VITAMIN A SUPPLEMENTATION: ITS EFFECTS ON ONTOGENY, LUNG DEVELOPMENT AND NEONATAL LUNG INJURY
- STUDIES IN A RAT MODEL

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ABSTRACT

Vitamin A (VA; retinol) is essential for normal vision, cell proliferation and differentiation, embryonic development, overall growth, reproduction, and immune function. VA deficiency can lead to premature birth, low-birth weight, and incomplete development of organs, such as the lungs, resulting in respiratory complication. Therefore it is speculated that retinol used to generate the biologically active metabolite, retinoic acid (RA), is needed to support the differentiation of rapidly growing tissues such as the lungs. The physiological requirement of neonates for VA is unknown. Previously we have shown retinol combined with RA (VARA) is able to increase retinyl ester (RE) formation and cellular uptake of VA in lungs, increase the total time that VA resides in VA storage organs (lung, liver, kidney, and intestine), and increase the distribution of VA into various tissues, including the lung. However, these findings, although beneficial, do not provide a comprehensive view of VA metabolism during ontogeny from birth to adult age. In this thesis research, I hypothesized that incremental supplementation of VARA will alter the ontogenic expression patterns of retinoid homeostatic genes responsible for retinol trafficking, metabolism, and catabolism; alter the expression of proliferative genes essential for proper lung septation and maturation; and attenuate bleomycin-induced bronchopulmonary dysplasia (BPD) lung injury in neonatal rats.

In the present project, several studies were carried out to 1) investigate ontogenic expression pattern of retinoid homeostatic genes from birth to adult age in VA storage organs: lung, liver, and kidney; 2) investigate how the expression pattern of retinoid homeostatic genes are altered after VARA supplementation; 3) determine if there are long-term effects of neonatal VARA supplementation on the tissues of adults; 4) explore effects of VARA supplementation on proliferative gene expression in postnatal lungs; and 5) examine therapeutic properties of VARA in treatment of lung injury resembling BPD in neonatal rats. The results our studies have shown
that expression of retinoid homeostatic genes during ontogeny as well as retinol concentration in the liver, lung, kidney, and plasma remain constant in neonates. In addition, VARA supplementation, significantly increased expression of retinoid homeostatic genes responsible for cellular uptake and storage of retinol, and terminal oxidation of RA in neonates, as well as adults. At the same time, VARA had a long-term effect on expression of these genes and total retinol concentration in liver of adult rats. Although VA is known to be important for secondary septation, our results determined that VARA supplementation did not alter expression of proliferative genes in lungs during the time of secondary septation and alveolarization in neonates. However, VARA remarkably increased plasma retinol concentrations, attenuated immune cell infiltration and hemorrhaging in lung tissues, as well as reversed early signs of fibrosis, promoted cellular proliferation, and restored secondary septation in the presence of neonatal lung injury.

Overall, our studies have shown potential benefits of VARA, in comparison to VA alone as studied previously. Our findings provided insights on 1) VA metabolism in neonates and long term effect of VA supplementation; 2) the effects of neonatal VA supplementation on proper lung development; and 3) VARA supplementation as a potential mode of therapy for BPD, or similar chronic lung diseases in preterm/term infants. By determining effects of VA-marginal nutritional status and VA supplementation and VARA administration during neonatal development, we will better understand neonatal retinol physiology and VA nutritional requirements. Potentially, the translation of these results will improve neonatal intensive care.
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Design and treatment schedule showing generation of SD offspring by mating female and male rats fed VAM diet, which was maintained throughout gestation and fed to animals that continued in the study after weaning until adult age. Pups were randomly assigned to receive oil or VARA treatments at five ages, as shown by X. The adult group marked 0 received VARA on neonatal days P1 and again on P4, P7 and P10, but not when as adults, and thus was used to assess any carryover effect of neonatal VARA supplementation at adult age. For all ages, the final treatment with oil or VARA was administered 6 h before euthanasia and tissue collection. Total retinol levels increased in lungs with age (ontogeny) and after VARA supplementation in plasma [B], liver [C], and lung [D]. Data are shown as mean ± SEM, n ≥ 5/group. One-way ANOVA with posthoc analysis was used to determine differences between oil-treated groups at different ages, indicative of developmental changes with ontogeny; means without a common letter differed significantly, P < 0.05. For each age, VARA versus Oil groups were compared by unpaired t-test; * indicates P < 0.05. In C, linear trends with age were determined for the Oil and VARA groups separately. 

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Figure 5.1. Animal experimental design

BLM was administered to rat pups through intraperitoneal injection beginning one day after birth (P1) until P5 at a concentration of 0.4 µg/µl adjusted to body weight (5 µl/g bw). Control group received 1X DPBS. Supplementation of VARA (or placebo) began at P6 every other day up to P14. Animals were euthanized for collection of plasma and lung tissues at P15 for analysis.

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Figure 5.3. Cytokine expression in neonatal lung after BLM treatment and VARA supplementation

BLM significantly increase IL-6 (A) expression at P5 in neonatal lungs. By P15, TNFα (B) expression significantly increased while IFNγ (B) expression significantly decreased in response to BLM treatment. VARA supplementation did not restore
expression of these cytokines levels comparable to control group. Data are shown as mean ± SEM, n ≥ 3/group. One-way ANOVA with multiple t-test posthoc analysis was used to determine differences at P15 between treatment groups. At P5, PBS vs. BLM groups were compared using multiple t-test. Means without a common letter differ significantly (a>b>c>d) indicates P < 0.05. .................................

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expression in alveolar tissue. Data are shown as mean ± SEM, n ≥ 3/group. One-way ANOVA with multiple t-test posthoc analysis was used to determine differences at P15 between treatment groups. At P5, PBS vs. BLM groups were compared using multiple t-test. Means without a common letter differ significantly (a>b>c>d) indicates P < 0.05.

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance
AP1: activating protein 1
BLM: bleomycin
BPD: bronchopulmonary dysplasia
CRABP: cellular RA-binding protein
CRBP: cellular retinol binding protein
CYP: cytochrome P450
EGFR: epidermal growth factor receptor
H&E: hemotoxylin and eosin
i.p: intraperitoneal
IL-1β: interleukin-1 beta
IL-6: interleukin-6
INFγ: interferon gamma
LBW: low birth weight
LIC: lipid interstitial cells
LRAT: lecithin:retinol acyltransferase
MIP-2: macrophage inflammatory protein-2
NF-κB: nuclear factor kappa-B
P: postnatal day
PDGFRα: platelet derived growth factor receptor alpha
PKC: protein kinase C

PcG: polycomb group

qPCR: quantitative polymerase chain reaction

RA: retinoic acid

RAE: Retinol Activity Equivalents, a term referring to vitamin A activity

RALDH: retinaldehyde dehydrogenase

RAR: retinoic acid receptor

RBP: retinol binding protein

RDH: retinol dehydrogenase

RE: retinyl ester

REH: retinyl ester hydrolase

ROH: retinol

RP: retinyl palmitate

RXR: retinoid X receptor

SD: Sprague Dawley

SEM: standard error mean

SP: surfactant protein

STRA6: stimulated retinoic acid gene 6

Thy-1: Thymocyte antigen 1

TNFα: tumor necrosis factor alpha

TTR: transthyretin

VA: vitamin A

VAD: vitamin A deficient

VAM: vitamin A marginal
VARA: vitamin a, retinoic acid

VEGF: vascular endothelial growth factor

VLBW: very-low birth weight

αSMA: alpha-smooth muscle actin
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Chapter 1

LITERATURE REVIEW

1.1 General Introduction to Vitamin A

1.1.1 History of Vitamin A

Vitamin A (retinol) is an essential micronutrient for all vertebrates, which collectively encompasses a group of retinoids that includes retinol and its metabolites: retinal, retinyl ester (RE), retinoic acid (RA), carotenoids, and \( \beta \)-carotene. The discovery and characterization of vitamin A spanning over a period of 130 years is accredited to the contribution of numerous scientists. Even before these discoveries, early Egyptians around 1500 B.C were using ox liver, unknowingly at the time, which is rich in vitamin A, to cure night blindness [1]. Based on investigations conducted by Mendel and Osborne in 1911 on the importance of milk for growth in young rats [2], F. G. Hopkins demonstrated a year later that unknown “accessory factors” other than proteins, fats, carbohydrates, and salts found in milk were necessary for growth of these rats. Hopkins later received a Nobel Prize for his work. A factor was then discovered in 1913 by biochemists E. V. McCollum and Marguerite Davis at the University of Wisconsin as an ether-soluble fraction present in certain fats and tissues, such as butter and egg yolk, that was required for growth and survival in young rats [3]. This discovery lead to further investigations of vitamin A and its importance not only in growth and survival, but also its essentiality for vision, reproduction, immunity, and cellular differentiation amongst other important functions. Through incremental exploration, the term “accessory factors” progressed to the term “fat-soluble A” and then to the final nomenclature, now known as “vitamin A,” by 1920 [4]. The characterization of
vitamin A was then elucidated in the 1930s as all-trans-retinol, which, in tissues, is mainly present in its stored form as RE. It was shown through metabolic studies that β-carotene can be converted to retinol in the intestine and have the same biological activity as dietary retinol or RE [5]. In the 1940s, the acid form of vitamin A, retinoic acid, was synthesized [4, 6] and shown to have essentially all of the properties of retinol except for vision [5, 7]. By the 1950s, the essential role of 11-cis-retinaldehyde in vision, also known as retinal, led to a Nobel Prize for this research [8]. Interestingly, these various discoveries provided the basis for further investigations on functions and properties of VA.

1.1.2 Functions and Properties of Vitamin A

Vitamin A (all-trans-retinol; VA) and its natural and synthetic analogs are collectively known as retinoids. There are different forms of retinoids present in the body. The natural forms of retinoids include retinol, retinal, RA, water-soluble metabolites and pro-vitamin carotenoids. The most abundant of these in mammals are retinol and its storage form, REs. [9]. Retinoids are essential for immune maintenance and function, reproduction [10], cell and tissue proliferation and differentiation, vision [6], maintenance of epithelial tissue, and embryonic development [1, 7, 10].

