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Vascular Dysfunction in a Rat Model of Prematurity After O2 Supplementation

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VASCULAR DYSFUNCTION IN A RAT MODEL OF PREMATURITY AFTER O2
SUPPLEMENTATION

by

Amanda Owens

A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Health and Human Physiology

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Thesis Mentor

Spring 2017

All requirements for graduation with Honors in the
Health and Human Physiology have been completed.

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Vascular Dysfunction in a Rat Model of Prematurity After Oxygen Supplementation

By Amanda Owens

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Introduction

Medical interventions in an individual's neonatal period may or may not have health implications in adolescence and into adulthood. Although certain interventions are necessary to help sustain the life of the neonate, there may also be complications that arise shortly after intervention in the newborn that can follow the individual into adulthood (Lau, 2011). Premature babies are often exposed to supplemental O_2 to help support the development of the respiratory system and drive alveologenesis. High supplemental O_2 exposure to the newborn can cause an increased risk for respiratory diseases such as bronchopulmonary dysplasia (BPD), which may cause problems in the adult lung, such as hypoxemia, asthma, and frequent respiratory infections (Vogel, 2015). There has been a lot of interest on understanding how these high O_2 exposure conditions during this critical time in neonatal development might affect lung development, but how it may affect other systems, such as the cardiovascular system, have not been extensively examined. Surviving prematurity is a relatively new phenomenon due in part to the clinical use of surfactant that affects approximately 13% of the births in the US, or 450,000 babies per year since 1991 (Reich, 2012). This means that high O_2 treatment following birth in premature babies may have widespread health implications on a significant proportion of the adult population. As this population begins to age, there is a critical need to address these potential health problems. Understanding the effects on the adult cardiovascular system regarding neonatal supplemental O_2 is essentially unknown.

One of the few previous studies that examined the effects of neonatal high O_2 exposure on the cardiovascular system stated that neonatal O_2 has been shown to cause alternations in cardiac function and structure. These include events such as increased

blood pressure and adverse left ventricular function (Velten, 2011). High O_2 exposure after birth may lead to the increase of reactive oxygen species (ROS) and oxidative stress, which has been linked to vascular dysfunction, such as stiffening of the large elastic arteries (Ydydorczyk, 2008, Panth, 2016, Meiners, 2016, Payne, 2010, Loperena, 2017). Arterial stiffness is associated with changes in the arterial wall that alters the extracellular matrix, such as decreased elastin and increased collagen and cross-linking (Palombo, 2016). Collagen, being the stiffer protein in comparison to elastin, decreases the elasticity of the vessel if more collagen is present. Vascular dysfunction such as stiffening of the large elastic arteries increases the risk of cardiovascular disease.

Pulse wave velocity (PWV) is the gold standard method of measuring arterial stiffness, and predicts future cardiovascular events and death (Wentland AL, 2014). A healthy artery is compliant and thus able to distend and recoil to buffer the flow (pulse wave) as it travels through the aorta. As arteries become dysfunctional, the arteries become stiffer and consequently less compliant, allowing blood to flow more quickly through the artery (AlGhatrif, 2016). This can cause end-organ damage to the kidneys, brain and heart and also impair blood flow delivery to the coronary arteries (Codreanu, 2011).

This study examines how supplemental O_2 treatment in neonatal rats contributes to vascular dysfunction, such as large elastic artery stiffness, in older rats. We hypothesize that newborn rats exposed to high supplemental O_2 after birth will have increased aortic stiffness in adulthood when compared to rats that did not receive supplemental O_2. The increase in aortic stiffness is believed to be caused by increased aortic oxidative stress and changes in structural proteins. To test this hypothesis, we will

determine aortic stiffness using PWV. Microscopy and Western blotting will also be used to determine protein changes within the aorta, which may be associated to aortic stiffness.

Methods

Animals

All animals were handled in accordance with NIH guidelines, and the Office of Animal Resources at the University of Iowa approved protocols. Male and female Sprague-Dawly rat pups (Charles River) were placed into environmental O_2 chambers immediately after birth. The experimental group (n=13) were exposed to 80% O_2 (a mixture of room air and 100% O_2) for 8 days, while the control group (n=7) were exposed to 21%, or room air O_2 levels, for 8 days. These animals were then allowed to age for 15 months, and then studied.

