THE ROLE OF HYPOXIA AND METABOLISM IN HIGH ALTITUDE RENAL SYNDROME

by

Anjana Murali

BA Anthropology, BS Psychology, University of Pittsburgh 2018

Submitted to the Graduate Faculty of

University of Pittsburgh in partial fulfillment

of the requirements for the degree of

Bachelor of Philosophy

University of Pittsburgh

2018

UNIVERSITY OF PITTSBURGH

Faculty of Arts and Sciences

This thesis was presented

by

Anjana Murali

It was defended on

April 13, 2018

and approved by

Dr. Michael Siegel, Department of Anthropology, University of Pittsburgh

Dr. Joseph Alter, Department of Anthropology, University of Pittsburgh

Dr. Pinelopi Kapitsinou, Division of Nephrology and Hypertension, University of Kansas Medical Center

Thesis Advisor: Dr. Sunder Sims-Lucas, Division of Pediatric Nephrology, University of Pittsburgh

THE ROLE OF HYPOXIA AND METABOLISM IN HIGH ALTITUDE RENAL SYNDROME

Anjana Murali, BA, BS

University of Pittsburgh, 2018

More than 140 million people permanently live in regions of high altitude. Due to the deficiency of atmospheric oxygen, the inhabitants of these high mountains are subject to compromised physiology and High Altitude Renal Syndrome (HARS). Symptoms of HARS include systemic hypertension, microalbuminuria, polycythemia, and hyperuricemia. While it has been reported that low levels of oxygen result in renal malformations during development, including decreased nephron number, glomerular filtration rate (GFR) is preserved in patients with HARS. This study investigated the roles of hypoxia and metabolism as environmental regulators of kidney development and as driving mechanisms of the disease state HARS. High altitude hypoxia (12% O_2) exposure during development alone did not introduce any significant pathology, as evidenced by unchanged proximal tubular morphology and tubular bioenergetic expression. However, the high altitude hypoxia did have a subpathological role in exacerbating kidney injury. As seen through cisplatin-induced AKI, kidneys previously exposed to hypoxia had dilated proximal tubules and proteinaceous casts. Moreover these kidneys were physiologically impaired as seen through significantly upregulated toxic levels of BUN and creatinine. Based on the results of the present study, people living in high altitudes may be more susceptible to secondary insults later in life due to hypoxia's subpathological role in renal disease progression.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMATICS

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Sunder Sims-Lucas, for believing in me and supporting my research endeavors since my freshman year at the Unviersity of Pittsburgh. I also thank Dr. Michael Siegel, Dr. Joseph Alter, and Dr. Pinelopi Kapitsinou for serving as members of my thesis committee and for always challenging me to be a better scientist.

They say it takes a village to raise a child and that couldn't be more fitting to describe my time at the Rangos Research Center. I am indebted to the entire Division of Pediatric Nephrology (Bates, Ho, and Airik laboratories) for nurturing me and helping me become a good scientist. I would especially like to thank the Sims-Lucas group members and our collaborators for all of their help and guidance in my pursuit of this thesis. The Goncharova laboratory was very kind in letting me use their hypoxia chamber for this project and Takuto Chiba was extremely generous with his help in raising mice and expertise in AKI models. Additionally, Kasey Cargill was instrumental in helping me generate figures and schematics for this thesis while Elina Mukherjee and Kevin Peaseley were fantastic friends and colleagues throughout my undergraduate research career.

I would also like to thank the Honors College at the University of Pittsburgh for making the Bachelor of Philosophy experience possible for me and all Pitt students. The Honors College not only challenged me to conduct a graduate level research project and defend my work in an oral defense, but they also provided me funding throughout the summer term and school year to make this project a reality. Lastly, none of this would have been possible without the tremendous support that I've received from my family and friends. I am eternally grateful for their continous love and understanding.

1.0 INTRODUCTION

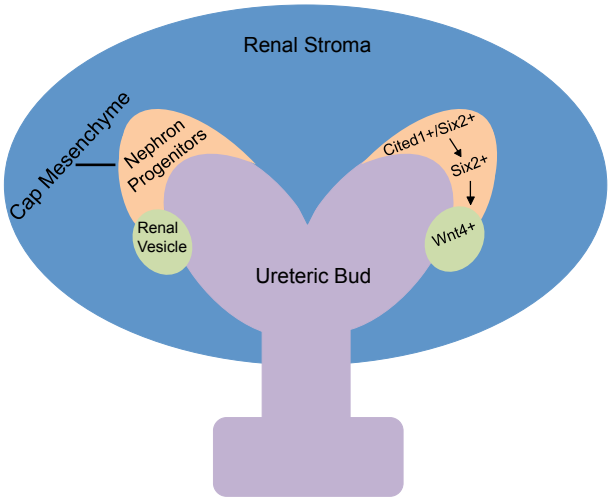
More than 140 million people (2% of the world's population) permanently live in regions of high altitude (which is defined as exceeding 2400 meters above sea level and has ~15% O_2) [1]. The three major populated high altitude regions include the Ethiopian Summits, Himalayan Mountains, and the Andean Mountains. Due to the deficiency of atmospheric oxygen, the inhabitants of these high mountains are subject to compromised physiology and High Altitude Renal Syndrome (HARS) [1]. Symptoms of HARS include systemic hypertension, microalbuminuria (moderate increase in the protein albumin in urine), polycythemia (increased concentration of hemoglobin), and hyperuricemia (excess of uric acid in blood) [2]. While it has been reported that low levels of oxygen result in renal malformations during development, including decreased nephron number [3], glomerular filtration rate (GFR) is preserved in patients with HARS. This would suggest that fewer nephrons in a hypoxia-adapted kidney receive increased workload to maintain normal GFR, and that this would cause a deleterious effect in the kidneys.

HARS is a recently identified disease, and therefore its etiology and molecular mechanisms are poorly understood. While adaptive responses to a variety of oxygen concentrations is a critical process during kidney development, its roles in gene signaling and cellular metabolism rates are largely unknown. Moreover, the effects of hypoxia on kidney function and hypertension have not been studied at altitudes that patients suffering from HARS live in. The major objective of this project was to interrogate the roles of hypoxia-induced signaling and hypoxia as environmental regulators of kidney development and as driving mechanisms of the disease state HARS.

2.0 IMPACT OF HIGH ALTITUDE HYPOXIA ON KIDNEY DEVELOPMENT AND FUNCTION

2.1 KIDNEY DEVELOPMENT AND HYPOXIA

The nephron is the functional unit of the kidney and is responsible for blood filtration, removal of toxic wastes, and regulation of several important physiological functions. Nephron endowment, or the number of functional nephrons in a developed kidney, is contingent on proper regulation of oxygen concentration in the developing metanephric kidney [3, 4]. As the ureteric bud grows towards the metanephric mesenchyme, which condenses around the ureteric bud tip, creating caps of nephron progenitor cells, and crosstalk between the nephron progenitors and ureteric bud tips signals for their self-renewal and branching, respectively [5]. The self-renewing nephron progenitor cell population is defined by Cited1+ and Six2+ signaling. As these cells mature, nephron progenitors sequentially lose Cited1



Scheme 2-1: Overview of kidney development: As development progresses, nephron progenitors condense around the ureteric bud tips to form the cap mesenchyme. This process is genetically defined by the expression of Cited1 (self-renewing), Six2 (self-renewing/poised for differentiation), and Wnt4 (differentiated).

K. Cargill, unpublished

and Six2 expression, then start expressing differentiation markers, such as Wnt4 (Figure 1). Progenitors that commit to differentiation undergo mesenchymal-to-epithelial transition and form the renal vesicle (first differentiated developing nephron structure), which then further develops into a functional nephron [5]. In humans, nephrogenesis results in 200,000 to 2,500,000 nephrons at birth [6]. This 10-fold range in nephron endowment has significant implications in an individual's susceptibility to disease because once nephrogenesis is complete; no new nephrons are formed [5]. For an individual who is endowed with fewer nephrons at birth, the loss of nephrons drastically increases their susceptibility to certain diseases such as chronic kidney disease and diabetes, compared to individuals with higher nephron endowment.

Oxygen concentration regulation is a critical process during kidney formation because the nephron progenitor cells develop under physiological hypoxia (~1-9% O_2) [7]. The hypoxia inducible factor (HIF) family of transcription factors regulates the major cellular oxygen sensing pathway [5]. Under hypoxic conditions, HIFs are upregulated to activate numerous target genes that are responsible for generating new vessels and regulating metabolism [4, 8]. However, once ingrowth, maturation and perfusion of the vasculature is complete, there is an increased oxygen concentration in the tissues, resulting in the degradation of HIFs by the E3 ubiquitin ligase von Hippel Lindau (VHL) protein [5, 8].

Although temporal changes in oxygen tension during kidney development are normal, and even necessary, chronic hypoxia can be detrimental to fetal development. For example, intrauterine hypoxia is an environmental stressor that is seen in cases of high altitude and can also result from environmental pollution and placental insufficiency [7]. Placental insufficiency is one of the

main causes of intrauterine growth restriction [9, 10], and is known to cause low birth weight with low nephron endowment in the babies born at high altitude [2]. Since the placenta is responsible for delivering nutrients to the fetus, an impaired placenta can restrict fetal growth. In response to hypoxia and decreased nutrients, blood flow in the fetus is preferentially redirected to the brain, heart, and liver, preventing the robust growth of other organs, including the kidney [11]. This redirection of blood flow is suggested to cause the observed low nephron endowment.

Mouse models for placental insufficiency are often use a chambered hypoxia devise, where pregnant dams are housed at 12% O_2 [7]. As it so happens, 12% O_2 is equivalent to the oxygenation that the majority of people living in high altitude receive (Table 1).

Table 2-1. Oxygen concentrations at varying altitudes

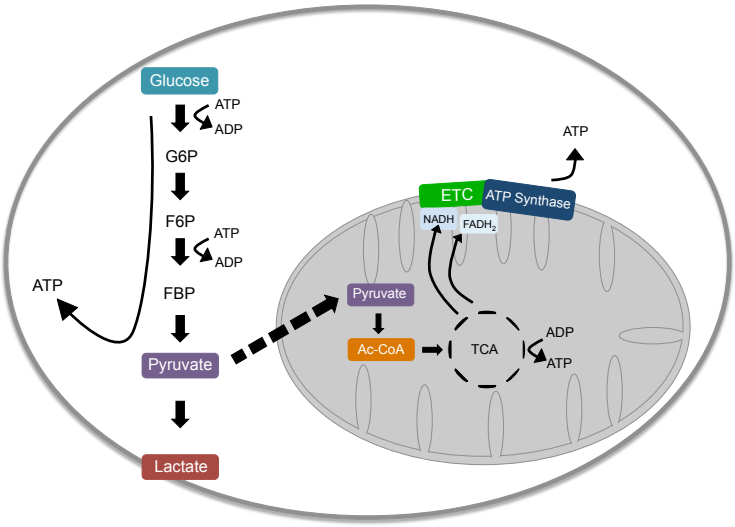
ALTITUDE (m)	EFFECTIVE O_2 %	ALTITUDE CATEGORY	EXAMPLE CITY
0	20.9	Low	Boston, MA
500	19.6	Low	
1000	18.4	Medium	
1500	17.3	Medium	Boulder, CO
2000	16.3	High	
2500*	15.3	High	
3000	14.4	High	
3500	13.5	Very High	
4000	12.7	Very High	Pikes Peak, CO
4500	11.9	Very High	Ethiopian Summits
5000	11.2	Extreme	
5500	10.5	Extreme	Kilimanjaro Peak
6000**	9.9	Extreme	

* Definition of high altitude living (lowest limit)

** Upper limit of the MAG-20 (high altitude hypoxia stimulator)

2.2 KIDNEY DEVELOPMENT AND METABOLISM

Changes in tissue oxygenation also affect rates of cellular metabolism in the kidney. The two main metabolic pathways activated during embryonic development are glycolysis and oxidative phosphorylation. Anaerobic respiration through glycolysis occurs in the cytoplasm and generates 2 ATP per glucose molecule. Glycolysis is the major metabolic process during early kidney development (embryonic day (E) 13.5) and appears to be a distinguishing metabolic feature of young nephron progenitors [12]. Nephron progenitors at stage E13.5 are in a significantly higher energy state compared with the nephron progenitors at E19.5 due to their need to continuously self-renew [12]. As nephron progenitors begin to differentiate and exit the



Schematic 2-2: Cellular respiration pathways: During glycolysis, glucose is converted into pyruvate yielding a net gain of 2 ATP per reaction. The pyruvate enters the mitochondria where it is converted into acetyl-coA (Ac-CoA) then is utilized in the TCA cycle. NADH and $FADH_2$ are bi-products of the TCA and used by the ETC to generate a net gain of 26 ATP. In the absence of oxygen, pyruvate is converted to lactate, which cannot undergo further oxidation in the mitochondria. G6P (glucose-6-phosphate), F6G (fructose-6-phosphate), FBP (fructose 1,6-bisphosphate).