Retinoids are most stable in the all-trans configuration [1]. All retinoids contain the chemical structure of four isoprenoid units joined from head-to-tail consisting of three parts: a trimethylated cyclohexane ring (β-ionone), a conjugated tetraene side chain, and a polar carbon-oxygen functional group [1, 9]. Retinol is most often in storage form in tissues. The esterification of retinol protects the hydroxyl group from oxidation [1]. Esters of palmitic acid, oleic acid, stearic acid, and linoleic acid are the most abundant forms of RE in the body. Despite their abundance, they serve no known biological function other than for storage [9]. Retinal’s
primary role is in the eye where it is necessary as rhodopsin for vision and serves as the
intermediate for the formation of RA from retinol. Although maintained at low concentrations in
tissues, all-trans RA, the most active form of vitamin A, is responsible for the transcriptional
regulation of genes necessary for the diverse multifunctional properties that vitamin A is known
for, including its role in lung development [1, 9, 11].

1.1.3 Vitamin A Deficiency and Toxicity

Vitamin A-sufficient plasma concentrations range from 0.7 – 2.8 µmol/L. Plasma
concentrations less than 0.35 µmol/L are considered vitamin A deficient [12]. Vitamin A
deficiency (VAD), depending on its severity, can result in fetal malformations during gestation,
often of the neural cord, limbs, and craniofacial structures as well as cardiovascular related
abnormalities seen in the anterior cardinal veins and sinuatrial venous. These abnormalities could
ultimately lead to death or fetal resorption [10]. Interestingly, these outcomes are similar to those
produced by excess vitamin A, suggesting that either a deficiency or excess of vitamin A or its
products at critical stages of development are teratogenic [13-16]. In infants and young children,
VAD also affects growth and survival. These conditions include xerophthalmia which can lead to
blindness; impaired immune system resulting in increased susceptibility to infections; and risks
such as severe diarrhea, measles, and malaria, stunted growth, and chronic lung disorders [1, 17].

Each year, approximately 9 million children under five years of age die worldwide; most
of these deaths occur in developing countries. Among these deaths, about one-third are attributed
to maternal and child malnutrition [18]. VAD has been recognized as a public health issue in
developing countries. Malnutrition of this micronutrient affects approximately 127 million pre-
school aged children, 7 million pregnant women [17] and accounts for approximately one-third of
the total deaths of children 5 years of age and younger [19, 20]. Over 120 countries, mainly
located in Africa and South-East Asia [21] (Fig. 1.1), are affected by VAD. The World Health Organization (WHO), The United Nations Children's Fund (UNICEF), and other global health organizations have designed and carried out several new and ongoing trials worldwide in which VA supplements have been given to pregnant and breastfeeding mothers, infants and/or young children. A typical form of supplementation includes a high dose of VA given orally in the form of concentrated retinyl palmitate, at a dose of 50,000-200,000 IU (15-60 mg RAE), depending on age and number of doses [17]. These clinical trials, in which supplements of VA have been administered to children 6 months to 5 years of age, have been shown to reduce child mortality by 23%, with reductions in all-cause as well as measles and diarrhea-associated mortality [19].

Despite public health programs aimed at eradicating VAD, a survival benefit has not been demonstrated in infants under 6 months[20]. Although VAD is rare in the U.S., physicians understand that alcoholics, the elderly, pregnant women, infants and young children are susceptible to experience some level of VAD; pediatric patients, because of their high demand for VA due to rapid growth and development, are especially recommended to consume enough VA to insure adequacy.
Figure 1-1: Heat map of vitamin A deficiency prevalence of preschool age children.

VAD has been recognized as a public health issue in these developing countries mainly in Africa and South-east Asia, affecting approximately 130 million pre-school aged children (3-5 years of age).
While VAD is detrimental to the body’s systems, so is VA toxicity, also called hypervitaminosis A. Hypervitaminosis A can become critical and even fatal depending on the level of toxicity. Signs of acute VA toxicity include pseudotumor cerebri, anorexia, anemia, rough and dry skin, and bone pain, while prolonged elevation of VA may lead to irreversible organ damage, especially of the liver [7]. As is the case with VAD, manifestations of VA toxicity during pregnancy can cause teratogenic birth defects such as malformations of the face, nervous system, heart, and thymus gland [7, 22]. Hypervitaminosis A occurs when plasma retinol exceeds the availability of plasma retinol-binding protein (RBP) to bind it, leading to unbound and elevated levels of free retinol in plasma, thus allowing for increased exposure of cell membranes and organelles to retinol, formation of excess retinoid metabolites and abnormal activation of retinoid receptors [23]. Although there is no antidote, hypervitaminosis A may be slowly reversible by restriction of VA consumption, with remediation of symptoms within a few hours to a few months. However, a patient with chronic hypervitaminosis A was reported to still experience symptoms of increased intracranial pressure and elevated serum retinol and RBP levels almost 2 months after cessation of VA supplementation [24]. Dietary vitamin E has displayed some therapeutic benefit in treating VA toxicity [25], and 2-hydroxypropyl-β-cyclodextrin has shown to be safe and useful to solubilize VA, which allows for urinary excretion as a method of ridding retinol from the circulation [26]. However, more research is needed to provide therapeutic interventions for chronic hypervitaminosis A.

1.2 Vitamin A Metabolism

1.2.1 Transport and Metabolism of Retinoids

No animal species is capable of de novo synthesis of VA. VA is consumed through diet
as retinyl esters (RE) from meat sources or mainly in the form of provitamin A carotenoid β-carotene from plant sources. Carotenoids enter enterocytes in the small intestine by passive diffusion and undergo cleavage in order to form retinal in the intestine, which is then reduced to retinal, whereas dietary RE are enzymatically hydrolyzed to retinol by pancreatic triglyceride lipase and phospholipase B in the intestinal lumen and complexed with bile acids prior to uptake into enterocytes [1, 5]. Retinol is then bound to the cellular retinol-binding protein type II (CRBP-II), enzymatically re-esterified by lecithin:retinol acyltransferase (LRAT). To meet the demand of extrahepatic tissues, RE is incorporated into chylomicron complexes consisting of triacylglycerides, phospholipids, other fat-soluble micronutrients, and apolipoproteins, and secreted from the enterocytes into the intestinal lymph. While in the circulation, chylomicrons undergo rapid hydrolysis of triacylglyceride, forming a chylomicron remnant [1]. The chylomicron remnant is cleared by hepatocytes and RE is subsequently hydrolyzed back to retinol and either re-esterified and transferred to hepatic stellate cells for storage or, based on extrahepatic tissue needs, the free retinol can be bound to RBP and carried into the plasma for uptake by other tissues. RBP facilitates optimal retinoid transport, metabolism and retention as well as protects the cellular environment from VA toxicity. In order for transportation in plasma, the retinol-RBP complex binds to a serum carrier, transthyretin (1:1), which prevents excessive glomerular filtration of retinol [5]. Membrane receptors for RBP such as stimulated by retinoic acid 6 (STRA6) present on target cells of extrahepatic tissues mediate the uptake of retinol. Once in target cells, retinol either undergoes esterification for storage or oxidation by retinol dehydrogenases to form retinal. Further irreversible oxidation by retinal dehydrogenases forms RA. RA (all-trans-RA or 9-cis RA) bound to cellular retinoic acid-binding protein 2 (CRABP2) transports RA to the nucleus and activates RA receptors, RAR and RXR. All-trans-RA binds to RAR with high affinity while 9-cis RA mainly activates RXR. CRABPs, similar to RBP, facilitate the transport and protection of their ligand; however, CRABPs, present in both the
cytosol and nucleus of cells, facilitate nuclear transport of RA. As a result of ligand-induced activation of RA receptors, receptors undergo conformational changes that result in dimerization of RAR/RXR, or RAR/RAR bound to retinoid responsive elements, RARE or RXRE, in the DNA of responsive genes [1, 27]. This in turn allows for the binding of the RNA polymerase II complex, thus enabling the activation of the target gene and transcription.

Because of the potent regulatory properties of RA, tissues have very low levels of RA, usually 100 to 1000 times more than that of retinol [9]. The metabolism of retinol to RA during development and in the adult only occurs in some cell types [27]. Tissue regulation of RA occurs by both retinal synthesis and RA catabolism. Cytochrome P450 enzymes are major contributors to the terminal oxidation of RA by converting excess RA into polar metabolites, such as 4-oxo-at-RA, 18-hydroxy-at-RA and 4-oxo-9-cis-RA, and then to water soluble compounds that are eliminated from the body as biliary metabolites [28].

The transcriptional properties of activated RARs through RA binding include gene repression, activation, or transrepression, which can occur through association of RARE and non-RARE binding sites as well as nongenomic activation [5]. The activation of the transcription of genes that possess RARE’s result in a direct response to RA known as the “primary response”; whereas genes that do not possess RAREs results in an indirect response to RA activated gene expression known as the “secondary response”. Some direct responses of RA involves the cell lineage-specific, epigenetic modifications due to receptor activation of RXR/RAR that occur through the interaction of various proteins including epigenetic gene silencers known as polycomb group (PcG) proteins [27]. A secondary response is a result of direct target genes of RA possessing different transcription factor genes that transcriptional activate their target genes, thus causing an indirect transcriptional response to RA [27]. One example of regulations on gene expression independently of an RARE includes the transrepression of activating protein 1 (AP1) and modification of nuclear factor kappa-B (NF-κB). AP1 controls cellular differentiation,
proliferation, and apoptosis while NF-κB induction is importation for an appropriate immune and inflammatory response [5]. Modulation of protein kinase C (PKC) activity is an example of RA action through nongenomic mechanisms where RA directly binds PKC isozymes thus decreasing PKCα activity [5]. Like AP1, PKC regulates cellular proliferation, differentiation, and apoptosis, as well as tumorigenesis. As a result, RA is known to act as two-edged sword where it contains the properties of both proliferative and apoptotic cellular activity as well as promote or suppress an immune response. Consequently, RA not only plays a role in various physiological functions such as limb, central nervous system, and lung development, but also under pathological conditions where RA has been used to treat cancer, skin diseases, and disorders resulting from premature birth [5] seen in the cases of leukemia, acne, and low-birth-weight related chronic lung disorders.