Pulse Wave Velocity

Aortic PWV was measured using protocol previously described (Fleenor & Sindler, 2011). Rats are anesthetized using a 2% isoflurane solution, and placed in supine position on a heating board with legs secured to ECG electrodes. A Doppler probe is placed at both the transverse aortic arch, as well as the abdominal aorta. The time between the R wave of the ECG to the to the foot of the Doppler probe is determined at both sites. Using this protocol, the aortic PWV was calculated by measuring the distance between the two probes divided by thoracic-abdominal pre-ejection time difference. PWV was taken at 12 months for both groups of rats, prior to them being sacrificed. PWV measures the time it takes with each beat of the heart for

blood to move through the aorta. Higher PWV indicates more arterial stiffening than lower PWV.

Microscopy

Aortic rings were stained with PicroSirius Red (PSR) to assess the location and the amount of collagen amounts in the aortic wall. Approximately 2mm sections of descending aorta were taken at time of dissection and prepped using optical cutting temperature (OCT). Samples were processed at the University of Iowa Central Microscopy Research Facility. OCT prepared aortic samples were cut into 7-micron sections via cryostat, and then placed on slides and stained with PSR stain for total collagen. Stained aortic sections were imaged at 10x with bright field illumination using a slide scanner. Sections were then analyzed using threshold analysis in ImageJ software.

Western Blotting

Aortic tissue samples were prepared in lysis buffer (Sigma R0278 RIPA Buffer and Roche complete protease cocktail). Protein content was quantified using BCA assay (Thermo-Scientific Pierce BCA Protein Assay Kit). Protein concentrations were normalized by diluting samples with the lysis buffer. Protein was loaded and run using SDS-page (BIO-RAD Mini-Protean TGX stain free, 4-20%,15 well, 15 µl gel) and transferred to PVDF. Membranes were blocked with 5% Bovine Serum Albumin (BSA) buffered saline with Tween (TBST) for 60 minutes, then washed 3x10 minutes with TBST. Membranes were then incubated in primary for one hour (abcam ab110282 Anti-3-Nitrotyrosine Mouse mAB 1:500 for aorta; Cell signaling GAPDH (14C10) Rabbit mAb 1:1,000 for aorta). The membranes were then washed with TBST 3x10 minutes, and incubated in secondary antibody (abcam ab6721 Goat Anti Rabbit IgG H&L (HRP) at 1:

10,000; Invitrogen 31430 Goat Anti Rabbit IgG at 1:10,000)for 60 minutes. The

membranes were then washed 3x10 minutes with TBST, wet with chemiluminescent

detection substrate (BIO-RAD Clarity Western ECL Substrate)

and imaged with X-Ray film (Research Products International, base blue, high

sensitivity). Associated WB is found on Fig. 5. At this time, the image is not quantifiable.

Future WB will be processed the same as described above, and future developed images

will be analyzed using ImageJ software for densitometry. NT abundance (at 25 and 55

kD) will be normalized to GAPDH, and to 21% O_2 controls as set as 1.0 arbitrary units

(AU).

Blood Pressure

A Millar conductance catheter was used to determine the left ventricular end-systolic

pressure. Rats are anesthetized with urethane (1.3g/kg, i.p.) and mechanically ventilated

with 100% O_2. The Millar conductance catheter was placed into the left ventricle to