K. Cargill, unpublished

self-renewing cycle, they become more dependent on oxidative phosphorylation [12]. Oxidative phosphorylation is a process in which pyruvate, the product of glycolysis, is further oxidized in the mitochondria in the presence of oxygen [13]. Through aerobic respiration, mitochondria are able to generate approximately 26 ATP per glucose molecule, which is done through coupling a proton gradient with ATP synthesis. During this process, electrons are passed through the electron transport chain (ETC), a collection of five protein complexes. Complexes I, II, and IV contribute to the proton gradient and oxidation of the electron carriers while complex V (ATP synthase) uses the energy of the diffusing protons to convert ADP to ATP [13]. During kidney development, either of these two metabolic processes can preferentially be employed to induce nephron progenitor self-renewal or differentiation. For example, inhibiting glycolysis can facilitate the epithelialization of the metanephric mesenchyme, and conversely, inhibiting self-renewal signaling can decrease glycolytic flux in young nephron progenitors [12].

The switch from reliance on glycolysis to oxidative phosphorylation is crucial for proper kidney development because it dictates the differentiation process and ultimately nephron endowment [12]. In fact, failure of terminal differentiation causes renal pathologies such as defective nephron formation or function [14]. It is important to note that mitochondria are most often dysregulated in disease states because they generate the most energy under aerobic conditions [13]. Together, this suggests regulation of metabolism is essential for proper kidney development.

2.3 PROXIMAL TUBULE DEVELOPMENT

The kidneys are the filtration units of the body, and the bulk of the filtration is the responsibility of the proximal tubules [15]. Proximal tubules are responsible for reabsorbing ~65% of the filtered load, and almost all of the filtered amino acids, glucose, solutes, and low molecular weight proteins [15]. These tubules also regulate acid-base balance by reabsorbing ~80% of filtered bicarbonate [15]. Proximal tubules are derived from nephron progenitor cells, which are a major type of kidney progenitor cell that gives rise to the glomerular and renal tubular epithelia [16]. As nephron progenitors epithelialize, the progenitor niche is depleted [17]. Near the end of nephrogenesis, remaining nephron progenitors undergo differentiation shortly after birth in the mouse (nephrogenesis is from E10-P3) and before birth in humans (nephrogenesis takes place from week 5-week 36) [16, 17]. The balance between progenitor self-renewal and differentiation is extremely important because no new nephrons are formed after the cessation of nephrogenesis, and the activity of nephron progenitors largely determined nephron number [17].

2.4 PROXIMAL TUBULES AND HYPOXIA

Physiological hypoxia is necessary for maintaining the nephron progenitor niche, however, hypoxia variably affects epithelial derivatives of the same progenitors. While proximal tubules are injured by hypoxia, glomeruli are relatively spared [18]. It is thought that proximal tubules are more susceptible to hypoxic injury because of their high aerobic energy demand for solute transport as well as low blood flow to the outer medullary region. Furthermore, obstruction of the tubules by sloughed dead cells and swelling of injured cells can also make them more susceptible to injury. The three different segments (S1, S2, and S3) of the proximal tubule are variably affected by hypoxia. Injury to the S1 and S2 segments of the proximal tubule is characterized by mitochondrial swelling, brush border alterations, and eventual disorganization of this region [19]. Morphologically, this is seen as accumulation of microvesicles just below an intact brush border and closely packed swollen mitochondria [19]. Meanwhile, hypoxic injury to the S3 segment is more severe and has two distinct markers of damage: cytoplasmic edema and tubular epithelial fragmentation [19]. Furthermore, the S3 segment of the proximal tubule, which is located in the outer medulla, is more susceptible to hypoxic injury than the collecting duct [20]. The heterogeneity in proximal tubule response to hypoxia is due to differences in the segment type function and exposure to vascular supply.

2.5 PROXIMAL TUBULES AND METABOLISM

The kidney is an organ with a high-energy demand, and consequently has a high density of mitochondria. Proximal tubules contribute greatly to the bioenergetics of a functioning kidney, as sodium is primarily reabsorbed via an active transport process. The Na+-K+-ATPase is expressed on the basolateral membrane of the proximal tubule [15]. Consequently, proximal tubules mainly rely on aerobic metabolism and have a high density of mitochondria. Compared to distal tubules, proximal tubules also have a greater mitochondrial volume to nuclear ratio because their cells are larger [21]. Unlike their distal counterparts, proximal segments contain mitochondria with a more oxidized state, and so they have little ability to partake in glycolysis. In response to the stress of hypoxia, mitochondria in proximal tubules rapidly depolarize while mitochondria in distal tubules maintain their potential by reversing the activity of the ATPase, as seen through multiphoton imaging of the kidney [21]. One possible explanation for this is the higher glycolytic ATP production in distal tubules. The oxidized state of the proximal tubules, along with the lower membrane potential can be explained by its greater workload and ATP hydrolysis [21]. Consequently, proximal tubules are especially vulnerable to hypoxia exposure and kidney injury.

2.6 NOTE

It is important to note that high altitude may generate complex responses which are not limited to HARS. Recent studies have actually shown that high altitude may exert beneficial effects. Specifically, high altitude (16% O_2) was inversely associated with all-cause mortality and cardiovascular events, such as myocardial infarction, stroke, and cardiovascular death, among dialysis patients, an outcome that was attributed to constant HIF activation [22]. It is thought that the upregulation of Hif-1a stimulates erythropoietin production and increases intestinal absorption of iron and its subsequent availability to the bone marrow. The mechanism of protection is thought to be caused by a downregulation of a risk factor for atherosclerosis: hepcidin (a hormone that regulates the entry of iron into circulation) [22]. It is also important to note that altitude did not significantly alter the rates of non-cardiovascular death [22].

2.7 SPECIFIC AIMS

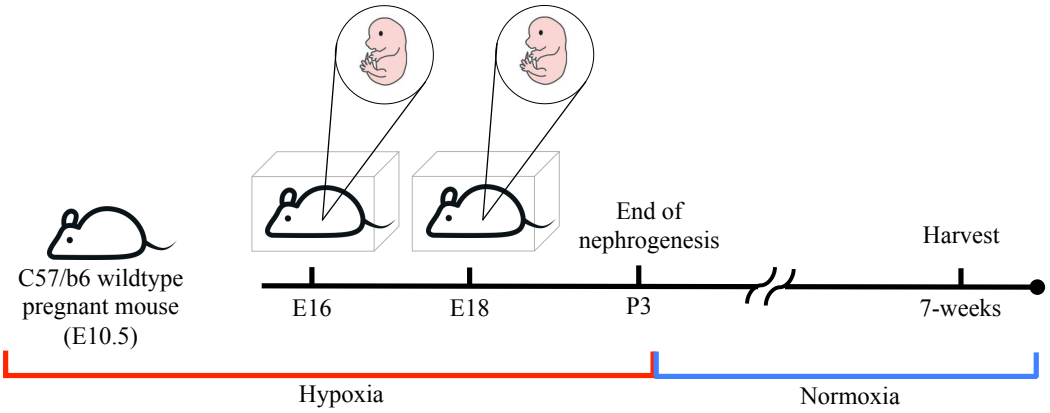
This chapter interrogates the formation and function of the kidney exposed to low levels of oxygenation that mimics high altitude exposure. At the early stages of the developing kidney, hypoxia is a normal state, prior to global kidney perfusion, and allows for abundant self-renewal of the nephron progenitors. Subsequent oxygenation allows the nephron progenitors to differentiate into mature renal structures. Therefore, environmental hypoxia during the later stages of nephrogenesis may hinder proper kidney formation. Since low nephron endowment is predictive of heightened susceptibility to disease, I hypothesized that the stress of chronic high altitude hypoxia would cause structural renal malformations and a decrease in renal fitness. Our laboratory has generated preliminary data from a genetic model of chronic physiological hypoxia indicating that the differentiation of nephron progenitors is highly dependent on oxygen concentrations and HIF expression, such that the progenitors that experience prolonged hypoxia do not differentiate properly. Additionally, we have found that progenitors experiencing hypoxia remain in a glycolytic state and are unable to switch to oxidative phosphorylation to drive differentiation. These led to my overarching hypothesis that mice exposed to low oxygen concentrations that simulate high altitude will have up-regulation of HIF signaling, causing structural kidney abnormalities. Two specific aims were proposed to test this hypothesis:

Aim 1: Determine the structural and molecular alterations that occur in the developing kidney following exposure to low environmental oxygen concentrations *in utero*.

Aim 2: Physiologically assess the renal fitness of animals exposed to the stress of high altitude hypoxia during nephrogenesis.

2.8 METHODOLOGY

Experimental Mouse Models



C57/b6 wildtype (WT) female pregnant mice (gestation age E10.5) were placed in a hypoxia chamber with a purge airlock system and CO_2 and O_2 control indicators designed for experiments in live rodents, with controlled and monitored humidity and gas composition. Exposure to hypoxia was initiated at embryonic day 11 (E11), which coincides with the induction of nephrogenesis, and continued until post-natal day 3 (P3), where there is the conclusion of nephrogenesis. The O_2 level was set at 12% for the group of hypoxia treatment, whereas the group of normoxic controls remained in ambient air (21%) O_2. For embryonic renal assessments, dams were sacrificed on E16 and E18 by cervical dislocation during isoflurane anesthesia. For the postnatal mice assessment, hypoxic pups were transferred to cages in ambient air at P3 and sacrificed at 7-weeks of age by cervical dislocation after isoflurane anesthesia. Kidney tissue was immediately collected and processed for analysis.

Tissue collection and histological assessment

Renal histology was assessed at E16, E18, and 7-weeks of age. At the indicated time points, dissected kidneys were fixed in 4% paraformaldehyde in PBS and processed in paraffin. Samples were sectioned at 4 μm thickness and stained with hematoxylin and eosin (H&E) for histological examination. Samples were imaged using a Leica DM 2500 microscope (Leica) and LAS X software (Leica).

Immunofluorescent staining and Real Time RT-qPCR

Immunofluorescent staining was performed on samples processed as described above. Sectioned samples were probed using primary antibodies or lectins (1:100) against NCAM (Sigma-Aldrich), Six2 (Proteintech), Phospho-histone H3 (Cell Marque), Endomucin (Santa Cruz Biotechnology), Tomm20 (Santa Cruz Biotechnology), Lotus tetragonolobus (LTL; Vector Laboratories), Oat1 (Alpha Diagnostic International), Dolichos biflorus agglutinin (DBA; Vector Laboratories), and Glut1 (Abcam). A Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was performed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. Real time reverse transcriptase quantitative PCR (RT-qPCR) was used to assess mRNA expression (data normalized to Rn18s).