1.2.2 Retinoid Homeostatic Genes

VA metabolism involves key enzymes that are responsible for the storage, trafficking, cellular uptake and the oxidation of retinoids (Fig. 1.2). The liver is the major site for the body’s reserve of VA, accounting for approximately 80% of retinol in the VA-adequate state, while the remaining 20% of retinol is accounted for in extrahepatic tissues such as the lung and kidney. Although the liver is a major storage organ for VA, the lung and kidney also have the capability of storing VA as RE and play a major role in maintaining VA homeostasis. Lecithin:retinol acyltransferase (LRAT) is responsible for the esterification of retinol which allows for storage of RE and maintenance of VA homeostasis [29]. LRAT enzymatic activity and expression are sensitive to VA status, especially in the liver and lung [30]. In the 1970s, it was shown that there exists a cell specific RBP receptor on the retinal pigment epithelium and on intestinal epithelial cells. It was not until 2007 that Kawaguchi and co-workers identified a transmembrane cell
surface receptor for RBP, STRA6, as responsible for retinol removal from the retinol-RBP complex in plasma and transport of retinol across plasma membrane for cellular uptake [31]. STRA6 is highly expressed in the brain, eye, testis, kidney, spleen, and female reproductive tract [31-33] as well as the lung [34]. Similar to LRAT, STRA6 expression is highly regulated by VA [33]. In fact, LRAT enhances STRA6 cellular uptake of VA while STRA6 works cooperatively with LRAT to increase RE accumulation [31, 35]. Both are significantly increased in expression level in the neonatal rat lung after VA supplementation [34].

The balance of maintaining VA homeostasis also involves the catabolism of RA. Retinol undergoes double oxidation by retinol dehydrogenase (RDH) and retinal dehydrogenase (RALDH), respectively, to form RA. As mentioned in section 1.2.1, cytochrome (CYP) P450 enzymes are responsible for the terminal oxidation of retinol and RA into inactive polar metabolites. Comparable to LRAT and STRA6, the expression of CYPs is also highly regulated by VA status. Ross et al [34] have shown that acute treatment with RA and synthetic analogs significantly increases expression of CYP26 family genes in the liver and lungs of adult and neonatal rats.

Megalin is a large transmembrane, multi-ligand protein that is important for recycling of retinol, among other vitamins such as vitamin D and vitamin B12 [36-38]. This endocytic receptor is expressed in a number of tissues including the kidney, lung, eye, and intestine. In rats, the kidney contributes to approximately 50% of the total circulating retinol-RBP pool by preventing urinary loss of retinol [36]. The kidney is also able to produce RBP. Studies have shown that, RBP synthesis is decreased in megalin knock-out mice [36]. Therefore, megalin is also necessary for RBP synthesis, furthermore confirming the importance of megalin in maintaining vitamin A homeostasis. Further research is needed to better understand the regulation of megalin by VA, and possible connections to STRA6, LRAT, and CYP26s.
Retinol is absorbed in the small intestine, incorporated into the liver, esterified by LRAT, and stored in the hepatic stellate cells. Based on extrahepatic tissue demand of VA, retinol is hydrolyzed and bound to RBP, and is transported through circulation as a retinol-RBP complex bound to serum carrier, transthyretin. Cellular uptake of retinol into extrahepatic tissues such as the lung and kidneys occurs through RBP receptors STRA6 and megalin respectively. Retinol is moved intracellularly via CRBP and either esterified by LRAT for storage or undergoes double oxidation to form RA by RALDHs. RA is translocated to the nucleus bound to CRABP to carry out transcription or undergoes terminal oxidation through CYP450 enzymes such as CYP26A1 and CYP26B1.
1.2.3 Neonatal Vitamin A Metabolism

Vitamin A is a necessary macronutrient for overall development, however there is limited knowledge on vitamin A metabolism in neonates. Like adult, the main storage organ for VA in neonates is the liver. However, extrahepatic concentrations of retinol in neonates are higher than that of adult extrahepatic tissues. A recent study by Tan et al [39] showed that, in neonatal rats compared to adult rats, extrahepatic tissues played a significantly greater role in the clearance of chylomicron RE from plasma. It is speculated that this higher distribution of retinol in extrahepatic tissues is an adaptive mechanism to insure vitamin A adequacy in all organs, which may be necessary for the rapid development of organs such as the lung or immune system. Neonatal rats also have a significantly higher recycling number (the number of times retinol recycles between plasma and tissues before undergoing irreversible oxidation) than adult rats and are in constant demand for retinol with dynamic transfer of retinol between plasma and tissue. It is speculated that the increased demand for VA in neonates may also play an important role in both maturation and maintenance of innate and adaptive immunity [40], especially since neonates are vulnerable to various infections. It is interesting to note that the retinoid homeostatic genes LRAT, CYP26, STRA6, and megalin are all expressed in neonatal rat tissues [41, 42]. Therefore, despite the immature state of the neonate, they are prepared for important processes necessary to maintain VA adequacy.

1.3 Supplement use of Vitamin A in Dietary Intake

The dietary requirement of VA established by the Institute of Medicine is based on a factorial model that allows for adequate storage of VA in adults, and is then scaled down for body weight in children and adolescents. The recommended intake for newborns is based on the mean
concentration of VA in breast milk of well-nourished mothers [43]. The recommended dietary allowance (RDA) is calculated using the Retinol Activity Equivalent (RAE), which is a unit of bioactivity where one µg RAE is equal to 1 µg of all-trans-retinol, 2 µg of all-trans-β-carotene in oil, 12 µg of β-carotene in foods, and 24 µg of other provitamin A carotenoids in foods [43]. The RDAs for infants and young children (newborn – 18 yrs.) range from 400 – 900 µg RAE/d, whereas in adults it is 700 µg RAE/d and 900 RAE/d for women and men, respectively.

Populations susceptible to VAD are children, mothers, and the elderly, especially those from a developing country where malnutrition is prevalent. In order to combat VAD, supplementation of VA is administered to help meet the nutritional requirement for VA and maintain adequate plasma retinol concentrations. The various routes of administration are described below.

1.3.1 Parenteral Administration of Vitamin A

In term infants without malabsorption, VA is well absorbed enterally. However, enteral supplementation of VA to very-low-birth weight (VLBW) premature infants has not been effective in restoring plasma levels of VA due to the immaturity of their gastrointestinal system, and therefore has not improved VAD related outcomes [44]. Although clinicians fear the pain, discomfort, and risk of infection associated with injections, parenteral administration of VA has been shown to be more effective and provides a more rapid response in improving VA status, especially in VLBW premature infants with severe respiratory complications. Despite enteral administration being the preferred route of VA administration, parenteral administration of VA is widely used both experimentally and clinically.
Intramuscular administration

Intramuscular (i.m.) VA supplementation is a route of administration used clinically to treat in mainly preterm infants born at a very-low-birth weight with severe VAD. These infants are at risk for a variety of infections due malnutrition and immature immune system. Several different dosage regimens have been designed for this mode of administration of VA in order to achieve adequate VA plasma levels, but the optimal i.m. dose is still unclear. These dose concentrations used clinically range from 5,000 IU VA to 15,000 IU VA [44]. Although one i.m. administration of VA is short lived, subsequent doses have deemed to be effective in treating or preventing VAD. However, this route is painful and clinicians conclude that this route is not worth the modest benefit in respiratory outcomes [12, 44]. Consequently, i.m. supplementation of VA is sparingly used clinically, but has been used extensively in research laboratories on animal models [45].

Intravenous administration

Although commonly practiced clinically, especially outside of the United States, to treat VAD in preterm infants [44], intravenous (i.v.) VA supplementation has not shown much efficacy. Many researchers would suggest that this mode of administration is inadequate. There is no evidence that i.v. dose of VA improved plasma levels or body stores of VA in very low birth weight (VLBW) infants. Because VA absorbs to the tubing, it is more efficient to administer VA intravenously coupled with a lipid emulsion. Even increasing the VA content of i.v. fat emulsion from 910 IU/kg/day by two or three-fold (2700 IU/day) [12] in VLBW infants that received Vitlipid (VA in lipid emulsion in conjunction multivitamins in an amino acid/dextrose mix) [46], there was still no difference in plasma retinol concentration or in clinical outcome [47]. Since no
single i.v. preparation of vitamin A is available, it is not practical to increase the current intravenous dose. Further research is needed to directly compare i.m with i.v. administration of VA in VLBW infants in order to effectively combat VAD.

1.3.2 Intratracheal Administration of Vitamin A

The latest novel administration of VA is through inhalation. Because the lung is one of the main organs affected by VAD, physicians and researchers seek the most optimal way for preterm infants to achieve adequate concentrations in the lung. With inhalation, the lung is in direct contact and can readily absorb REs into cells and is metabolized. This form of therapy has been effective in animal studies [48] and in humans [49, 50]. In a preliminary study conducted by Edris et al [50], VAD preschool age Ethiopian children were treated with inhalation of two aerosol puffs of retinyl palmitate (6,000 IU) every two weeks for three months. This study resulted in improved VA status of treated children in comparison to non-treated children. Meanwhile, daily inhalation (18,000 IU of retinyl palmitate) for 3 months in adult smokers improved metaplastic lesions of the respiratory epithelium. Further research is needed to determine if these benefits would be displayed in infants with pulmonary disorders.

1.3.3 Oral Supplementation of Vitamin A

Unfortunately, young children and pregnant mothers of developing countries are a vulnerable population susceptible to VAD at some point. VA supplementation has been provided to pregnant mothers in hopes to promote a healthy pregnancy, term births, and VA sufficient maternal supply to offspring. Pregnant mothers of a compromised VA status show benefits in receiving an oral supplementation. Studies have shown that these women were
protected postpartum when treated acutely with 200,000 – 400,000 IU of VA [51]. Although VA has the capability of storage and maintenance of adequacy after supplementation for at least 6 months [9], this capability decreases as the body’s constant dietary demand for VA increases.