obtain pressure volume loops. End systolic blood pressure was taken using the pressure-
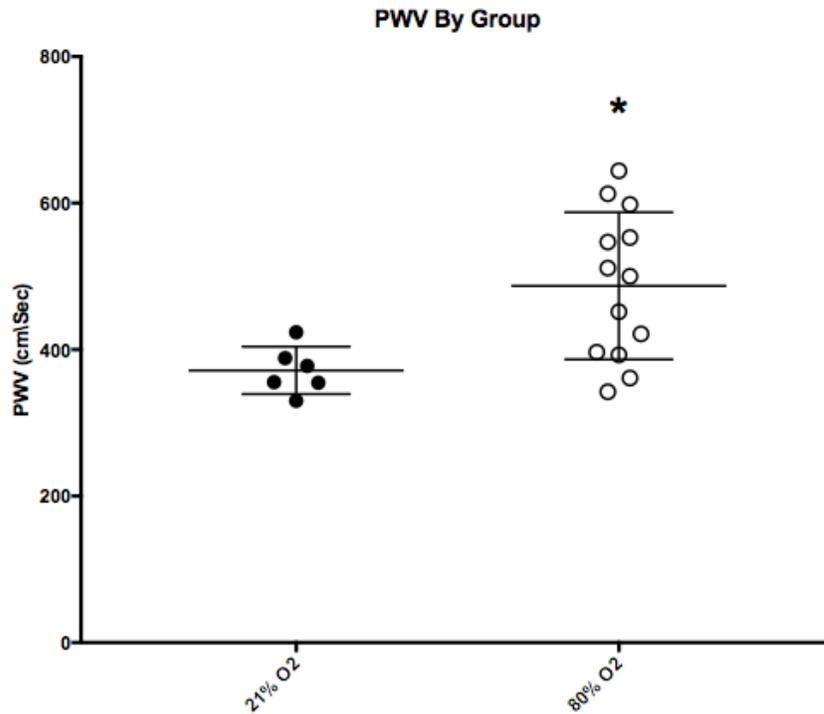
volume loops.

Results

Fig. 1 Aortic pulse wave velocity (PWV) in 21% and 80% oxygen exposure groups. Values are mean ± SEM. Unpaired t test used, * P < 0.05 vs. 21% control (n=6 for 21%, n= 13 for 80%)

Pulse wave velocity was significantly higher in rats exposed to 8 days of 80% O_2 than those exposed to 8 days of 21% O_2 at time of birth (values 80% vs 21% p=0.0147).

Because pulse wave velocity is an indicator of large arterial stiffness, this observation indicates that high oxygen exposure at birth leads to more stiffening of the arterial vessels as the subjects age.
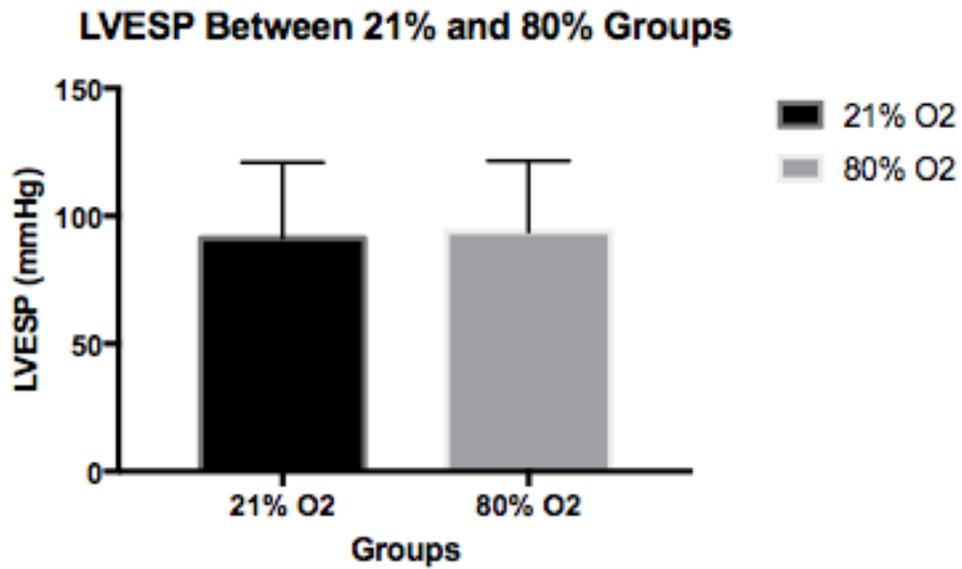
LVESP Between 21% and 80% Groups

