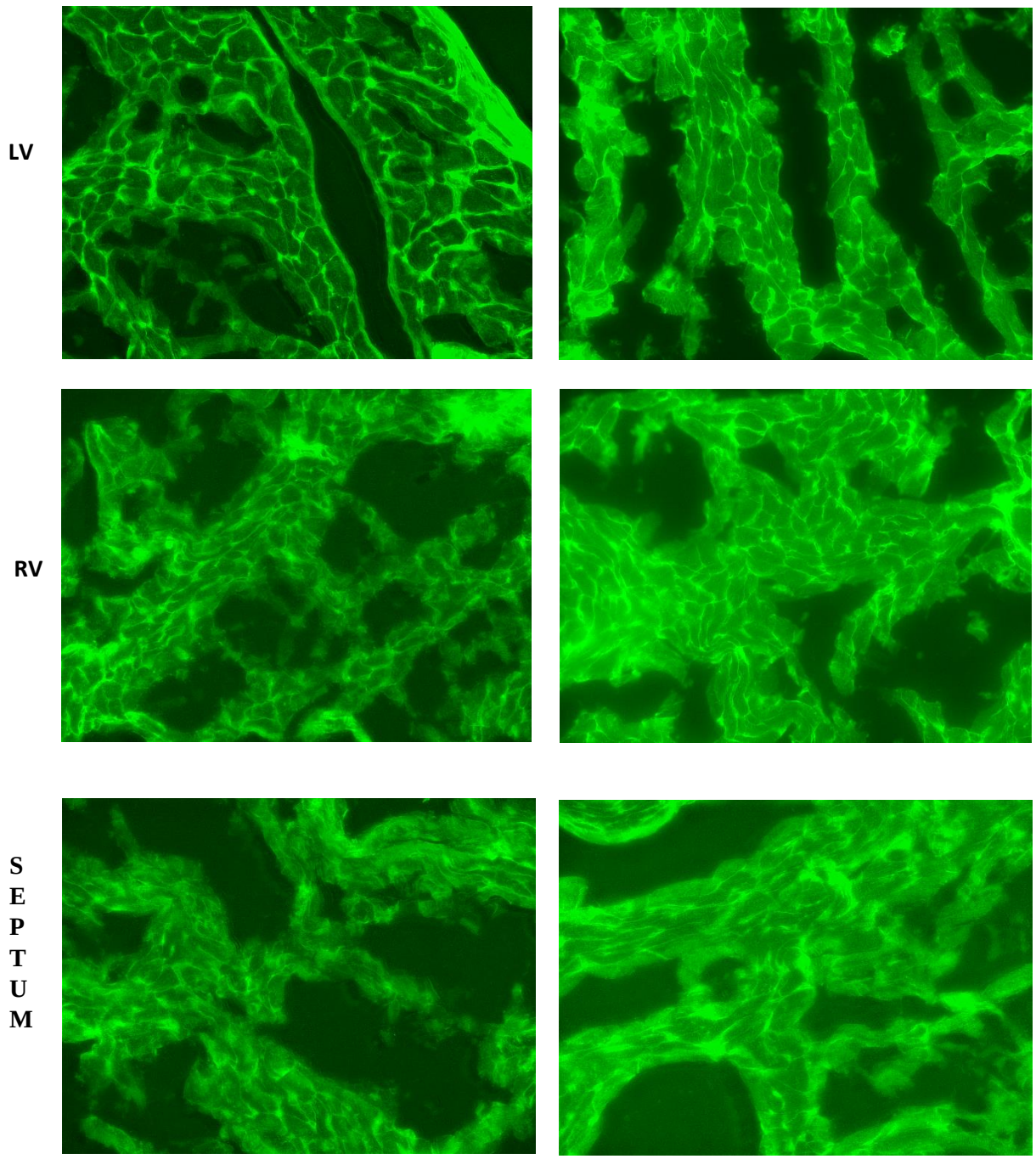

Figure 8 (B): Wheat germ agglutinin (WGA) image comparison of normoxia and hyperoxia LV, RV and septum.: From images it is evident that LV cardiomyocytes in guinea pigs are decreasing while RV and Septum are increasing in size after hyperoxia treatment as compared to normoxia controls.