Another mode of intervention is a bolus dose of oral retinol palmitate (50,000) in children between 6 – 59 months [21], which has reduced mortality by about 30%, and was up to 68% in Indonesia if given one dose in newborns within 2 days of birth. Preterm VLBW neonates, however, may not benefit from oral supplementation because of possible inadequate protein synthesis of the liver and immaturity of their fat digestion capabilities [52]. As a result, the benefit of VA supplementation in neonates has not shown consistent results. However, in comparison to enteral supplementation of VA, oral supplementation appears to be the most favorable and effective route of VA administration in preterm and term infants, especially in conjunction with i.m supplementation of VA [44]. Further research is needed to better understand the dose requirement at various times of postnatal development.

**Vitamin A Used Concomitantly with Retinoic Acid for Supplementation**

Retinoids play important roles in various physiological processes. As a result, retinoids have implications of being used in forms of treatment for various metabolic and dermatological diseases, infections, as well as cancer. Therefore, various combinations of retinol in conjunction with other retinoids, both synthetic and natural, have been utilized to optimize the best benefit. Ross et al [34] have shown in rats that VA and RA together at a 10:1 molar ratio (VARA), work synergistically to up-regulate total retinol concentration in plasma and tissues as well as alter the expression of genes necessary to maintain VA homeostasis. In addition, this admixture increases total RE in neonatal rat lungs significantly more than VA or RA alone in comparison to the liver [45, 53, 54]. Even with as little as 0.5% of RA added to a VA oral dose still results in a
significant increase of RE in lungs. Interestingly enough, with only 2% of RA in VARA STRA6 expression significantly increased in neonatal lung [55].

1.3.4 Fortification of Vitamin A into Foods

To help maintain VA adequacy outside of oral supplementation of VA is the fortification of various common foods. Food fortification is one of the most cost effective ways to address worldwide micronutrient deficiency. Examples of these biofortified foods are rice, milk, cereals [20], and seasoning such as monosodium glutamate [17]. There has also been a big push for implementing VA in agriculture. In 2005, The UN World Food Program (WFP) and other supporters established fortification in flour plants in Afghanistan, Angola, and Zambia [56]. Education, funding, and more research will help expand the implementation of vitamin A-biofortification in plants.

1.4 LUNG STRUCTURE AND DEVELOPMENT

1.4.1 Lung structure and function

The lung is one of the most important organs in the body because it is essential for respiration, providing oxygen to meet the body’s demands. Humans have two lungs, where the left lung contains 2 lobes and the right lung contains 3 lobes. The total gas-exchange surface area spans between 50 - 100 m², which is approximately the size of a tennis court. Although the structure of the lung is mainly for respiration, it carries other functions important such as for immunity and various metabolic processes [57].
The lung is divided into two main zones based on function: the conducting zone (upper airway) and the respiratory zone (lower airway). The conductive zone delivers inhaled air from the mouth or nose, down the trachea towards the respiratory zone. The respiratory zone is the distal end of the airway responsible for gas exchange [58]. Generation numbers 1 – 23 define progressive branching of the pulmonary airways. The upper airways of the lung begin at generation 0 at the trachea, which then splits into bronchi. The bronchi divide to into lobar and then to segmented bronchi. The airways continues to branch out, becoming narrower and shorter, with less to no cartilage and an increase in number into the respiratory zone. Beginning at the nose, the conducting zone ends at the terminal bronchioles.

Transition of the conducting airways into the respiratory zone begins the gas-exchange region of the lung. [59]. The terminal bronchioles of the conducting airways divide into respiratory bronchioles located in the respiratory zone [59]. Although both zones are important for the overall functioning of the lung, the respiratory zone makes up majority of the lung tissue and carries out the main duty of the lung, respiration. The respiratory zone is spongy and made up of delicate tissue septa containing capillaries. These septa subdivide the airspaces to form alveoli. The alveoli first appear off the walls of the bronchioles at approximately generation 17. There are approximately 500 million alveoli in an adult lung [59] and these account for about 90% of total gas exchange. With increasing generation number more alveoli are generated, eventually completely lining the airway. Respiratory bronchioles and alveolar ducts are responsible for the remaining 10% of gas exchange.

The alveolar tissue provides the framework that closely separates the air from the blood. The separation allows for a distinction between compartments, but the layer of epithelial and endothelial cells are thin enough to allow for close contact between the air and blood for efficient, effective, and optimal gas exchange. The lining of the alveolar wall consists of two types of epithelial cells: type I and type II alveolar pneumocytes. Type I cells are thinner than type II cells
and cover up to 95% of the total alveolar surface; this is the shortest route for gas diffusion [60]. Type II cells are cuboidal in shape, exist in clusters, and contain lamellar bodies, which is the major site of surfactant production, storage and secretion. Surfactant reduces alveolar wall surface tension and the collapse of these airspaces during respiration, as well as participating in immune function.

1.4.2 Stages of Lung Development

The human lung is steadily and continuously developing beginning at approximately three weeks gestation until about 8 years of age [61]. The development of the lung is divided into two phases: growth and maturation. Lung growth is important for structural development, whereas lung maturation is important for functional development. Successful development of the lung involves the completion of both phases. Any disturbance or perturbation of these phases may result in respiratory complications and, possibly, fatality.

At approximately three weeks, the fetal lung begins as an outpouch of the foregut endoderm through paracrine signaling between the epithelium and surrounding mesenchyme. Several days later, two tubular structures that are the framework of the main bronchi are formed. By 6 weeks of gestation, the bronchial tree begins to expand with repetitive branching. The further maturation of the lung consists of 4 major stages of development (Fig. 1.3): pseudoglandular stage, canalicular stage, terminal-saccular stage, and finally the alveolar stage [57, 58, 60, 62]. The timing at which these stages occur is not exact and are approximations, but occur in the order listed.
Figure 1-3. Phases of lung development
**Pseudoglandular stage**

In humans, the pseudoglandular stage occurs within weeks 5 – 17 of gestation. During this period, the lung primitive airways are fluid-filled epithelial tubes lined with cuboidal epithelial cells resembling branching exocrine glands. At this stage, the lung is too immature to support any efficient gas-exchange properties.

**Canalicular stage**

With possible overlap of this stage into the pseudoglandular stage, the canalicular stage occurs at gestation weeks 16 – 25 and is characterized by canalization of the airways. During this stage respiratory branching continues to expand in length and diameter. The completion of this period ends at approximately airway generation 17, including respiratory bronchioles. Each respiratory bronchiole gives rise to alveolar ducts thus forming primitive alveoli. At approximately week 24 of gestation, vascularization and angiogenesis take place, where the interstitial tissues separate the rapidly increasing number of capillaries from the respiratory epithelium. Airway epithelial cells are differentiated into peripheral squamous cells and proximal cuboidal cells. Despite the further development of the lung, premature birth at this stage will be of a great disadvantage to the fetus because of the very low diffusing capacity of gas exchange as a result of the large distance between the alveolar lumen and the capillary lumen.

**Terminal-saccular stage**

The terminal-saccular stage of lung development occurs from 24 weeks of gestation to birth. At this stage, the respiratory epithelium undergoes significant thinning due to apoptosis and differentiation of the mesenchymal cells, and the capillaries are pushed into the alveolar sacs,
thus greatly improving the potential for independent gas exchange. The capillaries proliferate rapidly and come into closer proximity to type I cells forming a complex, double, parallel network surrounding the saccules, also referred to as the primary setae. Toward the end of this stage, the fetal lung can support independent gas exchange.

Surfactant synthesis and storage begins in the lamellar bodies of the differentiate type II cells to sustain gas exchange with collapsing of the airspaces.

**Alveolar stage**

The final stage of alveolar growth occurs late in fetal life to about 8 years of age [61]. By 36 weeks of gestation, 10 – 15% of the adult number of alveoli is present, forming the majority of gas exchange surface area, and the number continues to increase until as late as 8 years of age. The primary setae give rise to secondary septa, which also contain a double capillary network. New alveoli are generated by subdivision of the secondary septa, this process is known as alveolarization. The alveolar stage includes microvascular maturation in which the double capillary networks of the septa are restructured to form a mature single network [63]. Although alveolarization ends at age 8, the human lung can regenerate alveoli after lung injury as a repair mechanism [64, 65].

**1.4.3 Immune Properties of the Lung**

Beginning in the upper airways is a complex network of mechanical barriers and various cellular and immunologic mechanisms along the trachea and in the nasooropharynx that help in the protection of the lower airways from potentially harmful inhaled particles and microorganisms. However, small particles may be able to pass through the conducting areas to
reach the alveolar surface, and therefore be harmful. Although mucociliary clearance and
coughing are defense mechanisms against these potentially harmful products, surfactants serve as
another line of defense in the respiratory zone, discussed later in this chapter. An impairment of
any of these protective mechanisms may cause an increase in inflammation of the lung.

The conducting airways are covered by a mucosal surface. These mucosal cells undergo
turnover approximately every 7 days [66]. Lymphocytes and plasma cells are distributed across
these airways in the submucosa and lamina propria areas, as well as surface macrophages. All of
these cells initiate immune response when needed. In response to possible debris or contaminated
absorbed fluid entering the lung, there is lymphatic clearance of the fluid, ingestion by surface
macrophages, and brush border cell absorption of this fluid. The integrity of the gap junctions
between the cells prevents submucosal deposition or penetration of airway antigens into the body.