Fig 3. Left ventricular end systolic pressure between 80% and 21% oxygen exposure groups. Values are $\pm$ SEM. Unpaired t test used. No significant difference between groups (P >0.05) n=10 for 80%, n=7 for 21%

Using a Millar conductance catheter, left ventricular end systolic pressure was determined by placing a catheter into the left ventricle, where *in vivo* systolic pressure was taken. There were no differences between the end systolic pressure between the 21% O_2 and 80% O_2 exposed groups.
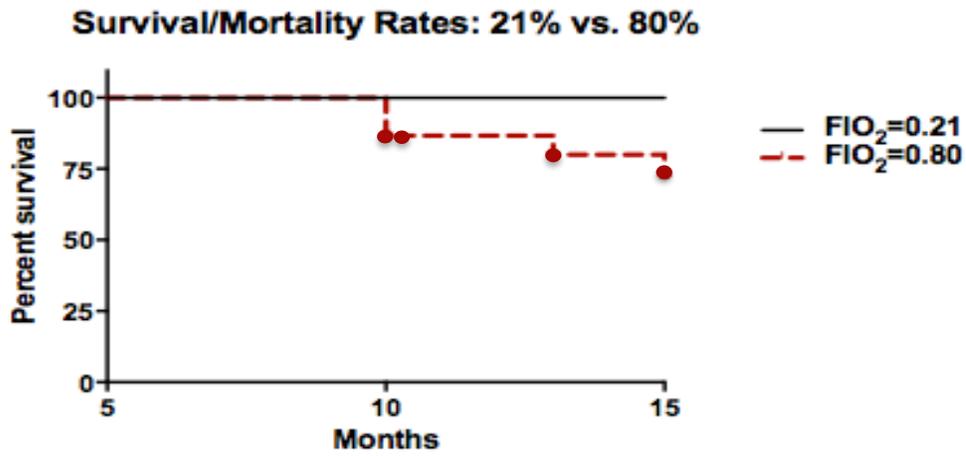
Survival/Mortality Rates: 21% vs. 80%

Fig 2. Survival/Mortality Rates for 21% and 80% groups.

The 80% O_2 group began to die starting at 10 months, and survival rates continued to decrease until 15 months. The 21% control groups noted no deaths, with survival rates staying at 100% until time of dissection and tissue harvesting.
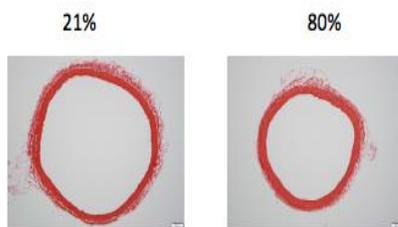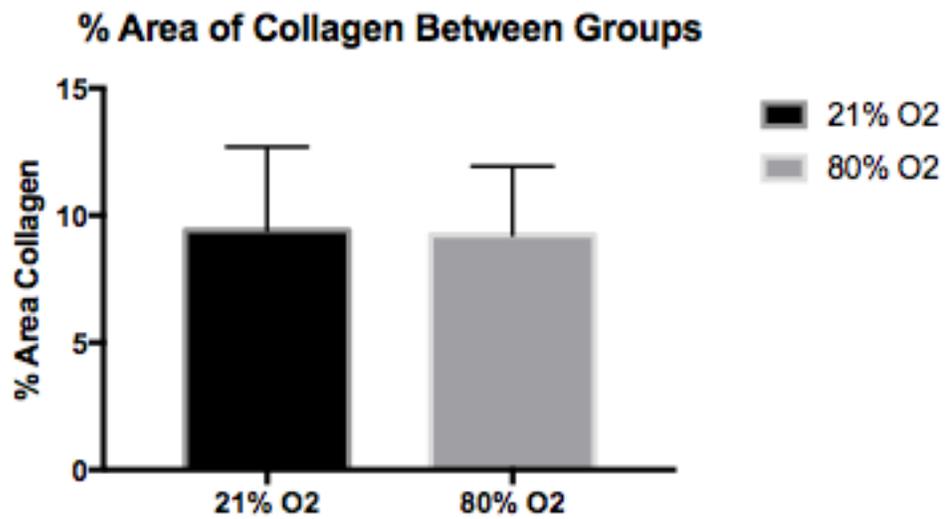
Fig 4. Area of collagen shown as percent normalized to whole aortic area. Unpaired t test used. No significant difference between the 21% and 80% O$_2$ groups (P>0.05) n=7 for 80%, n=3 for 21%