Cardiac Punctures and Blood Analysis

Intraperitoneal injections were performed on 7-week old mice that were born in the normoxic and hypoxic conditions. The blood samples were collected and analyzed by Kansas State

University for creatinine and blood urea nitrogen (BUN) levels, which are commonly used markers of murine renal function.

Statistical analyses

A minimum of 3 biological replicates was used for each experiment and at least one technical duplicate. When comparing two sample groups, statistical significance was determined using a two-tailed Student's *t* test ($\alpha = 0.05$). Significance was defined as *P<0.05, **P<0.01, and ***P<0.001. Where appropriate, data is presented as mean ± standard deviation analyzed and generated by GraphPad Prism.

2.9 RESULTS

Previously it has been determined that individuals exposed to low levels of hypoxia throughout gestation develop HARS. However, whether this is due to an underlying developmental kidney defect has yet to be determined. To assess this, we subjected wildtype pregnant mice to hypoxic conditions and compared their kidney development and function to mice in normoxic conditions during kidney development. Here we find that the overall kidney architecture is maintained in the hypoxia exposed group in both embryogenesis and adulthood, as compared to the to the normoxia group (Figure 2-1).

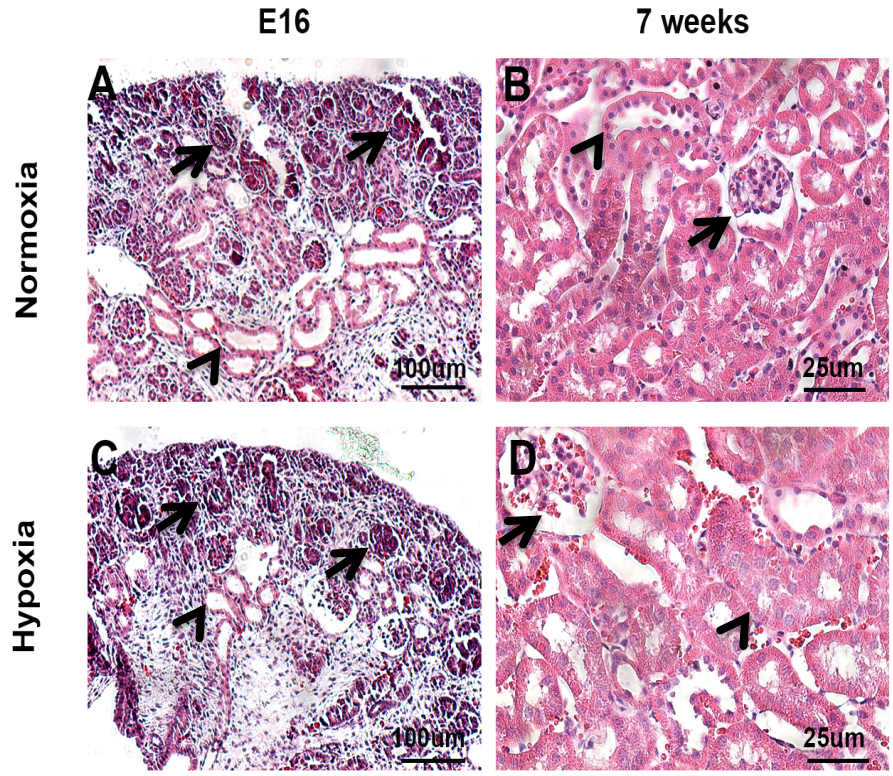


Figure 2-1. Kidneys exposed to hypoxia *in utero* are histologically normal. A thorough histological assessment was taken of kidney tissue in order to examine for structural changes. Hematoxylin (purple, nuclei) and eosin (pink, cytoplasm) staining revealed no pathological difference between normoxic and hypoxic kidneys neither at embryonic nor post natal ages. There appears to be the same amount of developing glomerular structures (arrows). Additionally, the proximal tubules remain in proper form with intact brush borders and no vacuolization or loss of nuclei (arrow heads).

To characterize the intrauterine effects of hypoxia on fetal growth, we measured the lengths of kidneys and embryos at E18. While hypoxia exposure stunted kidney and embryo lengths slightly, there is no difference in kidney to body length ratio (Figure 2-2).

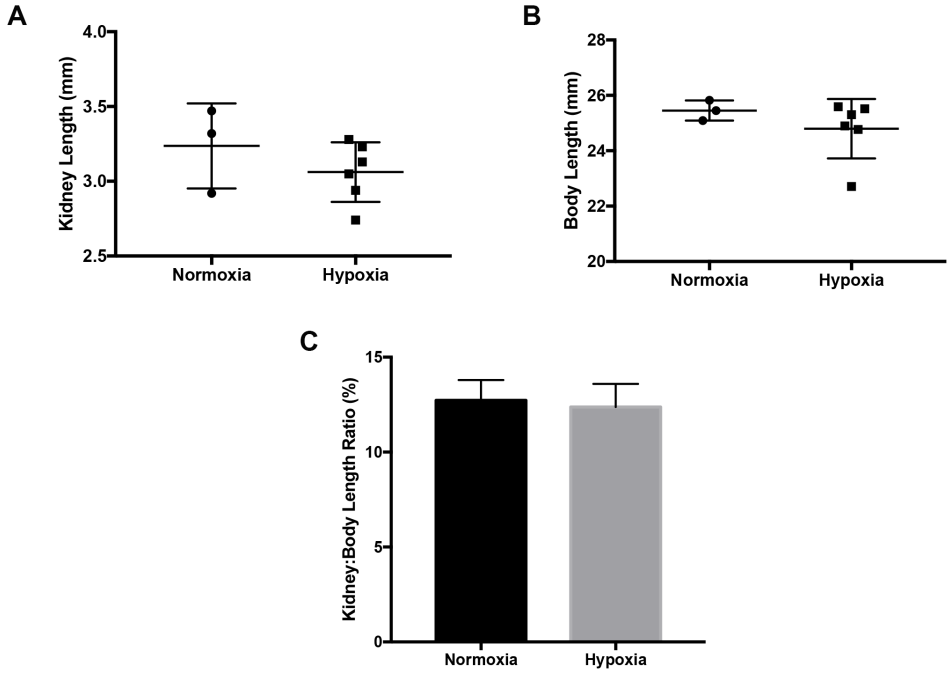


Figure 2-2. Kidney: Body Length ratio remains unaltered from exposure to high altitude hypoxia during nephrogenesis. E18 embryos were harvested from normoxic and hypoxic conditions and kidney and body lengths were measured using ImageJ software. There is no significant reduction in growth from 12% O_2 exposure *in utero*. (normoxia n=3, hypoxia n=6)

While there was no morphological difference from high altitude hypoxia exposure during nephrogenesis, we wanted to test for any functional differences from the intrauterine insult. We first weighed the adult mice from both normoxic and hypoxic conditions. Low body weights would support the idea of reduced nephron number in the setting of hypoxia, and thus make the mice exposed to hypoxic nephrogenesis more susceptible to kidney injury. However, we found that developmental high altitude hypoxia exposure did not affect nephron endowment (Figure 1-3). We also assessed renal fitness by analyzing BUN and creatinine levels from blood samples. At baseline, it was found that there is no difference in kidney function from a mild, hypoxic developmental insult (Figure 2-3).

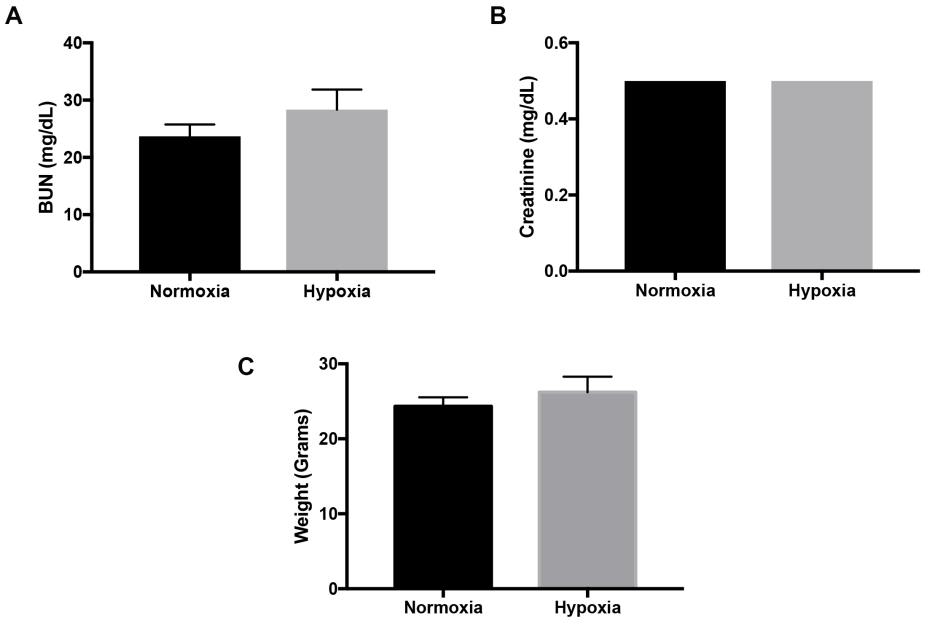


Figure 2-3. Kidneys exposed to *in utero* hypoxia function normally. Blood analysis of seven-week-old mice revealed no physiological abnormalities between normoxic and hypoxic conditions. Blood urea nitrogen and creatinine levels were normal under both treatment conditions. (normoxia n=3, hypoxia n=6)

High altitude hypoxia did not cause an overt phenotype in healthy mice in either embryogenesis or adulthood. However, this does not rule out the possibility of a subpathological role for hypoxia. Consequently, numerous tests were done to determine if there were any changes in the expression of genes relating to development, metabolism, and death. First, expression of nephron progenitors was stained for using Six2 immunofluorescence (Figure 2-4). An abnormality of this marker would indicate a misexpression of Six2 in non-progenitor, epithelialized portions of the developing nephron (marked by NCAM).

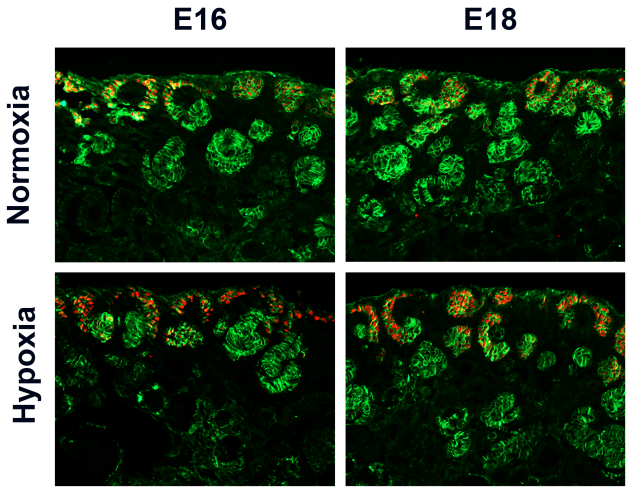


Figure 2-4. Exposure to high altitude hypoxia during development does not reprogram nephrogenesis. Immunofluorescence staining against Six2 (red, nephron progenitors) and NCAM (green, nephron progenitors and epithelialized structures) indicates that high altitude hypoxia exposure does not result in the misexpression of Six2, suggesting normal patterns of nephrogenesis in the maintenance of a nephron progenitor population.

Next we immunofluorescently co-stained for phospho-histone H3 (pHH3), a mitotic proliferation marker, and NCAM to visualize proliferation changes in the nephron progenitor region (Figure 2-5).