Moving into the lower airways, the epithelial layer becomes thinner and the cilia of
epithelial cells become shorter. The goblet cells and mucous glands that were present in the
upper airways are no longer present as airway generations approach more alveoli. However,
there are other secretory cells, phagocytic cells, and lymphocytes present as the next line in
defense. Research has shown that bacteria that are deposited on the alveolar surface are rapidly
ingested by alveolar macrophages within 30 minutes of capture [66]. Every one macrophage
works for and protects approximately three alveoli. In normal bronchoalveolar lavage (BAL)
fluids, the principal antibody is immunoglobulin G (IgG) as well as IgA and IgM in lesser
amount. It is speculated that once bacteria enter the macrophage, the microphage becomes
activated and coordinates with T lymphocytes, cytokines, and immune cells, prolonging
activation and thus improving phagocytic and intracellular bactericidal capabilities. The captured
microbe is killed, contained, or may require the help of recruited macrophages through
chemokines such as IL-8 and TNFα.
In addition to macrophages serving as “guards” of the lung, surfactant proteins also have immunoregulatory functions. Surfactant proteins are known for their ability to reduce surface tension at the respiratory interface of the lung, but have also been shown to function as a host defense. Mainly composed of phospholipids, there are four types of surfactant proteins: surfactant protein A (SP-A), surfactant protein B (SP-B), surfactant protein C (SP-C), and surfactant protein D (SP-D). SP-A and SP-D are the primary surfactants that possess host-defense functions by binding to bacteria and viruses, functioning as opsonins to enhance uptake of these cells with pathogens, activation ligands, and regulators of cell-surface receptors expression for microbial recognition. Surfactant proteins also regulate the production of inflammatory mediators such as tumor necrosis factor (TNF) and interferon gamma (IFNγ) [67]. In addition, surfactant also plays a role in enhancing the clearance of apoptotic cells by macrophages. Recent studies have shown that surfactants may also link innate and adaptive immunity in the lung through dendritic and T cell functions, where SP-A inhibits dendritic cell maturation and both SP-A and SP-D suppress T-cell activation in order to initiate an inflammatory response cascade that may be either helpful or damaging to the lung [67].

1.5 RETINOIDS IN LUNG DEVELOPMENT AND FUNCTION

1.5.1 Retinoid Metabolism in the Lung

Although the liver is the principal storage site for VA, other organs, including the lung, are capable of storing VA [68-70]. VA stores are high in fetal lung and decrease toward term, and are almost depleted in newborns [69, 71]. Depletion of these stores continues into early postnatal period, suggesting that the developing lung may depend on these local stores of VA during the period of active growth and differentiation that is associated with rapid alveolarization. Evidence
from animal models of postnatal VA supplementation suggests the rapid uptake of chylomicron REs into lung [72], as well as uptake of retinol from holo-RBP [39]. STRA6 is expressed in lung tissue and modestly up-regulated after treatment with RA [41]. CRBP1 is also expressed [73], as is LRAT, which likely accounts for the significant capacity of the lung to store RE [41].

Following cellular uptake by STRA6 (Fig. 1.4), retinol is bound to CRBP1 and deposited in a cell type similar to that of stellate cells in liver, known in the lung as lipid interstitial cells or lipofibroblasts, for storage [74], or it may be further oxidized to form retinal and RA through the actions of retinol and retinaldehyde dehydrogenases, respectively. Excess or unutilized RA can be further oxidized to polar metabolites for excretion by CYP26 enzymes, particularly CYP26B1 in lung tissue [41, 75].

Prematurely born animals, deprived of adequate stores of VA in the lungs before birth, may be susceptible to the adverse pulmonary effects of VAD. The fetal lung stores of VA can be augmented by prenatal maternal VA supplementation [76]. Conversely, prenatal interventions, such as maternal glucocorticosteroid treatment, can deplete the lung of VA [77]. In rodent models, retinoid deficiency caused by dietary restriction of the mother or offspring results in impaired septation, altered pulmonary tissue composition, and reduced elasticity [78, 79].
Cellular uptake of retinol into the lung from circulation occurs through transmembrane protein receptor STRA6 and undergoes double oxidation to form all-trans RA. RA binds and activates nuclear receptors in order to carry out transcription of lung specific genes such as extracellular matrix components, activate growth factor signaling pathways, enhance the expression of homeobox genes which all are important for the induction and formation of alveoli through secondary septation.

Figure 1-4. Vitamin A metabolism in lung.
RA, as a transcriptional regulator, binds to its nuclear receptors, which activates these nuclear RA receptors that then heterodimerize and bind to RARE in genes expressed in the epithelium of pulmonary tissue. RA is needed for proper lung development, maintenance, and repair [80]. Lung-specific genes regulated at least in part by retinoids include growth factors, homeobox genes, and extracellular matrix genes that activate cellular differentiation, regulate axial patterning, and promote secondary septation, respectively. This ultimately leads to alveolarization and vascularization, both of which necessary for increasing lung surface area for optimal gas exchange [81, 82].

RA treatment of neonatal rats exposed to hypoxia or treated with dexamethasone, both of which cause alveolar simplification, resulted in improved septation, suggesting an important role for RA in postnatal lung maturation [82-84]. In preterm and LBW infants, poor VA status may contribute to the development of chronic lung disease, such as bronchopulmonary dysplasia (BPD), which is a major cause of morbidity in preterm infants, associated with exposure to high oxygen concentrations, mechanical ventilation, and inflammation, and predisposing to chronic lung disease [81, 85]. Poor VA status (plasma retinol concentrations < 0.35 µmol/L) during the first month of life in LBW infants was reported to be related to increased risk of developing BPD and long-term respiratory disability [86]. Retinol therapy slightly but significantly improved outcomes in extremely LBW infants [87, 88].

1.5.2 Retinoids in Alveolar Septation

During the saccular stage of lung development, the airways expand to form sacculi. New ridges rise up from the primary septa to form secondary septa. These newly formed secondary septa subdivide the sacculi to form smaller units of airspace known as alveoli, in the process called septation. In humans, septation mainly occurs during the last stage of lung development,
the alveolar stage, which occurs between 36 weeks of gestation to early childhood, which in
rodents corresponds to postnatal day 4 – 14. Septation also referred to as alveologenesis or
alveolarization, has been shown to be a very integrated process that relies on cooperative
interactions between interstitial, epithelial, and vascular compartments of the lungs. RA
signaling through its activation of RA receptors (RARs) activates the growth factors that, in turn,
regulate transcriptional factors important in for promoting alveolarization.

RA storage granules are mainly in the fibroblastic mesenchyme surrounding alveolar
walls and have been shown to peak prior to alveolar septation to help prepare further
development [80, 89]. Synthesis of elastin by these alveolar interstitial fibroblasts, necessary for
initiation of secondary septation, peaks around the same time as RA storage in the lung.
Elastogenesis is important for alveolar septation and for normal expiratory lung recoil [89].
Several studies have shown that exogenous RA increases elastin synthesis and other extracellular
matrix components such as αSMA and collagen[89]. Deletion of RARα [90] or even mild VAD
[91] resulted in less elastin, fewer alveoli, and delayed alveolarization. Treatment with RA has
been shown to induce alveolar formation in rodents with impaired alveologenesis ([65, 92-95].
Lipofibroblast are stimulated by prostaglandins produced by type II cells, which provide a
substrate for type II cells to produce surfactant. Surfactant is important during septation to
provide stability to alveoli. Surfactant production also increases during the septation period, and
has been shown to be regulated by RA [96, 97]. Evidence that retinoids play a role in lung
development is well established and retinoids have therefore been tested as a form of therapeutic
intervention to prevent or decrease the incidence of various chronic lung disorders.
1.5.3 Retinoids in Lung Tissue Repair

It is well established that RA is essential for proper fetal and postnatal lung development. Results of clinical trials using VA supplementation to improve quality of life and prevent mortality in VAD populations have shown to be effective [21, 47, 98-102]. Unfortunately, the populations most affected are newborn to preschool age children, an age period in which the lung is still developing. It is understood that many chronic lung diseases and infections are associated with low levels in VA. Several studies have speculated and supported the idea that RA stimulates alveoli formation, which has led clinicians and researchers to wonder whether retinoids can be used pharmacologically to treat human lung diseases.

Bronchopulmonary Dysplasia

Lung disease such as BPD, asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis of the lung affect both children and adults, but are more commonly prevalent in undernourished children. BPD is the most common form of chronic lung disease in preterm infants and has been associated with low levels of plasma retinol. Various factors such as oxygen toxicity, airway infection due to prolonged tracheal intubation, or hyaline membrane disease influence development of BPD. As a result of this trauma to the lung, there is an arrest in lung maturation and inhibition of further healing [103].

As mentioned, VA is important for the proper development of the lung and researchers have shown therapeutic benefits in treating lung injury in rodent models. In rodents, RA treatment has been shown to prevent alveolar hypoplasia and increase alveoli number in the neonatal lung [65, 92], regenerate alveoli in adult lungs [104, 105], attenuate hyperoxic lung injury in neonates [54, 95], and partially rescue failed septation induced by glucocorticosteroid in
rodent models [94]. VAD alters the extracellular matrix composition of the lung, and Esteban-Pretel et al have shown that RA administration reverses this disturbance [106]. Although RA did not effectively treat cigarette smoke-induced emphysema in guinea pigs, it prevented the development of alveolar destruction by cigarette smoke in rats [107]. While much work has been done in animal models to test the potential benefits of RA in repairing lung injury, various studies have displayed this same benefit in humans, especially preterm infants who are susceptible to a wide variety of lung disorders. Clinical prevention and treatments of BPD and other lung disorders with the use of retinoids are summarized in Table 1.1.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Treatment</th>
<th>Result</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchopulmonary</td>
<td>Intramuscular retinyl palmitate 2000 in very low birth weight neonates (26 - 30 weeks gestation) on postnatal day 4 and every other day for 28 days.</td>
<td>Improved VA status, decreased incidence of BPD, lung infection and associated morbidity by promotion of regenerative healing from lung injury.</td>
<td>[100]</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>Intramuscular administration of 5000 IU of VA three times a week for four weeks.</td>
<td>Reduced VAD, decreased the risk of chronic lung disease in extremely-low-birth-weight infants</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>Addition of intramuscular injections of VA to routine pulmonary care of extremely low birth weight infants (early surfactant utilization and nasal continuous positive airway pressure)</td>
<td>Post-VA compared to Pre-VA decreased the incidence of moderate to severe BPD by 11%.</td>
<td>[109]</td>
</tr>
<tr>
<td>Cystic Fibrosis</td>
<td>Oral supplementation of</td>
<td>Attenuate inflammation</td>
<td>[99]</td>
</tr>
<tr>
<td><strong>Acute Respiratory Infection</strong></td>
<td><strong>β-carotene (maximum 50 mg/day) for three months and 10mg/day for another three months in adolescents between 6 - 17 years old.</strong></td>
<td><strong>and acute pulmonary exacerbations associated with CF</strong></td>
<td><strong>VA supplementation (200,000) at the start and end of 6 months in children aged 12 to 54 months.</strong></td>
</tr>
</tbody>
</table>
Chapter 2

DESIGN AND METHODOLOGY

2.1 Animal Model

2.1.1 Sprague Dawley Rats

The strain of rat used for the various studies described in later chapters is the Sprague Dawley rat. Sprague Dawley (SD) rats are an outbred multi-purpose breed of albino rats that have been used extensively in scientific research. SD rats are known for their calmness and ease of handling. In addition, these rats have been characterized previously for their response to dietary VA. Ross et al [45, 111-114] have shown that SD rats fed a VA-marginal or -deficient diet resembles that observed in humans of a VA compromised status. Therefore, these rodents have graded, reduced levels of plasma retinol and tissue VA, similar to values found in human population with low VA intake.