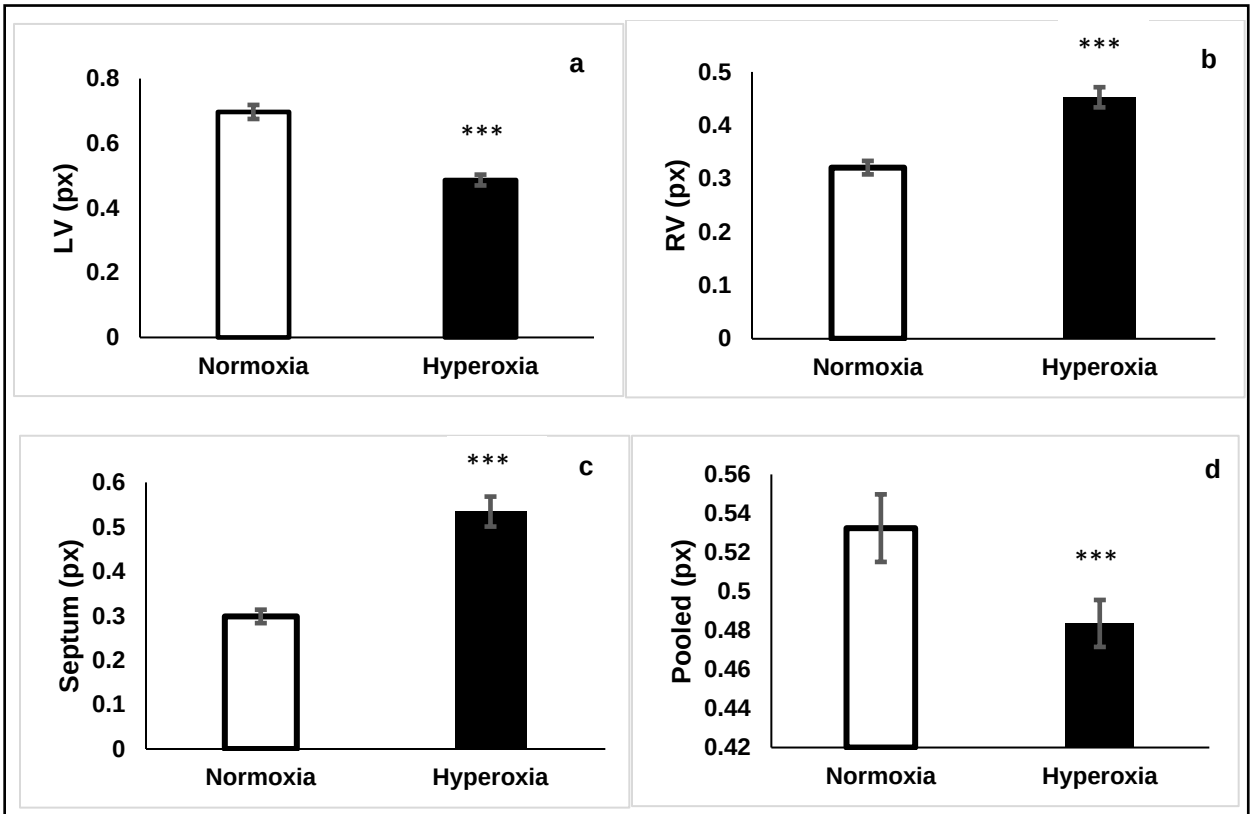

Figure 8 (C): Wheat germ agglutinin (WGA) Staining revealed significant changes in cardiomyocyte size after hyperoxia treatment. Area of cross section of Cardiomyocytes from normoxia and hyperoxia LV (a), RV (b), Septum (c) and all pooled data (d). All the values presented in the bar are mean (±SE) of n=6. *** represents p-value ≤0.0005 compared to normoxia control.