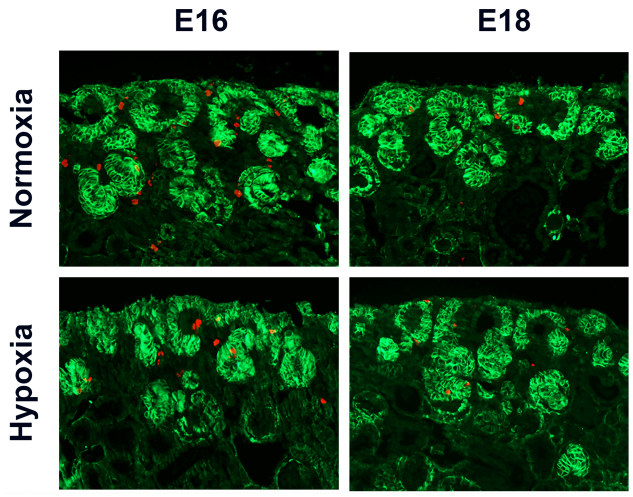


Figure 2-5. Exposure to high altitude hypoxia during development does not change proliferation patterns. Immunofluorescence staining with NCAM (green, nephron progenitors and epithelialized structures) and pHH3 (red, proliferating mitotic cells) reveals similar cell proliferation between normoxic and hypoxic kidneys.

We also we investigated the severity of the high altitude hypoxia exposure by measuring apoptosis through a TUNEL assay (Figure 2-6).

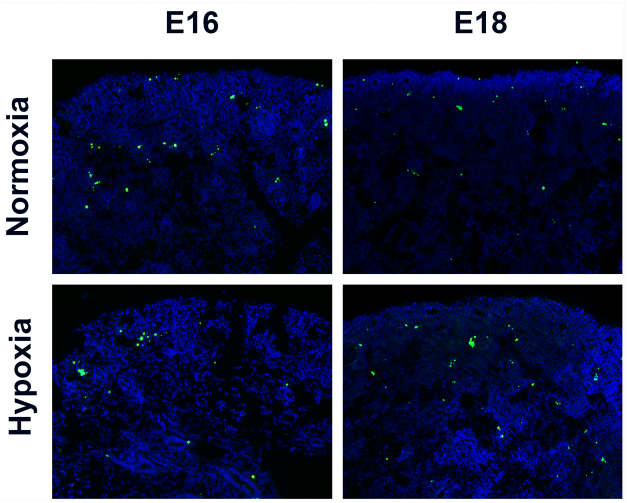


Figure 2-6. High altitude hypoxia exposure does not induce an apoptotic phenotype in the developing kidney. TUNEL staining reveals similar apoptosis levels between normoxic and hypoxic kidneys, suggesting normal cell death patterning.

Real-time RT-qPCR was then done on E18 whole kidneys from the normoxic and hypoxic conditions in order to evaluate mRNA abundance of genes related to hypoxia (*Hif-1a*), kidney injury (*Kim1*), and metabolism (*Pfkfb3* for glycolysis, *Glut1* for glucose transport, and *Tomm20* for mitochondrial density) (Figure 2-7).

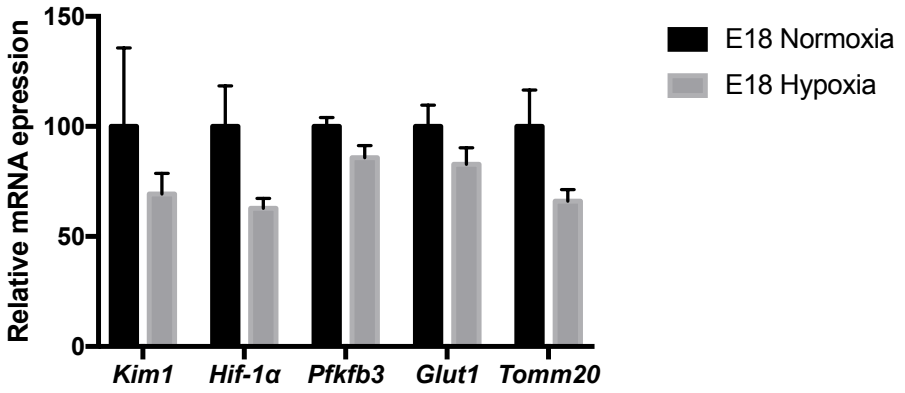


Figure 2-7. High altitude hypoxia exposure during development does not lead to dysregulation of genes in embryonic kidneys. Real Time RT-qPCR indicates that there is no significant difference in gene expression genes related to kidney injury, HIF signaling, nor metabolism of glucose between E18 normoxic and hypoxic whole kidneys. (normoxia n=3, hypoxia n=3)

We also performed a series of Real-time RT-qPCR assays to assess the dysregulation of genes in adult kidneys that were subject to high altitude hypoxia during nephrogenesis. Since these kidneys were subject to an insult during development but were then moved to ambient air after the cessation of nephrogenesis, we didn't expect much dysregulation. First, we measured the expression of important markers of kidney injury (*Kim1, Ngal*), de-differentiation (*Vimentin*), and apoptosis (*Bnip3, Bnip3L*). As suspected from lack of morphological and functional defects from high altitude hypoxia exposure during development, we found no dysregulation of injury gene expression (Figure 2-8).

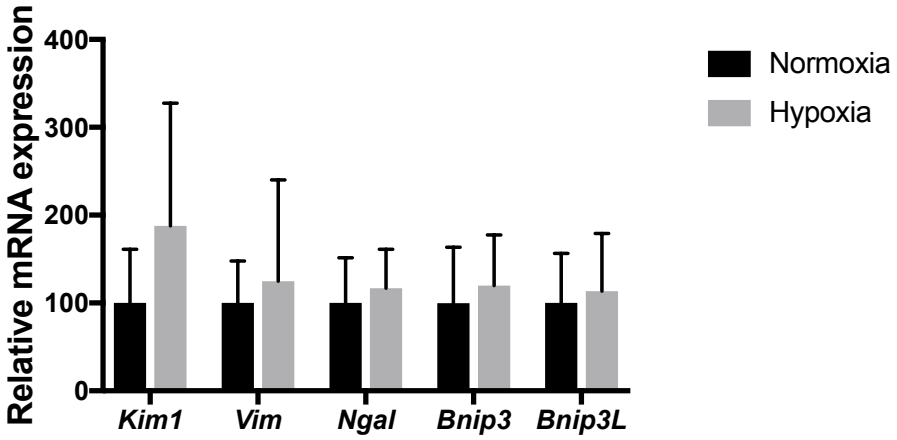


Figure 2-8. Developmental hypoxia treatment does not dysregulate markers of kidney injury in adults. Real Time RT-qPCR indicates that there is no significant difference in gene expression among markers of kidney injury (*Kim1 and Ngal*), de-differentiation (*Vim*), or apoptosis (*Bnip3 and Bnip3L*). (normoxia n=3, hypoxia n=6)

We also assessed the expression of important genes implicated with mitochondrial function and metabolism (Figure 2-9). We specifically analyzed genes involved with mitochondrial density (*Tomm20*), glucose transport (*Slc2a1/Glut1*), and glycolysis (*Ldha, Pfkfb3, and Pkm*).

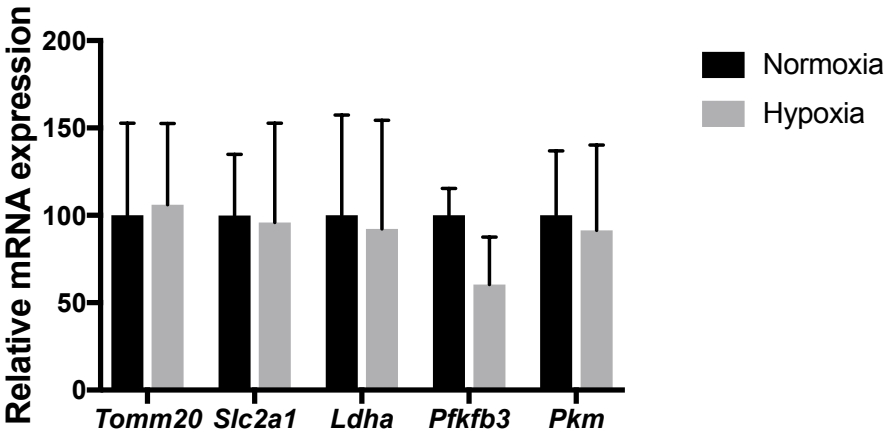


Figure 2-9. High altitude hypoxia exposure during nephrogenesis does not significantly alter the metabolic gene expression of adult kidneys. Real Time RT-qPCR indicates that there is no significant difference in gene expression among glycolytic genes (*Ldha, Pfkfb3, Pkm*) or genes involved with mitochondrial density (*Tomm20*) and glucose transport (*Slc2a1*).

To thoroughly investigate morphological changes caused by exposure to high altitude hypoxia during nephrogenesis, we examined proximal tubule structure in adult kidneys. The fluorescent marker LTL was used to stain proximal tubule brush borders, while Oat1, a proximal tubule transmembrane protein, was used to stain the basal membrane in order to analyze the heath of proximal tubules (Figure 2-10)

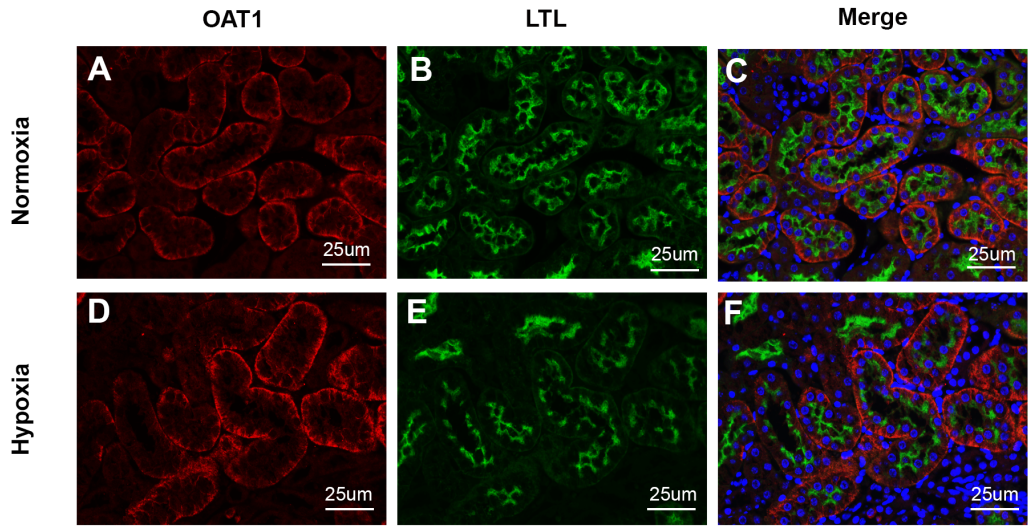


Figure 2-10. Proximal tubules in the adult kidney show no morphological differences after an *in utero* exposure to hypoxia. Immunofluorescence staining for Oat1 (red), a transmembrane protein on the basolateral side of the proximal tubule, serves as a marker for identifying proximal tubules. LTL staining (green) shows normal proximal tubule brush border formation.

To assess changes in cell death after hypoxia exposure, an apoptotic stain was done on the adult kidneys as well. Analysis of the TUNEL assay indicates that there was actually less death in kidneys exposed to hypoxia during development (Figure 2-11). However, this is not a quantitative measure of apoptosis so nothing conclusive can be drawn from this.