When normalized to the whole aorta, the percentage of collagen in the 21% O$_2$ and 80%
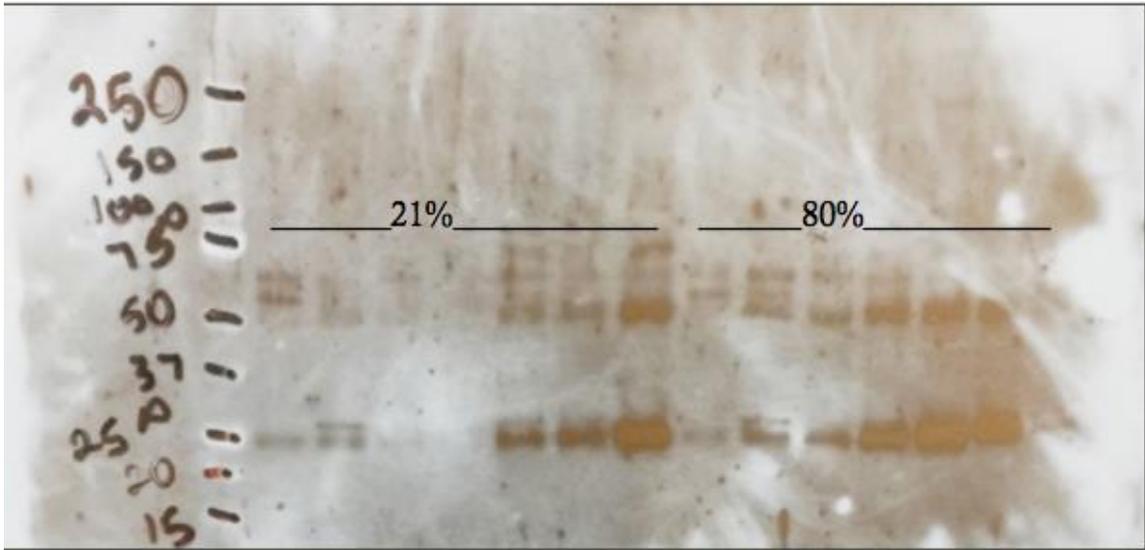
O$_2$ groups was not different.

Fig 5. Preliminary Western blot probing for NT.

A preliminary Western blot probing for NT suggests that there may be an increase in the marker of oxidative stress in the 80% O_2 rats. However, this blot cannot be analyzed, so results are currently inconclusive.

Discussion

The main objective of the project was to determine that if in a rat model of prematurity, rats exposed to 80% O_2 upon birth would be predisposed to an increase risk of CVD. As was expected, this project demonstrates that rats exposed to 80% O_2 had increased aortic stiffness, which as stated previously is a predictor of future CV events and death. These compliment the unexpected finding that rats exposed to 80% O_2 after birth had a 23% increase in mortality rate when compared to our control group that received room air (21%) O_2.

Prematurity and increased mortality rate

A study relating rat age to human age found that the approximate human age of the 15-month-old rats that were studied in this project were 35-40 years old (Sengupta, 2013). The population of people that were born in the early 1990's and subjected to this supplemental oxygen treatment are now reaching their late 20's. It is concerning that these rats, that were subjected to the same treatment as these adults that were born prematurely, are dying at such an early age, considering the normal lifespan of SD rat is between 24-36 months (Ohta, 2015). Neonatal oxygen supplementation may contribute to premature death and potential cardiovascular events in individuals as they age into middle adulthood.

Aortic stiffness accelerated arterial aging into adulthood in those born premature

There was a significant increase in PWV of the 80% O_2 exposed groups compared to the 21% control. Increased PWV in the experimental group suggests a link between this population and their increased risks of major cardiovascular events as they age. This provides first evidence about the deficits regarding the cardiovascular health of adults born prematurely. As stated previously, high PWV due to increased aortic stiffening can cause end organ damage to the heart, kidney, and brain, as well as impair blood flow delivery to coronary arteries (Codreanu, 2011). Not only do these adults suffer respiratory deficits due to supplementation of O_2 after their premature births, they also have impaired arterial health as they age into middle adulthood.