From our data, it is evident that as shown in **Figure 8 (B&C)** from WGA staining, after hyperoxia treatment in older guinea pigs (**Figure 8C_a)** LV cardiomyocyte size is significantly decreasing while (**Figure 8C_b)** RV and (**Figure 8C_c)** septum cardiomyocyte sizes are significantly increased. Whereas, when we combine all (**Figure 8C_d)** LV, RV and septum data, cardiomyocyte size overall in hyperoxia hearts are significantly smaller than normoxia cardiomyocytes. All the values presented in the bar are mean (±SE) of n=2. *** represents p-value ≤0.0005 compared to normoxia controls.

3.1.4 Lungs wet/dry Weight ratio:

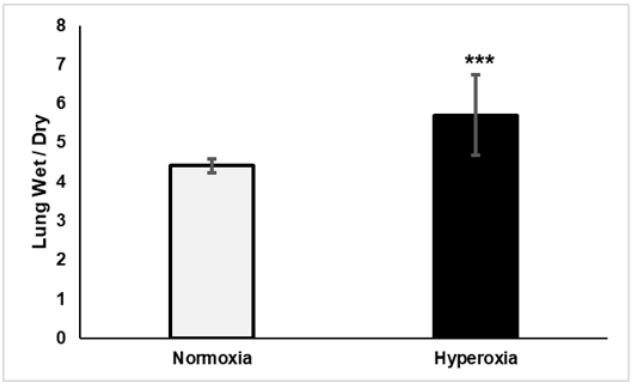

Figure 9: Physical parameters of lungs wet to dry ratio upon hyperoxia exposure. All the values presented in the bar are mean (±SE) of n=6. *** represents p-value ≤0.0005 compared to normoxia control.

Hyperoxia is known to induce lung edema due to accumulation of lung fluids [14]. We also examined that if 72h of hyperoxia treatment in older guinea pigs cause lung edema similar to the previous reports in mice. Briefly, confined lungs weighed to get wet weight and dried up overnight at 130 °C in a vacuum oven. Dry lung weights were at that point recorded and the proportion of the wet to dry weights was calculated. Our data showed that older guinea pigs treated with 72h of hyperoxia had significant lung edema which is evident by significantly higher wet/dry weight ratio in hyperoxia treated lungs compared to normoxia controls as shown in **Figure 9**. All the values presented in the bar are mean (±SE) of n=6. *** represents p-value ≤0.0005 compared to normoxia control.

3.2 Electrophysiological Parameters:

Electrocardiogram:

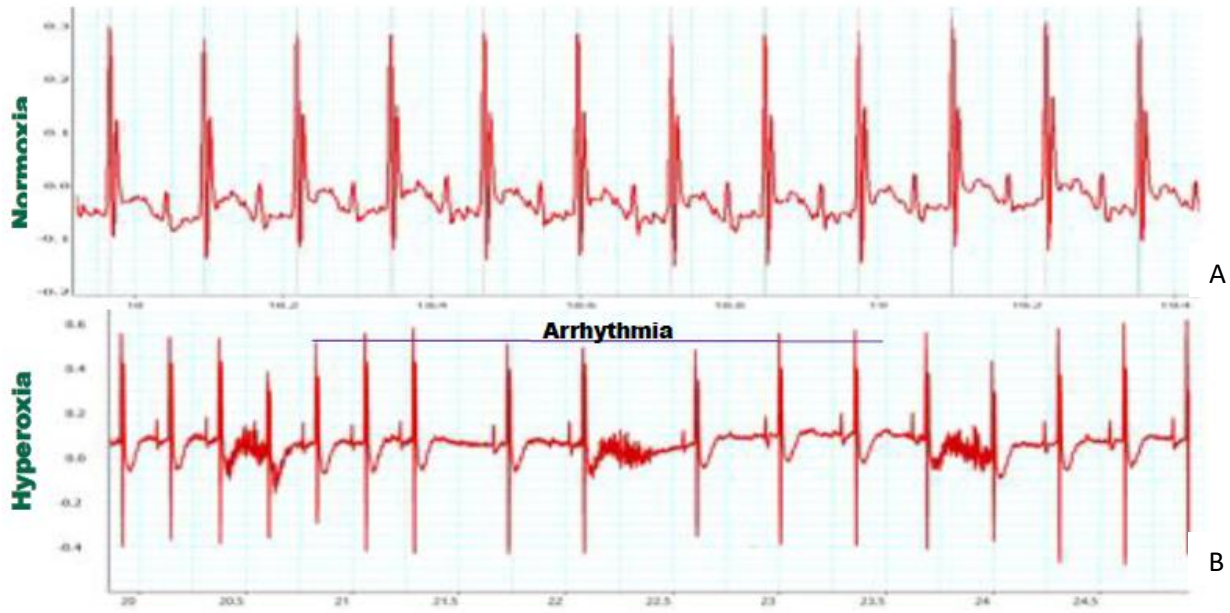

Figure 10: Schematic Electrical impairment and arrhythmias in hyperoxia treated Guinea Pig hearts. Brady-arrhythmia and repolarization defects were shown upon hyperoxia exposure in Guinea Pig: (A) Representative ECG (electrocardiogram) of Guinea Pig in normal air environment. (B) represents recordings from hyperoxia treated Guinea Pig having.

We took ECG recordings of normaxia group of guinea pigs before euthanization and organ collection and also of the hyperoxia group of guinea pigs right after hyperoxia treatment. Surface ECG was collected in lead-II mode under mild anesthesia and data was analyzed using LabChart Pro software as described in our previous studies [61]. We observed arrhythmias characterized by missed beats and slower heart rate in hyperoxia treated older guinea pigs when compared with age-matched normoxia (**Figure 10**).

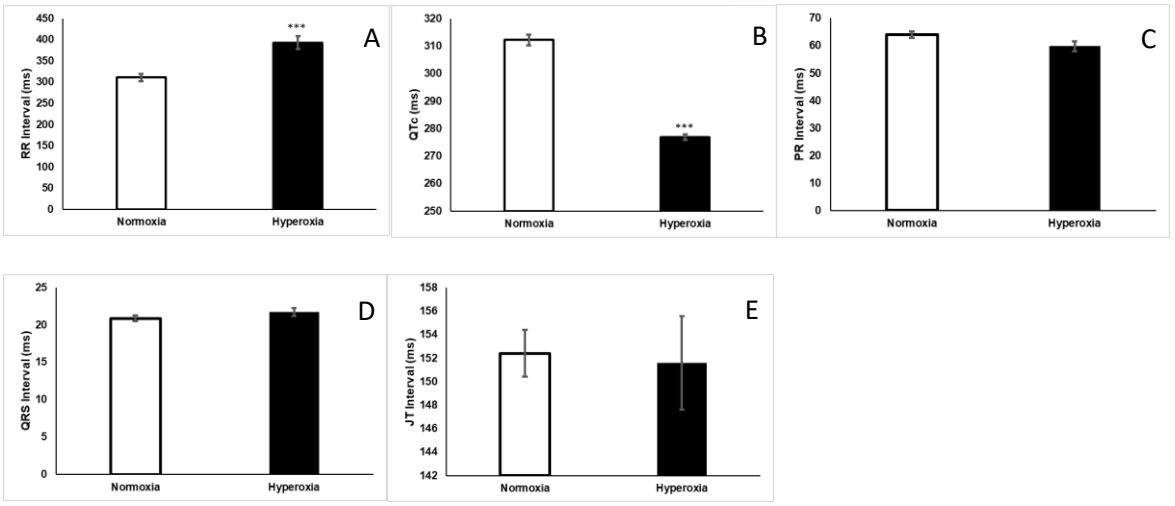

Figure 11 (A, B, C, D and E): Guinea pigs showed prolonged RR intervals (A) because of brady-cardia similar to what we reported in mice. Surprisingly, shorter QTc (B) intervals with no significant change in (C) PR, (D) QRS and (E) JT interval were observed in guinea pigs under hyperoxia. All the values presented in the bar are mean (±SE) of n=6. *** represents p-value ≤0.0005 compared to normoxia control.