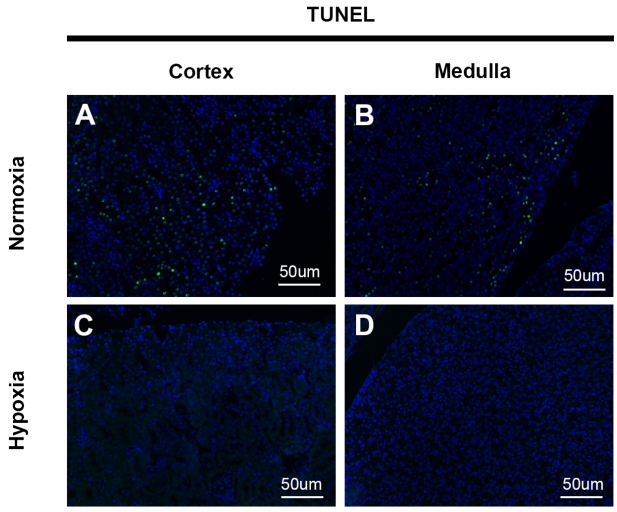


Figure 2-11. There is decreased cell death in adult kidneys after exposure to a hypoxic insult during development. The TUNEL assay shows apoptotic cells in only the normoxic kidney. The hypoxic kidney shows no cell death in either the cortex or the medulla.

To determine if *in utero* hypoxic exposure affects metabolic health of the adult kidney, we assayed for metabolism differences. First, we stained adult sections with Tomm20, a marker for mitochondrial density (Figure 2-12).

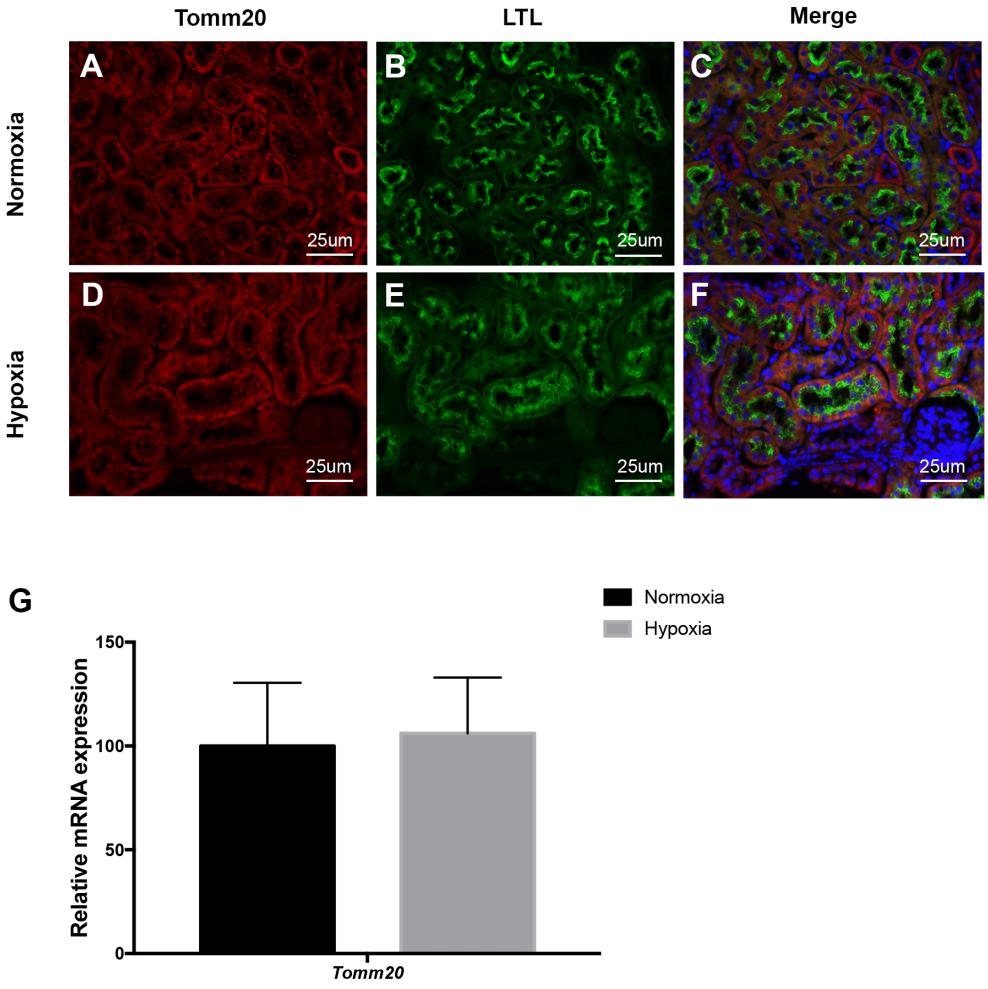


Figure 2-12. *In utero* high altitude exposure did not change mitochondrial density in the adult kidney. Immunofluorescence staining for Tomm20 (red), an outer-mitochondrial membrane protein, shows equivalent expression in the normoxic and hypoxic conditions. This trend was confirmed with real time RT-qPCR. LTL staining (green) was done to show the localization of the mitochondrial protein in the proximal tubules.

Next we stained for Glut1, a glucose transporter gene, in order to see if the mechanism for glucose metabolism was altered (Figure 2-13). Since mitochondrial density remained the same for kidneys exposed to developmental hypoxia we didn't expect to see a difference but wanted to be thorough in our metabolic assessment.

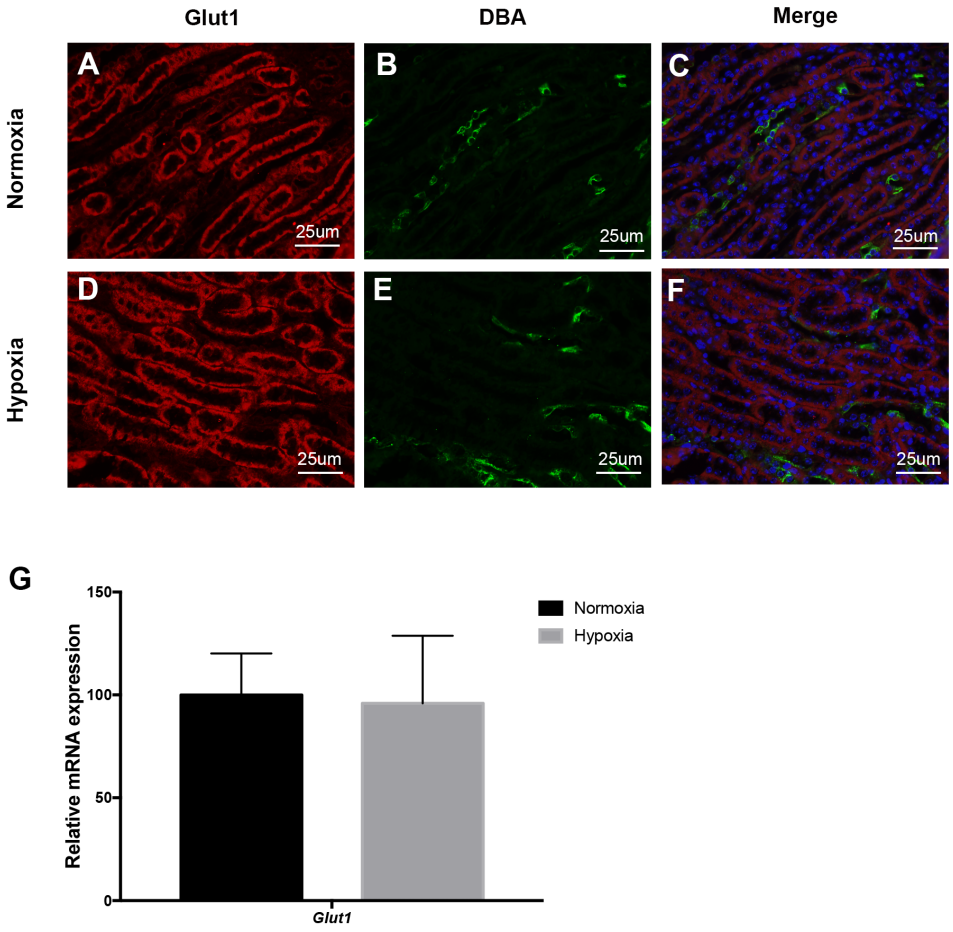


Figure 2-13. Exposure to developmental hypoxia does not alter the expression of Glut1 in adult kidneys. Immunofluorescence staining for Glut1 (red), a glucose transporter, shows equivalent expression in proximal tubules of the kidney. This trend was confirmed with real time RT-qPCR. DBA staining (green) for collecting ducts was done to reveal the expression of Glut1 in the proximal tubules specifically (DBA-negative tubules).

2.10 DISCUSSION

The data presented shows that exposure to *in utero* hypoxia that simulates high altitude during nephrogenesis does not overtly change the morphology or function of the kidneys. We assessed the kidneys using a myriad of assays including histology and real time PCR, but found no statistical differences in RNA or protein expression. We also assessed the blood urea nitrogen and serum creatinine (indicative of kidney function) and found these measures to be unchanged. This suggests that the 12% O_2 environment is not sufficient to stress the mice into developing HARS-like symptoms such as hyperuricemia (excess of uric acid in blood).

In attempts to characterize genetic changes from high altitude hypoxia exposure during nephrogenesis, we analyzed gene expression of kidney injury markers and apoptosis but found them to be largely unchanged after exposure to hypoxia.

Exposure to high altitude hypoxia did not result in metabolic dysfunction either. Mitochondrial density appears to be the same between normoxic and hypoxic kidneys and so does expression of glycolytic genes.

From this we can conclude that there is no overt tissue or functional pathology in kidneys that are exposed to hypoxic conditions *in utero*. However, we hypothesize that hypoxia may have sub-pathologically insulted these kidneys, reprogrammed them to be more susceptible to injury and this will be examined in Chapter 2.

3.0 EFFECTS OF IN UTERO HIGH ALTITUDE HYPOXIA EXPOSURE ON ACUTE KIDNEY INJURY

3.1 RATIONALE

Although clinical studies have shown that HARS occurs in resident populations at high altitude, we observed no morphological or pathological alterations in the kidneys of mice exposed to high altitude simulated hypoxia, compared to the normoxic group, as seen in Chapter 1. To address this ***we hypothesized that the mice exposed to 12% low oxygen concentrations*** **in utero** ***are susceptible to secondary insults later in life***.

To test this, we utilized a cisplatin-induced kidney injury model that directly targets the proximal tubules, as these are derivatives of the nephron progenitors that have been shown to be hypoxia-sensitive for normal development. Cisplatin is a platinum based nephrotoxin that is commonly utilized as an anti-cancer medication. However, cisplatin is highly damaging to proximal tubules, where it damages their DNA and mitochondria, thus causing cell death.

3.2 CISPLATIN-INDUCED ACUTE KIDNEY INJURY (AKI)

Cisplatin is an antineoplastic drug used to treat many solid-organ cancers [23]. Despite its effectiveness, it results in severe nephrotoxicity, the most common of its presentations being acute kidney injury (AKI). AKI is defined as a sudden reduction in glomerular filtration rate [24]. While clinical care for AKI has improved over the years, it has a mortality rate of ~40-80% in the intensive care unit [25]. Moreover, AKI can accelerate the onset of end-stage renal disease (ESRD) [25]. Cisplatin is known to cause DNA damages by binding to nuclear DNA and also diminishes metabolic activity by damaging mitochondria [23]. Since cisplatin is hydrolyzed to a positively charged metabolite, it accumulates within negatively charged mitochondria and therefore disproportionately affects proximal tubule function, which requires high mitochondrial function [23]. Renal cisplatin toxicity is also associated with apoptotic and necrotic cell death predominantly in the S3 segment of the proximal tubules and inflammation.[26].