Procedures were approved by The Institutional Animal Use and Care Committee of The Pennsylvania State University. Rats were housed in a room maintained at 22°C with a 12:12-h dark-light cycle, and food and water were freely available. In each study, a VA-marginal (VAM) purified diet (0.35mg retinol equivalents/kg diet, AIN-93G diet prepared by Research Diets, New Brunswick, NJ) as previously described [39] was fed to mating-adult SD female and male rats.
2.1.2 Mating and Feeding of Dams and Offspring

SD adult female and male rats were fed a VAM diet during mating (Fig. 2.1). Male and female rats were co-housed for _14_ days. After mating, pregnant dams were maintained on VAM diet to reduce the transfer of VA in milk from mother to pups during lactation [115]. This same diet was fed from weaning to adulthood. In this way, VA status of pups fed VAM diet resembles the status of at-risk infants in developing countries or low birth weight infants in the US. At birth, pups were randomly assigned to age of observation (P1, P4, P10, and adult) and treatment groups, either VARA supplementation or placebo (canola oil).
Design showing generation of SD offspring by mating female and male rats fed marginal VA diet, which was maintained throughout gestation and postnatally.

Figure 2.1. Mating of SD rats fed vitamin A marginal diet.


2.2 Dose Preparation

VA in the form of all-trans-retinyl palmitate was generously provided by Vitamin Angels (Santa Barbara, CA) or purchased from Sigma-Aldrich (St. Louis, MO). All-trans-RA was also purchased from Sigma-Aldrich. VARA was a mixture of VA and one-tenth the amount of RA [39]. Dosage for VA was selected to resemble the amount (50,000 IU; equivalent to 15 mg retinol per approximately 2.5 kg), that has been shown in VA supplementation trials and clinical interventions in human newborns to reduce morbidity and mortality associated with VAD [20, 101, 116, 117]. By converting international units to mass units with the factor of 0.548 µg retinyl palmitate (RP)/IU, we calculated the VA doses to be 6 µg retinol/g (or 10.96 µg RP), which was scaled to body weight of neonatal rats. The dose of RA was equivalent to 0.6 µg/g, which is similar to the dose previously shown to promote lung septation in neonatal rats when injected daily during the period of lung septation [65, 92, 93]. We therefore calculated 500 µg RA ip/kg body weight and adjusted that amount to take into account that only about 80% of dose is absorbed after oral delivery. As described in Section 1.3.4, VA and RA were mixed at a molar ratio of 10:1 at a concentration of 0.05 M and 0.005 M, respectively. At this concentration, 0.4 µl were administered per g body weight to achieve the desired doses. Canola oil was used as placebo (control), the solvent for VARA. Retinoids were stored in -20°C and protected from air and light to prevent oxidation.

Dose delivery

The timing of oral dose administration is shown in Fig. 2.2A and Table 2.1. The first dose was delivered on postnatal day 1 (P1; equivalent to 32 weeks gestational age in human infants regarding lung development, and used as a baseline in our studies), P4 (equivalent to a
term human infant, and the beginning of lung septation in rodents), P7; P10 (equivalent to a 2-year old child, mid-septation in rodents), and 2 months (human equivalent to a young adult, complete lung septation in rodents). On the day of dosing (P1, P4, P7, P10 and 2 months), each pup received an oral dose, which was delivered directly into the mouth using a small micropipette, as previously described [53, 54, 118]. The volume of each dose was ~0.4 µL/g body wt., determined by weighing. After dosing, each pup was returned to the mother. Weaned rats were fed the same VAM diet as mother. Rats were euthanized 6 h after the final treatment with VARA or placebo. Therefore, at P10, pups would have received an oral treatment at P1, P4, P7, and 6 h prior to tissue collection at P10 for a total of 4 treatments (Table 2.1). Animals described, as “VARA carry over” were adult rats that had received oral doses at the neonatal ages mentioned above (P1, P4, P7, and P10), but were not treated prior to euthanasia as an adult.
Experimental Design of neonates nursed by dams fed VAM diet was received oral dose of oil (placebo) or VARA supplementation and at postnatal day (P)1, P4, P10 and adult age. For all ages, the final treatment with oil or VARA was administered 6 h before euthanasia and tissue collection.

Fig. 2.2. Experimental Design
**Table 2-1 Treatment schedule**

Treatment schedule of SD rats that received oral dose of VARA or oil (placebo) from birth (P1) to adult age. The adult group marked “O”, also referred to as the “Adult VARA Carryover” group received VARA supplementation as neonates (P1, P4, P7 and P10) but not at adult age in order to assess any carry-over effect of neonatal VARA supplementation.

<table>
<thead>
<tr>
<th>Ages Examined</th>
<th>P1</th>
<th>P4</th>
<th>P7</th>
<th>P10</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P10</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X O</td>
</tr>
</tbody>
</table>
2.3 Tissue Collection

After rats were euthanized at designated times, heparinized blood was collected from the vena cava for plasma preparation and stored at -20°C. The liver, lung and kidney have the capability of storing VA as RE and play a major role in maintaining VA homeostasis. Therefore we collected VA storage organs: lung, liver, and kidney where these organs were removed, trimmed and weighed, and then frozen in liquid nitrogen and stored at -80°C prior to retinoid analysis (excluding kidneys) and gene expression analysis. The lung was also used for histology.

Lung Inflation and Histological Preparation

Unlike the human lungs, which have 3 lobes in the right lung and 2 lobes in the left lung, rat lungs have only 1 lobe of the left lung and 4 lobes of the right lung. The right lung was isolated following suture ligation of the right mainstem bronchus, dissected, snap-frozen in liquid nitrogen and stored at -80°C prior to retinoid analysis and gene expression. To inflate the left lobe of the rat lung, the trachea was cannulated with an angiocatheter to allow entry of perfusion-inflation fixative at constant pressure (30 cm H₂O) with 10% neutral buffered formalin [119], used for paraffin sectioning necessary for immunohistochemistry and morphology. Inflated lung tissue was placed in ¾ filled glass vials containing 10% formalin, left overnight and then transferred to 70% ethanol prior to dehydration through a series of alcohols and Histo-Solve™ (Thermo-Scientific, Waltham, MA), then embedded in paraffin prior to sectioning. Serial 5 µm thick sections were cut and placed on glass slides. Slides were stained with hemotoxylin and eosin (H&E) for histopathological and morphometric analysis. Sections were viewed using Olympus BX51 or Olympus 1X70 Florescent Light Microscopes (Olympus America Inc.;
Lehigh Valley, PA). Images were analyzed using Ilastik software for stained image coding and ImageJ for quantitative analysis.

2.3.1 Immunohistochemistry

**Hart’s Elastin Staining**

Hart’s Elastin staining is a method used to detect elastin fibers in tissues. To stain for elastin using a Hart’s Elastin staining kit (Poly Scientific, Bayshore, NY), lung tissue sections were deparaffinized in xylene and rehydrated through a series of alcohols and rinsed with distilled water. Sections were then oxidized in 0.25% aqueous potassium permanganate for five minutes and rinsed in distilled water before toning in 5% aqueous oxalic acid. Once sections were clear, they were washed in warm running tap water for three minutes and then transferred to distilled water. Sections were then soaked in working resorcin-fusion solution (overnight in the dark). After resorcin-fusion solution incubation, sections were washed again in warm running tap water for ten minutes and then rinsed in distilled water. Sections were counterstained with tartrazine for 3 minutes, dehydrated through a series of alcohols, and mounted in xylene based mounting media to preserve staining.

**Masson’s Trichrome Staining**

Masson’s Trichrome staining is a common method of detecting collagen deposition, which is useful in the study of diseases of connective tissue and muscle characterized by fibrotic and dystrophic changes. For detection of collagen, lung tissue sections were deparaffinized in xylene and rehydrated through a series of alcohols and then rinsed with distilled water. Sections
were then fixed in Bouin’s solution (Sigma St. Louis, MO) overnight to improve staining quality. After fixation, sections were rinsed in running tap water for 10 minutes to remove yellow coloring and then stained in Weigert’s iron hematoxylin working solution for 10 minutes. Sections were rinsed well in running tap water for 10 minutes followed by an acidified water wash. Biebrich scarlet-acid fuchsin solution was then used to stain slides for 5 minutes, washed in distilled water for several times, and transferred to 1% phosphomolybdic-phosphotungstic acid solution for 1 hour. After rinsing sections with distilled water, sections were transferred to aniline blue solution for 10 minutes, and then rinsed in tap water briefly. Slides were transferred back to 1% phosphomolybdic-phosphotungstic acid solution for three minutes, washed in distilled water, incubated in 1% acetic acid solution to render the shades of stained colors to become more delicate and transparent, and finally washed in distilled water. After quick dehydration of sections, slides were mounted in DPX Mountant (Sigma, St. Louis, MO) to preserve staining.