Further evaluation of electrocardiogram parameters revealed significant changes in RR interval (**Figure 11A**) and QTc intervals (**Figure 11B**) in hyperoxia treated guinea pigs. We observed significant increase in the RR interval indicating a decrease in heart rate (brady-cardia) among the hyperoxia treated older guinea pigs similar to what we reported in mice. Interestingly, we observed significant reduction of QTc intervals in the hyperoxic treated older guinea pigs compared to age-matched normoxia controls (**Figure 11B**). Additionally, there were no significant changes in the PR, QRS, and JT intervals (**Figure 11 C to E**) in the hyperoxic treated older guinea pigs compared to age-matched normoxia controls.

3.3 Real-time RT-PCR:

As we observed arrhythmias and repolarization defects in hyperoxia treated guinea pigs, we investigated if these electrophysiological changes are due to dysregulation of key ion channel genes such as potassium channel genes. For that we utilized quantitative real-time RT-PCR analysis. Although we didn't find any significant differences in gene expression of key ion channels like Kv2.2, ERG and KvLQ as shown in **Table 2**, which were known to express in guinea pig hearts [62, 63], we found marginal difference in Kv1.4 and Kv4.2 and significant difference in Kv4.3 and Kv2.1 in hyperoxia treated guinea pig hearts.

Table 2: Real-Time RT-PCR analysis of potassium channel genes

	Mean (±SE)	
Gene	**Normoxia**	**Hyperoxia**
Kv1.4	1.32 (±0.42)	6.1 (±2.2)ŧ
Kv4.2	1.10 (±0.32)	53 (±22.8)ŧ
Kv4.3	1.44 (±0.5)	3.3 (±0.5)*****
Kv2.2	2.10 (±1.01)	1.2 (±2.3)
Kv2.1	1.21 (±0.35)	2.5 (±0.3)ŧ
ERG	1.80 (±0.67)	2.3 (±0.1)
KvLQ	1.44 (±0.70)	2.2 (±0.6)ŧ

Note: ŧ represents marginal differences and * shows significant differences.

3.4 Mortality:

The hyperoxic treatment also led to the death of Guinea pigs. The mortality rate of was found to be 50% which means 3/6 Guinea pigs died of the hyperoxic treatment, which is considered to be very high mortality rate.

CHAPTER FOUR

DISCUSSION

Mechanical ventilation is often related with greater in-hospital death rate [64], with long-term utilize being for the most part related with destitute survival rates in ICU patients [65]. Previously in our lab we have done a significant amount of work on effects of hyperoxia in rodents and now for the first time we are presenting our data in relatively bigger animal (Guinea Pigs) to bring more translational value to our studies as guinea pigs are known to be closer to human compared to rodents. Our lab already illustrated that adult male C57BL/6J wild-type mice showed significant LV hypertrophy, arrhythmias, QTc and JT prolongation, and potassium channel dysregulation, as a result of 72 h hyperoxia exposure [6]. Although aged guinea pigs have a lot of similarities with young mice data after hyperoxia treatment, we also observed some distinct physiological features in older guinea pigs under hyperoxia compared to mice. Surface ECG evaluation showed essentially slower heart rate (sinus bradycardia) and skipped beats (sinus delay) in all the hyperoxia treated guinea pigs compared to normoxia group, demonstrating cardiac arrhythmia (**Figure 10**).

It is conceivable that changed parasympathetic and/or sympathetic tone may intercede these changes as both autonomic nervous system components direct heart rate and rhythm [66].