3.3 SPECIFIC AIMS

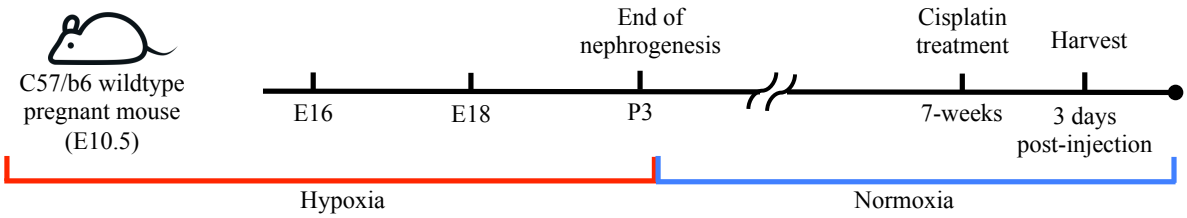
This chapter investigates cisplatin-induced acute kidney injury (AKI) after hypoxia exposure during pregnancy. To our knowledge, no literature has reported on the effects from hypoxia during pregnancy on AKI susceptibility in the adult kidneys. Two specific aims were proposed to test our hypothesis:

Aim 1: Determine the degree of secondary cisplatin injury on kidney morphology after *in utero* hypoxia exposure.

Aim 2: Determine the effect of *in utero* hypoxia on renal functioning after secondary cisplatin-induced injury.

3.4 METHODOLOGY

Experimental Mouse Models



C57/b6 wildtype (WT) female pregnant mice (gestation age E10.5) were placed in a hypoxia chamber with a purge airlock system and CO_2 and O_2 control indicators designed for experiments in live rodents, with controlled and monitored humidity and gas composition. Exposure to hypoxia was initiated at embryonic day 11 (E11), the induction of nephrogenesis and continued until post-natal day 3 (P3). The O_2 level was set at 12% for hypoxic animals, whereas normoxic controls remained in ambient air (21%) O_2. After P3, hypoxia-exposed pups were transferred to cages in ambient air. At 7 weeks of age, the pups were injected with 20mg/kg body weight of cisplatin (APP NDC 63323-103-64; working solution in normal saline). The pups were sacrificed three days later by cardiac puncture during isoflurane anesthesia. Kidney tissue was immediately collected and processed for analysis.

Tissue collection and histological assessment

Renal histology was assessed at 7-weeks of age in normoxic and hypoxic mice. At the indicated time points, dissected kidneys (7 weeks) were fixed in 4% paraformaldehyde in PBS and processed in paraffin. Samples were sectioned at 4 μm thickness and stained with hematoxylin

and eosin (H&E) for histological examination. Samples were imaged using a Leica DM 2500 microscope (Leica) and LAS X software (Leica).

Immunofluorescent staining and Real Time RT-qPCR

Immunofluorescent staining was performed on samples processed as described above. Sectioned samples were probed using primary antibodies or lectins (1:100) as follows: Lotus tetragonolobus (LTL) (Vector Laboratories), Oat1 (Alpha Diagnostic International), Dolichos biflorus agglutinin DBA (Vector Laboratories), Glut1 (Abcam), and Tomm20 (Santa Cruz Biotechnology). A Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was done to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. Real time reverse transcriptase quantitative-PCR (RT-qPCR) was used to measure mRNA expression (data normalized to Rn18s).

Cardiac Punctures and Blood Analysis

Intraperitoneal injections were performed on 7-week old mice that were injected with cisplatin working solution with and without hypoxia priming. The blood samples were collected and analyzed by Kansas State University for creatinine and BUN levels, which are commonly used markers of renal function.

Statistical analyses

A minimum of 3 biological replicates was used for each experiment and at least one technical duplicate. When comparing two sample groups, statistical significance was determined using a two-tailed Student's *t* test ($\alpha = 0.05$). Significance was defined as *P<0.05, **P<0.01, and ***P<0.001. Where appropriate, data is presented as mean ± standard deviation analyzed and generated by GraphPad Prism.

3.5 RESULTS

While we did not find any morphological or structural alterations in the adult kidney after a primary insult of hypoxia exposure during development, we anticipated that hypoxia plays a subpathological role in making kidneys more susceptible to secondary insult. Both normoxic and hypoxic mice were injected with cisplatin at 7-weeks of age in order to characterize a secondary stress phenotype. Histological assessment shows severe architectural differences between adult kidneys that developed in normoxic and hypoxic conditions (Figure 3-1).

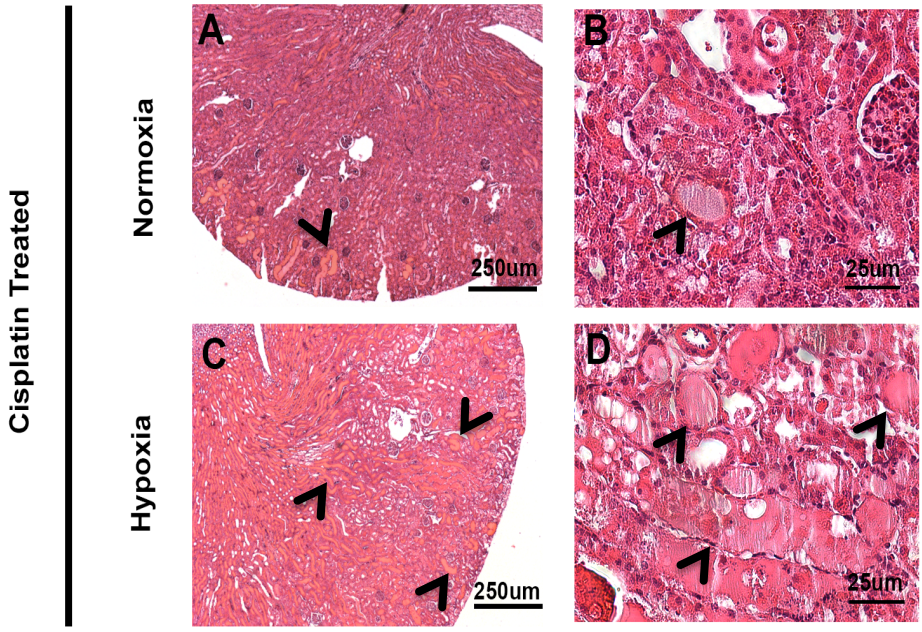


Figure 3-1. Adult kidneys exposed to 12% hypoxia *in utero* are more susceptible to cisplatin-induced AKI. Hematoxylin (purple, nuclei) and eosin (pink, cytoplasm) staining revealed renal pathology due to cisplatin treatment in both normoxic and hypoxic development conditions. Both kidneys show damage with dilated tubules and proteinaceous casts (arrow heads). However, the hypoxic kidney shows significantly more pathology with the presence of more casts and loss of nuclei in renal tubules.

Consistent with the morphological changes, developmental exposure to hypoxia decreased the filtration capacity of proximal tubules after cisplatin injury (Figure 3-2).

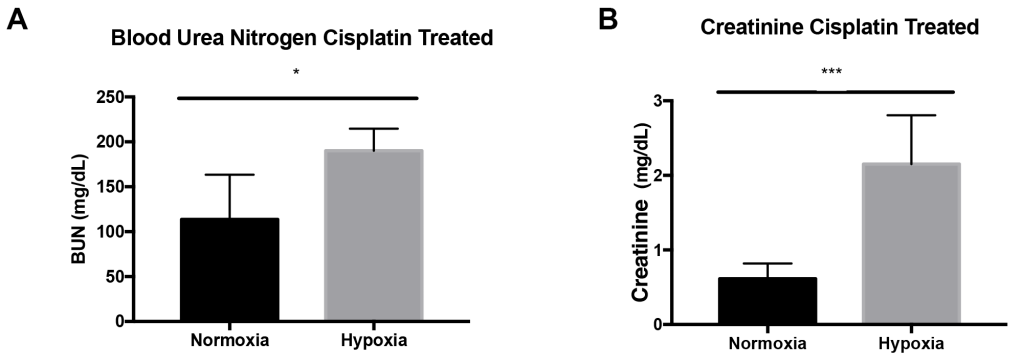


Figure 3-2. Kidneys exposed to high altitude hypoxia are more impaired functionally. Blood analysis of 7-week-old mice revealed severe physiological problems as indicated by high levels of urea nitrogen and creatinine in normoxic and hypoxic mice. Despite both treatment groups of mice showing decreased renal fitness, the mice in the hypoxic condition had further exacerbated damage than those in the normoxic condition. (normoxia n=7, hypoxia n=4)

We also performed a series of real-time RT-qPCR assays to assess the expression of genes in impaired adult kidneys that were previously subject to high altitude hypoxia during nephrogenesis. First we assessed the mRNA expression of important markers of kidney injury (*Kim1, Ngal*) and de-differentiation (*Vimentin*) (Figure 3-3). Since there were no morphological or functional defects from high altitude hypoxia exposure during development, we were curious to see what injury genes were dysregulated in the adult kidneys after a secondary stress model.

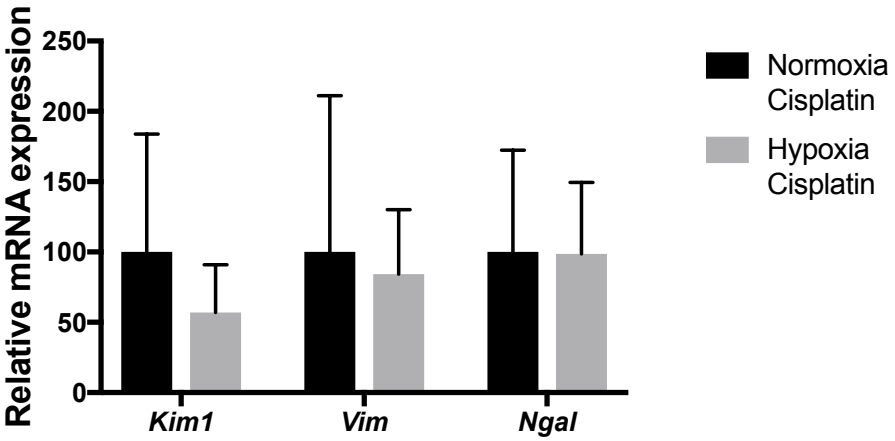


Figure 3-3. Kidney injury markers were not significantly different in impaired adult kidneys with previous exposure to high altitude hypoxia during development. Real Time RT-qPCR indicates that there is no significant difference in gene expression among kidney injury (*Kim1* and *Ngal*) and de-differentiation (*Vim*) markers. (normoxia n=3, hypoxia n=3)

We also assessed the expression of important genes associated with metabolism. We specifically analyzed genes involved with glycolysis, an anaerobic form of respiration (*Hk2, Ldha, Pfkfb3,* and *Pkm*) (Figure 3-4).

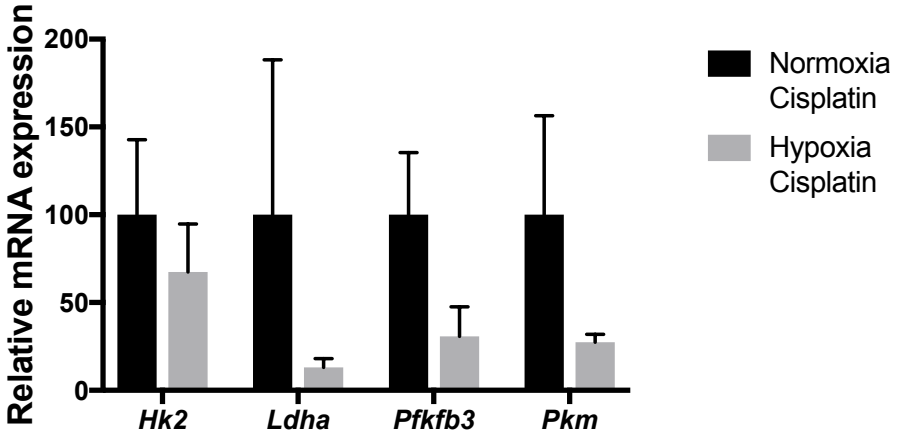


Figure 3-4. Primary high altitude hypoxic insult during development significantly reduces the metabolic gene expression of cisplatin-impaired adult kidneys. Real Time RT-qPCR indicates that glycolytic genes (*Hk2, Ldha, Pfkfb3,* and *Pkm*) are trending downregulated in cisplatin-induced AKI with hypoxia preconditioning. (normoxia n=3, hypoxia n=3)

To further characterize morphological changes caused by exposure to high altitude hypoxia during nephrogenesis, we examined proximal tubule structure in adult kidneys. LTL was used to stain proximal tubule brush borders and the sections were co-stained with Oat1, a proximal tubule transmembrane protein, in order to analyze proximal tubule health (Figure 3-5).