**Ki-67 Antibody Staining**

The Ki-67 nuclear protein is a marker for cell proliferation. To detect proliferation, tissue sections were deparaffinized, rehydrated and washed in tap water. To unmask epitopes for enhanced binding of Ki-67 antibody, paraffin sections were then boiled in citric acid, pH 6, for 10 minutes and cooled in the acid bath for an additional 20 minutes. Sections were washed in PBS and incubated in diluted normal goat serum to block (VectaStain ABC Elite Kit Rabbit IgG, Vector Laboratories, Burlingame, CA) for at least two hours at room temperature. After incubation, excess was blotted out and replaced with Ki-67 1° antibody (ThermoFisher Scientific, Waltham, MA) at a 1:500 dilution in antibody diluent (Abcam, Cambridge, UK) for overnight incubation at 4°C. Following overnight incubation, sections were washed in PBS + 0.1% Tween

20 (PBST) and incubated in 3% hydrogen peroxide for 10 minutes for quenching of endogenous peroxidase activity. Sections were then washed again in PBST prior to 7-minute incubation in biotinylated rabbit 2°-antibody solution (VectaStain ABC Elite Kit Rabbit IgG, Vector Laboratories, Burlingame, CA). After incubation, sections were washed again in PBST prior to a 30-minute incubation in VectaStain Elite ABC Reagent (VectaStain ABC Elite Kit Rabbit IgG, Vector Laboratories, Burlingame, CA) to enhance staining. Sections were washed in Tris Buffer Solution + 0.1% Tween 20 (TBST), stained with 3,3′-Diaminobenzidine (DAB) peroxidase substrate solution (Vector Laboratories, Burlingame, CA), and washed in tap water to stop DAB activity. TBST was used to wash sections again before dehydration through a series of alcohols, and then mounted with DPX Mountant (Sigma, St. Louis, MO) to preserve staining. Absence of primary antibody served as the negative control. All negative control slides showed no immunostaining.

2.3.2 Lung Morphometric Analysis

To measure changes in lung morphology in response to treatments described above and in later chapters, we selected at least 6 random 5-µm paraffin-embedded tissue sections of every lung tissue from each experimental group at specified stages that were separately stained with H&E or Ki-67. Three randomly selected areas of each section were observed using an Olympus BX51 or Olympus 1X70 Florescent Light Microscope (Olympus America Inc.; Leheigh Valley, PA) and photographed using Olympus OM-1 35mm SLR Film Camera and Cell Sans Software (Olympus America Inc.; Leheigh Valley, PA) or ProgRes® CFSCAN camera and ProgRes® Capture Pro 2.7 (Jenoptik, Jena, Germany). Each file was analyzed in a blinded manner.
**Ilastik Coding**

Ilastik is an open-source software tool that enables the investigator to train a machine-learning algorithm to identify which pixels of an image belong to a class of interest based on examples provided on the region of interest. For morphometric analysis of H&E stained lung tissues, images of sections at 20X or 40X magnification were first classified using Ilastik (Fig. 2.3A). In order to provide adequate training of the program to code for objects of interest, a total of four images were selected for coding. Features and scales (color/intensity, edge, texture, and pixel diameter) were then selected (Classification → Select Features) to be used to train the classifier in order to separate the visual attributes of the selected objects (e.g., Tissue area) from the background (e.g., Airspace). Colors were selected to label and code for objects and background. Each added label (color) should correspond to an object type (i.e., alveolar tissue) for separation of other object types and overall background (i.e., airspace) (Fig. 2.3B). After marking objects and background with proper labels onto images, satisfactory coding was determined based on the least amount of uncertainty displayed (Overlay → Uncertainty). Coded images were then commanded to train and predict (Fig. 2.3C). Once the classifier was trained, it was applied to new images by batch processing without requiring additional coding (Automate Classification → Batch process). This batch process was carried out using the developed algorithms to classify all images from each experimental group.

For Ki-67 staining, one color was used to code for a definite nuclear staining, another color to code for surrounding tissue, and a final color to code for airspace. Images were then saved and opened in ImageJ for conversion to binary images necessary for computational analysi
Figure 2.3 Lung morphometric coding and segmentation

To execute computational analysis of stained lung tissues, original lung image (H&E stained, A) was used to train an Ilstik classifier to segment the alveolar tissue (B, red) from the airway lumen (B, green) based on color/intensity, edge, texture, and size features. The segmentation accuracy was visually inspected (C) to ensure that the tissue and lumen were properly delineated.
ImageJ Imaging and Quantification

ImageJ is a public domain image processing scientific program designed for various multidimensional images. For our H&E image analysis, ImageJ was used to convert coded images to black and white binary images. To accomplish this, coded images were opened in ImageJ and converted to 8-bit grayscale images (Image → Type → 8-bit). After conversion, a threshold was created for the binary image (Process → Binary → Make Binary), ready for computational analysis (Fig. 2.4).
The resulting segmented image (A) using Ilastik software was converted to a binary image B) and exported as a binary image labeled as 1 (black; alveolar tissue) or 0 (white; airway lumen) for computational analysis done by MatLab software.
For Ki-67 stained sections, images were both converted to binary images and quantified for percentage area stained for Ki-67 relative to total tissue area. To accomplish this, two images were developed by Ilastik. One image was coded for alveolar tissue and airspace, similar to coding method for H&E stained lung tissues. The original image prior to coding for alveolar tissue and airspace image was then coded for Ki-67 staining and other (alveolar tissue + airspace). Thus resulting in two images developed from one image with two different coding labels. Each sample from experimental groups was subjected to this method of coding and separation. Images coded for Ki-67 vs. alveolar tissue + airspace were converted to 8-bit grayscale images and converted to a binary image by adjusting an optimal threshold until the stained Ki-67 was red (image → adjust → threshold). Once the threshold was set and the image was converted to a binary image, ImageJ was set to measure “Area”, ”Area Fraction”, “Limit to Threshold” and “Display Label” (analyze → set measurements). The total image area and the percent of the image stained for Ki-67 based on the set threshold was measured (analyze → measure) and displayed in the results window. The second image that was coded for alveolar tissue relative to airspace was analyzed in the same way as the first image. The results of the area stained by Ki-67 divided by the total tissue area by total image area (Ki-67/Tissue area)* (Total image area) provided the Ki-67 quantification of each stained lung.

**Computational Analysis**

For morphometric analysis of H&E stained lung tissues, images of sections at 20X magnification were first segmented using the open-source image segmentation toolkit Ilastik ([http://ilastik.org/index.html](http://ilastik.org/index.html)). Four images were used to train an Ilastik classifier to segment the alveolar tissue from the airway lumen based on color/intensity, edge, texture, and size features.
The accuracy of the segmentation of each of the four images was assessed using the Ilastik classifier uncertainty map. The segmentation accuracy was also visually inspected to ensure that the tissue and lumen were properly delineated. The resulting trained classifier was then used in batch mode to automatically segment the image stack from each experimental group. The segmented images were then exported as a binary image stack labeled as 1 (alveolar tissue) or 0 (airway lumen).

Given the segmented image stack, a morphometric analysis was performed to quantify the cross-sectional area, perimeter, and thickness of the alveolar tissue in each section. The segmented images were first processed in MATLAB using a 7×7 edge-preserving median filter to remove random “salt and pepper” noise in the segmentation (Fig. 2.5). The processed images were then analyzed using a custom image processing program in MATLAB, originally developed and validated by [120] for morphometric analysis of the size and shape of respiratory airways [120-122] and other biological flow conduits [123]. Given the image resolution, the cross-sectional perimeter and area of the alveolar tissue in each section were calculated directly from the segmented images (see Craven et al. [120] for details of the methodology). The average thickness of the tissue in each section was calculated using the engineering concept of hydraulic diameter, which is used to characterize the effective diameter or width of noncircular flow conduits [124]. Here, the hydraulic diameter of the segmented alveolar tissue was calculated [120] and is used to quantify the average thickness of the tissue in each section. The average thickness values calculated in this manner were confirmed by comparing with the average of multiple manual measurements of tissue thickness made using ImageJ (http://imagej.nih.gov/ij/) on a subset of images.
Figure 2.5. Removal of segmentation “noise”

Binary image (A) was processed in MATLAB using a median filter to remove random “salt and pepper” noise in the segmentation (B) prior to computational analysis. White area represents alveolar tissues, and black area represents airspace lumen.
2.4 Retinoid Analysis

Plasma and portions of lung and liver tissues were cut, weighed, homogenized, and extracted for lipids in 100% ethanol. Tissues were processed using the Folch, Lee, and Sloan Stanley wash procedure [125]. Extracts underwent alkaline hydrolysis to convert RE to retinol by a saponification process (5% (wt:v) KOH in ethanol containing 1% pyrogallol). After the final wash, extracts were dried down under argon and re-dissolved in hexanes. A known amount of an internal standard, trimethylmethoxyphenyl-retinol (TMMP-retinol) was added to each sample and then dried under argon and reconstituted in 100 µl of methanol. These reconstituted samples were separated on a reverse-phase C-18 column eluted with a gradient of 92.5:7.5 methanol:water at a flow rate of 0.5 mL/min using ultra performance liquid chromatography (UPLC) monitored by UV detection (Waters Aquity UPLC System). The areas of the peaks for TMMP-retinol and retinol were analyzed by Millennium software (Waters) and tissue total retinol concentrations were calculated. In tissues, >90% of total retinol is esterified, therefore total retinol the sum of RE + retinol, represents mostly RE in our studies. Retinal was not detected.

2.5 Gene mRNA Level Determination

2.5.1 Designing Primers

Primers are designed to amplify a specific DNA sequence from a genomic or plasmid template DNA sequence. Each PCR requires a unique set of primers. For Studies 1, 2, and 3, the species (Rattus norvegicus; rat) and mRNA sequences of interest were found in the National Center for Biotechnology (NCBI) nucleotide database. The coding region for the gene of interest was copied and transferred to NBCI Basic Local Alignment Search Tool (BLAST) in order to
find regions of local similarity between sequences and find statistically significant matches to sequence databases. Once a desired sequence was identified, the sequence was copied and transferred to the Primer3 program to design the primer set (forward and reverse). The selection of the sequence depended on the length (20 nucleotides), G and C content (40 – 60%), and melting temperature ($T_m$) 60°C. To insure that the proper primer set was selected, the selected sequence was checked again in BLAST to ensure that this sequence is found in the desired gene of interest. Other primers used had been previously published and were checked using NCBI BLAST prior to usage.