QT interval is the time from the end of the Q wave at ECG to the end of the T wave. QTc is the interval corrected for the heart rate. It comprises the QRS complex, the ST segment, and the T wave and it is a measure of ventricular repolarization. Alterations in QTc interval results into certain arrhythmias depending upon whether the interval is prolonged or shortened. In previous experiments with mice, we were the first to demonstrate that the QTc prolongation was seen in the

hyperoxia treated mice which was validated by qrt-PCR downregulation of Kv 4.2 and Kv 1.5 [6]. Here, with guinea pigs, we observed Qtc shortening in the hyperoxia treatment group which was further validated by the qrt-PCR results showing upregulation of the Kv 4.2 and Kv 2.1 ion channels. In humans it has been shown that hypoxic treatments such as climbing high altitudes resulted into prolongation of QTc interval [67]. Therefore, it is plausible that shortening of the QT interval by hyperoxia treatment is due to upregulation of the Kv 4.2 and and 2.1 in guinea pigs which results into bradyarrhythmia and skipped heartbeat. Hence it signifies the pathophysiological effect of hyperoxia in guinea pigs.

The heart is exceedingly delicate to oxygen [68], and hyperoxia treated rodent hearts experience systemic hypoxia due to accumulation of lung liquid[69]. It is likely that this may reduce cellular oxygenation, tissue digestion system and lead to hoisted redox stress as watched through altered pyridine nucleotide levels. The increase in the sum of decreased pyridine nucleotides (NADH, NADPH) in the cell causes reductive push[70]. Diminished body mass and expanded or decreased heart mass are the commonly observed physical changes with hyperoxia presentation, as showed in our past research [6, 60, 71], and in the current study (**Figure 6 & 8**). Although we did not degree the nourishment admissions, diminished cellular energy supply may be a likely donor of body weight misfortune, as concomitant enteral sustenance weakens hyperoxia initiated weight loss in animal models, expanded heart weight in hyperoxia-treated mice results primarily from left ventricular hypertrophy (LVH), through increased expressions of MHC-6/MHC-7 [6]. LVH has too been appeared to create in young mice after neonatal hyperoxia presentation [72]. Whereas, aged guinea pigs, unlike mice, don't exhibit cardiac hypertrophy, as a result of hyperoxia. Furthermore, these observations observed in male guinea pigs were comparable to female mice data, which have been appeared to be ensured from cardiac hypertrophy, possibly due to estrogen cardio-protective

nature [73]. In future we will expand our research in female guinea pigs and see if they show any cardiac hypertrophy with hyperoxia treatment. Although we cannot report a clear component for higher mortality in male guinea pigs test subjects in this research, we observed 50% mortality in hyperoxia-treated guinea pigs. If we compare this mortality rate with our previous studies in rodents, we found no mortality rate in male mice, but in female mice the mortality rate was exactly same 50%[60].

CHAPTER FIVE

CONCLUSION

Guinea pigs showed distinct physiological features under hyperoxia compared to mice (rodents). Although guinea pigs lose body weights under hyperoxia, no significant change in heart size or weight. Whereas we observed significant decrease in cardiomyocyte size in hyperoxia treated hearts compared to normoxia. ECG analysis revealed brady-cardia in guinea pigs after hyperoxia, but with shortened QTc intervals with no significant change in PR, QRS, and JT intervals. Evaluation of mRNA expression profiles of some key ion channel genes reveal up-regulation of Kv4.3 and 2.1 in hyperoxia treated mice hearts, which may be partly inducing arrhythmias and QTc shortening in guinea pigs. Further investigation on hyperoxia-induced electrical remodeling using more precise and specific techniques may give more insights on cardiac pathophysiology under these conditions. Although, we observed some similarities between mice and guinea pigs, hyperoxia exposed guinea pigs showed some distinct features. The data obtained from this study will not only improve our understanding on hyperoxia related in-hospital mortality but will also help us to understand possible mechanisms through which hyperoxia induce cardiac pathophysiology conditions. In addition, it will open new avenues for targeted therapy. As guinea pigs are closer to human than rodent, data obtained from this study will bring more translational value to our hyperoxia experiments. Our research offers novel insights into the cardiotoxicity and resulted electrophysiological consequences caused by introduction to high levels of oxygen.

CHAPTER SIX

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