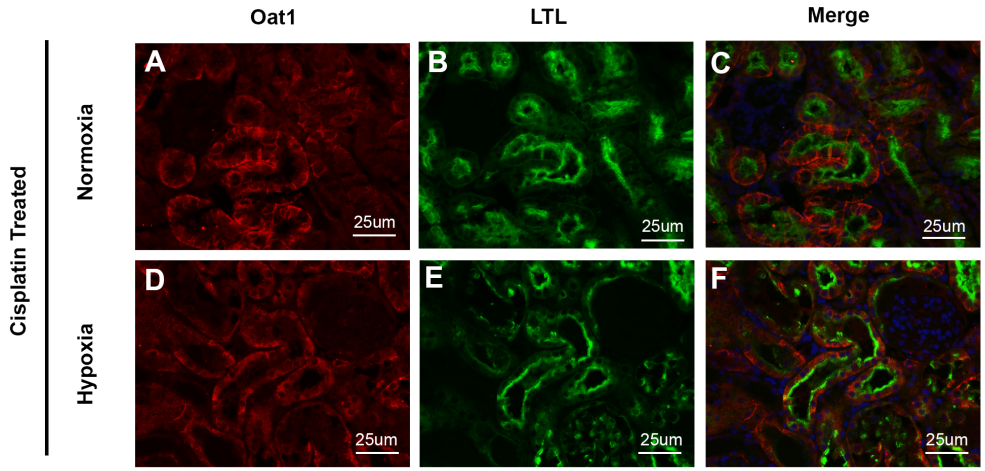


Figure 3-5. High altitude hypoxia exposure during development causes proximal tubule dilation in cisplatin impaired adult kidneys. Immunofluorescence staining for Oat1 (red), a transmembrane protein on the basolateral side of the proximal tubule, serves as a marker for identifying proximal tubules of the kidney. LTL staining (green) shows tubular dilation in the hypoxic kidney compared to the normoxic one, and the dilation is specific to the proximal tubules of the kidney.

Since the proximal tubules were dilated in kidneys exposed to hypoxia during development, we wanted to assess whether there was excess cell death in these kidneys. To measure this we used a TUNEL assay to stain cells in late-stage apoptosis (Figure 3-6). However, we observed decreased apoptotic cell death in kidney exposed to *in utero* hypoxia, which is consistent with results reported above in kidneys without cisplatin treatment (Figure 2-6).

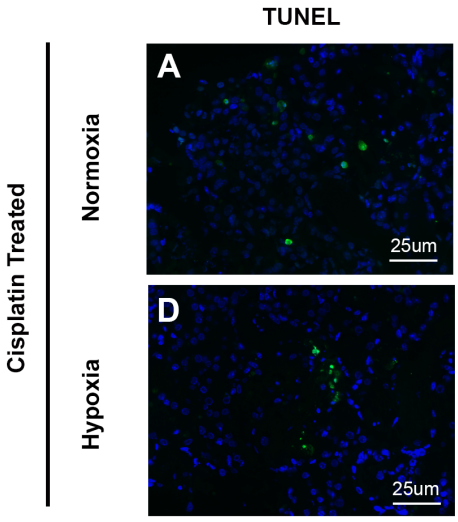


Figure 3-6. Kidneys previously exposed to hypoxia during development do not have as much nuclear cell death from cisplatin injury. The TUNEL assay shows apoptotic cells in both normoxic and hypoxic conditions. However, the normoxic condition treated with cisplatin shows sporadic apoptotic cells with more death localized to nuclear cells, suggesting DNA fragmentation. Meanwhile, the hypoxic condition has a clustered pattern of apoptosis with less death seen in nuclear cells.

In order to elucidate a mechanism for tubular dilation in the hypoxic condition, we decided to assay for metabolism protein expression differences due to *in utero* hypoxia exposure. First, we stained adult sections with Tomm20, a marker for mitochondrial density (Figure 3-7).

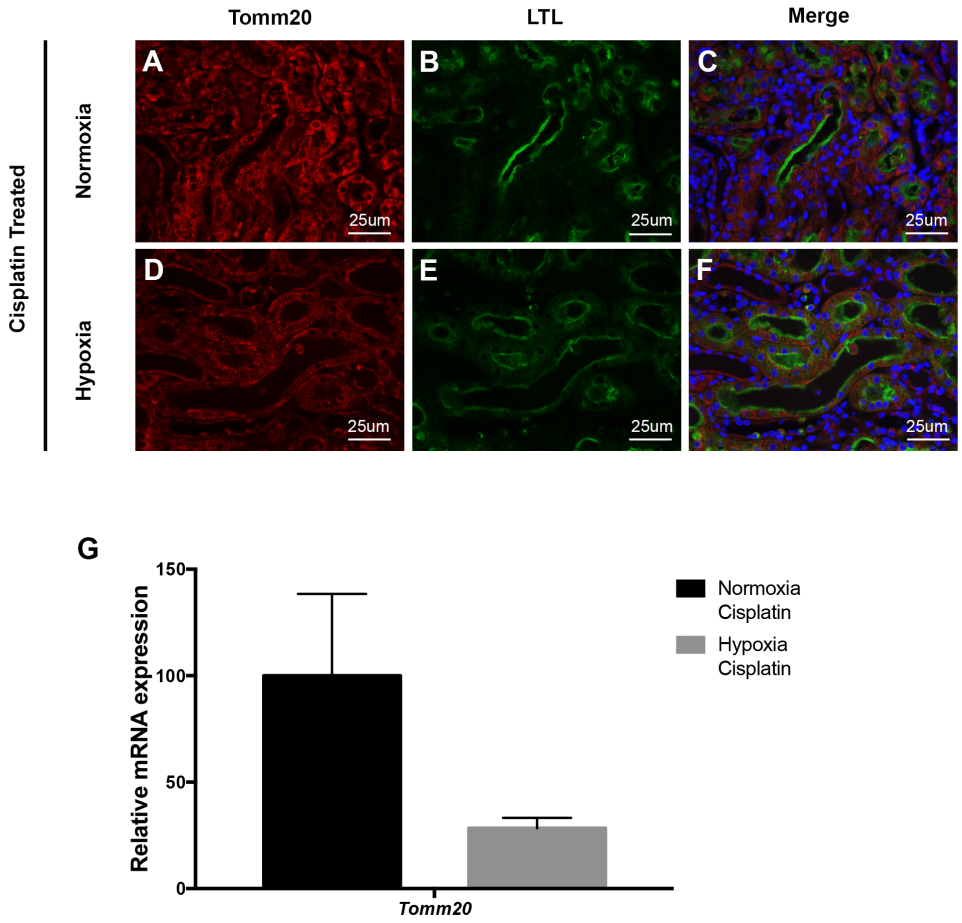


Figure 3-7. High altitude hypoxia exposure during development reduces mitochondrial density after cisplatin-induced AKI. Immunofluorescence staining for Tomm20 (red), an outer-mitochondrial membrane protein shows decreased expression in the hypoxic condition. This trend was confirmed with real time RT-qPCR. LTL staining (green) was done to show the localization of the mitochondrial protein in the tubules of the kidney.

Additionally we stained for Glut1, a glucose transporter gene, in order to see if the mechanism for glucose metabolism was altered (Figure 3-8). Since mitochondrial density was decreased for kidneys exposed to developmental hypoxia we expected to see a reduction in glucose transporters as well.

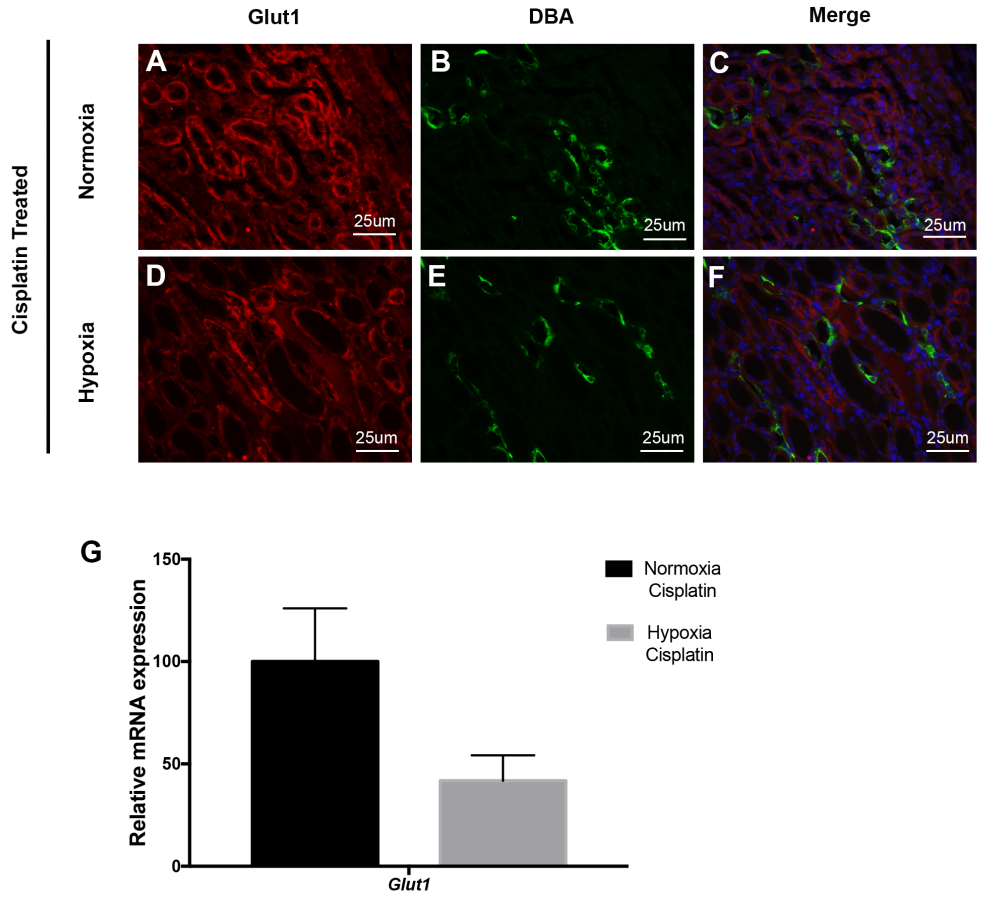


Figure 3-8. High altitude hypoxia exposure during development reduces glucose transport after cisplatin-induced AKI. Immunofluorescence staining for Glut1 (red), a glucose transporter, shows decreased expression in proximal tubules of the kidney. This trend was confirmed with real time RT-qPCR. DBA staining (green) for collecting ducts was done to reveal the downregulation of Glut1 in the proximal tubules specifically (DBA-negative tubules).