No difference in BP between 80% and 21% O_2 exposed groups

The lack of difference in LVESP suggests that the blood pressure is not the cause of the difference in PWV of the two groups. Arterial stiffness, measured by PWV, can be

associated with changes in BP. Because there was no difference, along with our other findings, it is concluded that the arterial stiffness is not caused by BP, but rather by structural changes in the aorta itself.

Collagen composition in aorta requires more inquiry

There was no difference found in the amount of collagen between the 21% and 80% O_2 exposed groups. Previous studies have shown that high O_2 during the neonatal period caused an increase in collagen in the walls of the aorta in rats during their 4th week of life (Huyard, 2014). It is known that the amount of collagen in the extracellular matrix increases due to age (Jani, 2006). There may have been a difference in the amount of collagen between the two groups if we assessed aortic collagen at an earlier time point in the rat's life. However, the differences in collagen between the groups may have been lost due to this age-associated increase in collagen that happened concurrent with the high oxygen exposure. PWV data and BP data suggest that there are structural differences between the 21% O_2 exposed groups and the 80% O_2 exposed groups.

Markers of oxidative stress need further assessment

Currently, there is not a Western blot that analyzable to assess the amount of NT between the 21% and 80% O_2 exposed groups to determine whether there was a difference in oxidative stress. However, a preliminary blot suggests that oxidative stress markers may be increased in the aorta of 80% O_2 rats. If this holds true, this could mean that there may be cell damage due to reactive oxygen species, which complements what has been previously studied (Fleenor, 2014) with aging alone. However, as of now, the results are inconclusive. The previous studied found that oxidative stress increased aortic

wall stiffness and collagen I protein in very old mice. This further illustrates that the high

PWV is due to structural changes in the aorta.

Further Analysis

It is worth determining the amounts of different isoforms of collagen protein,

because the PSR stains for Collagen 1 and 3 within the whole artery, however it has been

shown that specifically Collagen 1 increases with aging (Fleenor, 2014). Additionally,

other proteins could be changing such as elastin, which could be reduced contributing the

aortic stiffness that we observed in the 80% O_2 exposed rats. Structural changes of the

aorta caused by changes in the balance of collagen and elastin. Furthermore, oxidative

stress can modify these proteins within the aortic wall by increasing crosslinks in the

extra cellular matrix via advanced glycation end products (AGEs), which could be

measured via Western blot analysis in the future (Fleenor, 2014). Left ventricular

weights of the experimental group could be measured to determine whether changes in

heart remodeling are due to physiological or pathophysiological changes in the

myocardium, such as increased fibrotic tissue. Further studies should also include how to

continue to treat infants born prematurely, but using a different intervention that will not

cause such respiratory and cardiovascular stress as these individuals age. It is obvious

that oxygen exposure is necessary to sustain the lives of the newborns, and that

withholding oxygen would increase the mortality of newborns that are born prematurely.

However, there is an urgent need to understand the advanced cardiovascular

abnormalities so that treatment options can be development to reduce this increased risk

of CVD that these individuals will have, even before the reach middle-age. Ultimately,

there are two problems that need to be addressed. The first is how may we treat those

who are currently living that were exposed to this supplemental oxygen after their premature births, and second, how may we reduce the complications of high oxygen exposure? The first question may be answered in the future when more studies in this area have been conducted. A possible solution to the second may be giving premature newborns nitrite, which could be administered to alleviate the conditions that supplemental oxygen causes, as nitrite has been shown to reduce vascular oxidative stress (Sindler, 2011). This nitrite supplementation could possibly be administered in conjunction with high oxygen, or administered later in life to alleviate the risk of CVD.

This work shows extreme biomedical importance since 13% of babies are currently born premature, and the number of people who are living that were affected by oxygen exposure that have accelerated aging.

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