3.6 DISCUSSION

Histological assessment of kidney formation showed severe tubular injury due to cisplatin in both normoxic and hypoxic conditions. However, the degree of injury was significantly exacerbated in kidneys that were exposed to high altitude hypoxia during nephrogenesis, as seen by the cyst formation and tubular dilation that was not seen in normoxic mice. As a result, this study shows high altitude simulation exacerbates cisplatin-induced acute kidney injury. Mice that were raised in high altitude-like conditions for the duration of nephrogenesis had significantly higher levels of BUN and creatinine after cisplatin administration, suggesting that these mice were more susceptible to injury.

While hypoxia exposure during development was not protective against renal fitness, it may be protective against nuclear fragmentation (as measured by TUNEL). Cisplatin injury normally targets proximal tubular cells and is known to cause nuclear fragmentation; however this phenotype was predominantly only seen in kidneys formed in normoxia. Hypoxia exposure during nephrogenesis seems to have a protective effect against nuclear fragmentation, as there were very few nuclei that stained for late stage apoptosis. However, compared to the normoxic kidneys suffering from AKI, the hypoxic ones showed patchy, non-nuclear patterns of apoptosis. This either suggests that these cells are necrotic or that previous hypoxia exposure is protective against nuclear cell death.

Developmental exposure to hypoxia coupled with cisplatin-induced acute kidney injury had detrimental effects on the bioenergetics of the kidney. First of all, there was a severe reduction in mitochondrial density, which makes sense because cisplatin damages mitochondria, which are abundant in proximal tubules. This would suggest that aerobic respiration is limited in cisplatin-injured kidneys, thus anaerobic respiration should be upregulated. However, previous hypoxia exposure resulted in decreased expression of glucose transporters and significant down regulation of glycolytic genes, suggesting a compounding dysregulation of metabolism with cisplatin injury and hypoxia. This may be in part due to cisplatin-induced DNA damage preventing proper RNA transcription. If hypoxic mice cannot efficiently use oxidative phosphorylation or glycolysis, then the question remains how they can be energetically active and not yet dead.

4.0 OVERALL DISCUSSION

This study is one of the few analyses of High Altitude Renal Syndrome (HARS) using a mouse model. The idea behind using 12% O_2 was to recapitulate environmental conditions to stimulate the disease state that humans living in high altitudes suffer from. The study of high altitude disease states has not been extensively examined since the 1970s and needs to be updated. The present study attempted to establish a mouse model for HARS and characterize the stress of 12% O_2 tensions on kidney development and adult function.

At the embryonic level, intrauterine high altitude hypoxia did not introduce any significant pathology. This is most likely due to the fact that nephrogenesis normally occurs under hypoxia, so a mild maternal hypoxic stress did not reprogram fetal nephrogenesis. Furthermore, pups that were exposed to high altitude hypoxia during nephrogenesis did not show any problems with renal function. In terms of human disease and babies born at high altitude, this suggests that stress from low oxygen tensions is not sufficient to cause any significant pathology. However, since nephron endowment has a 10-fold range in human kidneys, these babies could still be at risk for developing kidney disease later in life if they were born with fewer nephrons.

Hypoxia exposure during development alone did not alter normal nephron function, as evidenced by unchanged tubular morphology and tubular bioenergetic expression. Although humans living at altitudes with 12% O_2 develop HARS, it is likely that mice are more robust animals and can survive with more hypoxic stress. However, despite the fact that mice do not show symptoms of HARS, it is likely that this mild environmental hypoxia exposure is

subpathological and can have implications upon a secondary renal insult. In fact, as seen in this study, hypoxia exposure during development exacerbated cisplatin-induced kidney injury by causing dilation of proximal tubules and accumulation of proteinaceous casts. This consequently severely hindered physiological renal fitness, as seen through significantly upregulated toxic levels of BUN and creatinine, and eliminated two major forms of bioenergetics.

A new clinical study published in the *Journal of Nephrology* in April 2018 characterized the higher prevalence of HARS in human patients at altitudes equivalent to 12% O_2 tensions. This cross-sectional study investigated differences in the prevalence of kidney function among healthy high altitude and sea level dwellers without any known history of hypertension, diabetes, or chronic kidney disease. It was found that high altitude dwellers who were previously healthy had worse kidney function, a higher prevalence of proteinuria, and a lower prevalence of metabolic syndrome compared to people living at sea level. This study is interesting for two major reasons. Firstly, it shows a growing interest in studying disease states, specifically HARS, caused by decreased oxygen tensions at high altitudes—a field of study, which has largely been abandoned since the 1970s from the anthropological perspective. Secondly, this study serves as validation for the secondary stress model established in this thesis. The patients analyzed in the clinical study were 40-60 years of age, which suggests that HARS symptoms don't manifest until later in life. This bolsters the idea that a 12% hypoxia environment plays a subpathological role

Based on the results of the present study, people living in high altitudes may be more susceptible to cisplatin-induced AKI later in life because exposure to high altitude hypoxia (12% O_2) plays a subpathological role in renal disease progression. There are a significant number of people living at high altitude worldwide and characterization of disease states correlated with high altitude are necessary to ensure the well-being of these populations.

4.1 CAVEATS/ALTERATIONS

The effects of high altitude hypoxia seen at embryonic ages are hard to differentiate from normal tissue hypoxia. To better assess the renal consequences of high altitude hypoxia, renal fitness measurements must be taken of adult mice that were born and raised in hypoxia. Regarding the hypoxia exposure, it did not seem like the level of oxygenation was hypoxic enough to cause any significant morphological or functional differences as a primary insult. However, that was intentional for this project since we wanted to recapitulate the stress of living in high altitude. In order to truly characterize the disease state of HARS it would be necessary to create a mouse model that actually presented with HARS symptoms.

Furthermore, the assessments for this project were all done on whole kidney as this was a preliminary phenotypic assessment for the HARS mouse model. One caveat of whole kidney analysis was that any significant differences in relative mRNA expression or protein presence might have been diluted. As a result, isolated analysis of nephron progenitors and proximal tubules, for embryonic and adult analyses respectively, would have given more accurate results. Another limitation of this study is that we did not assess the relationship between HIF protein levels and 12% environmental hypoxia. While relative mRNA expression among HIF isoforms were not significantly different between normoxic and hypoxic conditions, HIFs undergo post-transcriptional modifications and therefore evaluating protein expression would be a better assessment of their function. Finally, this study attempted to characterize metabolic gene dysregulation through relative mRNA expression and immunofluorescence staining. However,

no characterization of metabolic function was done. As a result, an exciting future direction would be to test metabolic activity of these kidneys through Seahorse Extracellular Flux.

4.2 FUTURE DIRECTIONS

Understanding the molecular, cellular and structural renal adaptations to low oxygen environments is essential for providing therapies for patients suffering from HARS. One of the most pressing future direction for this project is assessing nephron endowment after 12% environmental hypoxia exposure. While this study was approached from a phenotypic standpoint, it was limited by the fact that no mechanism of the subpathological role of high altitude hypoxia exposure in secondary injury was ever established. A reduction in glomeruli number after high altitude hypoxia exposure would be a start to elucidating a mechanism for this phenotype. Secondly, more manipulations of environmental oxygen concentrations need to be tested to see if a mouse model portraying HARS symptoms can be established. A model of symptomatic HARS would be critical for assessing drug therapies for this disease state. Finally, all of the analyses for this project were conducted in male mice and one mouse strain, to reduce variability. Therefore, an important future direction for characterizing HARS would be to analyze sex differences in this model because female mice are more prone to cisplatin-induced injury.

5.0 REFERENCES

1. Arestegui, A.H., et al., *High altitude renal syndrome (HARS).* J Am Soc Nephrol, 2011. **22**(11): p. 1963-8.
2. Hurtado, A., et al., *Cardiovascular and renal effects of chronic exposure to high altitude.* Nephrol Dial Transplant, 2012. **27 Suppl 4**: p. iv11-6.
3. Mugford, J.W., et al., *High-resolution gene expression analysis of the developing mouse kidney defines novel cellular compartments within the nephron progenitor population.* Dev Biol, 2009. **333**(2): p. 312-23.
4. Haase, V.H., *The VHL/HIF oxygen-sensing pathway and its relevance to kidney disease.* Kidney Int, 2006. **69**(8): p. 1302-7.
5. O'Brien, L.L. and A.P. McMahon, *Induction and patterning of the metanephric nephron.* Semin Cell Dev Biol, 2014. **36**: p. 31-8.
6. *<Human_nephron_number__implicat.PDF>.*
7. Hemker, S.L., S. Sims-Lucas, and J. Ho, *Role of hypoxia during nephrogenesis.* Pediatr Nephrol, 2016. **31**(10): p. 1571-7.
8. Bernhardt, W.M., et al., *Expression of hypoxia-inducible transcription factors in developing human and rat kidneys.* Kidney Int, 2006. **69**(1): p. 114-22.
9. Eleftheriades, M., G. Creatsas, and K. Nicolaides, *Fetal growth restriction and postnatal development.* Ann N Y Acad Sci, 2006. **1092**: p. 319-30.
10. *<1. Development of the placental villous tree and its consequences for fetal growth.pdf>.*

11. Cosmi, E., et al., *Consequences in infants that were intrauterine growth restricted.* J Pregnancy, 2011. **2011**: p. 364381.

12. Liu, J., et al., *Regulation of Nephron Progenitor Cell Self-Renewal by Intermediary Metabolism.* J Am Soc Nephrol, 2017. **28**(11): p. 3323-3335.

13. Zhang, H., et al., *Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia.* J Biol Chem, 2008. **283**(16): p. 10892-903.

14. Saifudeen, Z., *A role for p53 in terminal epithelial cell differentiation.* Journal of Clinical Investigation, 2002. **109**(8): p. 1021-1030.

15. Zhuo, J.L. and X.C. Li, *Proximal nephron.* Compr Physiol, 2013. **3**(3): p. 1079-123.

16. Nishinakamura, R., *Stem cells and renal development in 2015: Advances in generating and maintaining nephron progenitors.* Nat Rev Nephrol, 2016. **12**(2): p. 67-8.

17. Brown, A.C., S.D. Muthukrishnan, and L. Oxburgh, *A synthetic niche for nephron progenitor cells.* Dev Cell, 2015. **34**(2): p. 229-41.

18. Turman, M.A. and C.M. Bates, *Susceptibility of Human Proximal Tubular Cells to Hypoxia: Effect of Hypoxic Preconditioning and Comparison to Glomerular Cells.* Renal Failure, 2009. **19**(1): p. 47-60.

19. Shanley, P.F., et al., *Hypoxic injury in the proximal tubule of the isolated perfused rat kidney.* Kidney International, 1986. **29**(5): p. 1021-1032.

20. Wilson, P.D. and R.W. Schrier, *Nephron segment and calcium as determinants of anoxic cell death in renal cultures.* Kidney International, 1986. **29**(6): p. 1172-1179.

21. Hall, A.M., et al., *Multiphoton imaging reveals differences in mitochondrial function between nephron segments.* J Am Soc Nephrol, 2009. **20**(6): p. 1293-302.

22. Winkelmayer, W.C., et al., *Altitude and the risk of cardiovascular events in incident US dialysis patients.* Nephrol Dial Transplant, 2012. **27**(6): p. 2411-7.

23. Miller, R.P., et al., *Mechanisms of Cisplatin nephrotoxicity.* Toxins (Basel), 2010. **2**(11): p. 2490-518.

24. Kapitsinou, P.P. and V.H. Haase, *Molecular mechanisms of ischemic preconditioning in the kidney.* Am J Physiol Renal Physiol, 2015. **309**(10): p. F821-34.

25. *<Acute Renal Failure in Critically Ill Patients.pdf>.*

26. *<Cisplatin-Induced Renal Apoptosis- Caspase 3-dependent and independent pathways.pdf>.*