### 2.5.2 mRNA Analysis

Total RNA (2 µg) was extracted from the liver, lungs, and kidneys of individual pup and adult rats using a hand-held power homogenizer into 1 mL of Trizol® reagent (Life Technologies, Carlsbad, CA) as previously described in literature [126] and product protocol. Complementary DNA (cDNA) of the isolated RNA was prepared using reverse transcriptase (Promega, Madison, WI) [34]. The diluted reaction product was used for quantitative real-time PCR (qPCR) analysis using 2X Real SYBR Green PCR Master Mix (BioRad, Hercules, CA) or PerfeCta® SYBR® Green Supermix, ROX™, and qPCR was performed using the DNA Engine2 Opticon (BioRad, Hercules, CA) [55] or Applied Biosystem StepOnePlus™ (ThermoFisher Scientific, Waltman, MA).

Primers designed to detect rat mRNA expression in Chapter 3 were: 5’-CGGACCCATTTTACCCACTA-3’ (forward) and 5’-CAGACTGCAGGAAGGGTCAT-3’ (reverse) for *Lrat* (NM_02280); 5’-TTGAGGGCTTGGAGTTGGT-3’ (forward) and 5’-AACGTTGCCATATTCTCCTCGC-3’ (reverse) for *Cyp26B1* (NM_181087); 5’-
CCGATCCTGGACAGTTCTA-3' (forward) and 5'-CCACCTGGATAATGGCTGT-3' (reverse) for \textit{Stra6} (NM_0010029924); 5'-ACGGCGCAATGCCGCTGACT-3' (forward) and 5'-TGCCCCAATGCCCATAGGTAACGA-3' (reverse) for \textit{megalin} (Lrp2; NM_030827) [127]; 5'-GCACTCATGGGAGAGAGAGAG-3' (forward) and 5'-GGGGGCTTGTCTTCTTCTTGT-3' (reverse) for \textit{Cyp26A1} (NM_130408); 5'-ACCGCCGCAATGCCGCTGACT-3' (forward) and 5'-TGCCCCAATGCCATAGGTAACGA-3' (reverse) for \textit{megalin} (\textit{Lrp2}; NM_030827) [127]; 5'-GGAATACTGCAGGCTGAA-3' (forward) and 5'-GGGGGCTTGTCTTCTTCTTGT-3' (reverse) for \textit{Rbp4} (NM_013162); 5'-AATCAAGGAAGCTGAGGAA-3' (forward) and 5'-CACCCAGTTCTCCTTCTTCTTGT-3' (reverse) for \textit{Raldh-1} (NM_022407); 5'-AGAAGGATGGGACGCTTCTGA-3' (forward) and 5'-GTCCAAGTCAGCAATGCCATAGGTAACGA-3' (reverse) for \textit{Raldh-2} (NM_053896); 5'-CGACCTGGGAGGCTTGTATTA-3' (forward) and 5'-CTCTTCTTGGCGAACTCCAC-3' (reverse) for \textit{Raldh-3} (NM_153300); housekeeping genes \textit{Gapdh} (NM_017008) and 18S rRNA.

Primers designed to detect rat mRNA expression in lungs for \textbf{Chapter 4} were: 5'-CACGGGGTCCATGCCACTAAGCAT-3' (forward) and 5'-ATCCGGATTCTGGCTTGTGTCGC-3' (reverse) for \textit{Pdgfra} (NM_012802); 5'-CCATGAACCTTCTGGCTTCTTTCG-3' (forward) and 5'-GGTGAGAGGTCTAGTTCCCGA-3' (reverse) for \textit{Vegf} (AC_000077); 5'-GGCATGAACTACCTGGAAGAC-3' (forward) and 5'-TTCTCCTCAGCAGGCACCAAGCA-3' (reverse) for \textit{Egfr} (NM_031507); 5'-TCAACCTGTTCACCTTGAC-3' (forward) and 5'-AACACAAAGAGGGTGTTG-3' (reverse) for \textit{elastin} (eln) (NM_012722); 5'-GCTTCTTCTTCTTCCCTGGAG-3' (forward) and 5'-AGATGGTGGAAGGGGCTTCTC-3' (reverse) for \textit{a-sma} (NM_031004); 5'-TACCAGAGCAGGAGGCACA-3' (forward) and 5'-CAATATCTGGCAATGGGCTGGT-3' (reverse) for \textit{Sp-a} (NM_001270645) [128]; 5'-CCATCCCTCTGCCCCCTTCTG-3' (forward) and 5'-CACCCTTGGGAATCACAGCTA-3' (reverse) for \textit{Sp-b} (NM_138842) [128]; and 5'-TCCCAGAGCCAGTGGTTG-3' (forward) and 5'-CACGATGAGAAGGCTTGTG-3' (reverse)
Primers designed to detect rat mRNA expression in lungs for Chapter 5 were: 5'-ACAAGGCTGCCCCGACTAT-3' (forward) and 5'-CTCCTGGTGATGAAATGGCAAATC-3' (reverse) for *Tnfα* (NM_012675); 5'-GTCACACTCCATCTGCCCTTCAG-3' (forward) and 5'-GGCAGTGGGTGCAACAACAT-3' (reverse) for *IL-6* (NM_012589); 5'-TACCTATGTCTTTGCGTGGAG-3' (forward) and 5'-ATCATCCCACGAGTCACAGAGG-3' (reverse) for *IL-1β* (NM_031512); 5'-AGTCTGGAAGAACATTATTTAACTCAAGTACAT-3' (forward) and 5'-TGTCCTCTCAAGTATTTTCTGTTAC-3' (reverse) for *IFNγ* (NM_138880); 5'-ATCCAGAGCTTGACGGTGAC-3' (forward) and 5'-AGGTACGATCCAGGCTTCCT-3' (reverse) for *Mip-2* (NM_053647); 5'-TTGCCCTTCTCAAGCCAGATGC-3' (forward) and 5'-AGCAGCGCTCTCCTATCTTG-3' (reverse) for *Thy1* (NM_012673). 5'-CCGATCCTGGACAGTTCCTA-3' (forward) and 5'-CCACCTGTGTAAGTGCTGTGTT-3' (reverse) for *Stra6* (NM_0010029924); 5'-CGGACCCATTTTACCCACTA-3' (forward) and 5'-CAGACTGCAGGACGGTCAT-3' (reverse) for *Lrat* (NM_02280); [128]; housekeeping gene *Gapdh* (NM_017008).

To calculate mRNA expression of selected genes, we used the comparative CT (ΔCT) method [129, 130], in which CT is the threshold cycle number (the minimum number of cycles needed before the product can be detected). The arithmetic formula for the ΔCT method is the difference in threshold cycles for a target (i.e. megalin) and an endogenous reference (i.e. housekeeping gene 18S). The amount of target normalized to an endogenous reference (i.e. megalin in controls) is given by 2-ΔΔCT. The mRNA expression levels were normalized by calculating using the reference genes 18S and GAPDH. Data was then normalized to the average value for the P1 oil group, set at 1.00 prior to
2.5.3 Statistical Analysis

Statistical analyses were conducted using Prism 6.0 software (GraphPad). Data are presented as group means ± SEM. Differences between age groups for retinol content in plasma, liver, and lungs; gene expression in lungs, liver, and kidneys; and morphology in lungs were tested by one-factor ANOVA with Holm-Sidak multiple comparisons post-hoc test followed by a linear trend test. Two-factor ANOVA with Holm-Sidak multiple comparisons and Student’s t-test were used to analyze the overall effects of oral treatments and the statistical difference between VARA versus placebo at each age. In addition, Student’s t-test was used to analyze gender difference in plasma retinol concentration and gene expression in tissues of the VARA adult carryover group. For all comparisons, data were normalized to the mean mRNA value of the control group (P1 oil), set at 1.00, and the mean values of the other groups were converted accordingly to determine fold change. When variance terms were unequal, values were log10-transformed prior to statistical analysis. To account for outliers, the Rout test was used to detect multiple outliers based on the statistical variation of the data. Differences with $P < 0.05$ were considered significant.
Chapter 3

DEVELOPMENTAL EXPRESSION PATTERN OF RETINOID HOMEOSTASTIC GENES

3.1 Abstract

Vitamin A (VA, retinol) is essential for proper development in early life. The physiological requirement of neonates for VA is unknown and the response of retinoid homeostatic genes in major VA-metabolizing organs, liver, lung and kidney, to supplementation with VA has not been studied systematically during ontogeny. In the present study, we determined the developmental trajectory of VA metabolism in placebo-treated VA-marginal rats, and the response of plasma, liver, lung, and kidney to supplementation with VARA, a combination of VA and 10% all-trans-retinoic acid, from birth to adulthood. Rat dams and their post-weaning offspring were fed a VA-marginal diet, while pups received an oral dose of oil or VARA on post-natal (P) day P1, P4, P7, P10, and as adult (2 months); tissues were collected 6 h after final dosing. During neonatal ontogeny (oil group), Lrat, Rbp4, Raldh-1 and Raldh-3 mRNA levels did not differ, but were higher in adults, while Cyp26B1, Stra6, megalin, and Raldh-2 did not differ in perinatal to adult stages. VARA significantly up-regulated total retinol in plasma and tissues, and the expression of Lrat, megalin, Cyp26A1/B1, and Stra6, which are important for VA storage, VA recycling, RA terminal oxidation, and retinol uptake, respectively. However, VARA decreased the hepatic expression of Rbp4, responsible for VA trafficking, and Raldh-1 and Raldh-2, but not Raldh-3, which function in RA production. Our results define changes in retinoid
homeostasis between the neonatal and adult periods and show that VARA promotes retinoid storage and alters retinoid homeostatic gene expression in both neonates and adults.

3.2 Introduction

Vitamin A (VA; retinol) is an essential micronutrient for several biological processes, including vision, embryonic development, cell differentiation, growth and development, and regulation of the immune system. The activity of VA depends on the oxidative metabolism of retinol to form retinal and retinoic acid (RA). RA functions as the most active metabolite of VA by activating nuclear transcription factors that regulate the expression of several genes, including several that are involved in the metabolism of retinol, known as retinoid homeostatic genes. VA deficiency (VAD) is a common form of micronutrient malnutrition affecting preschool-age children and pregnant women worldwide [51, 117], and VA supplementation of young children is promoted by The World Health Organization as a means to reduce young child mortality [117]. Nonetheless, there are still significant gaps in understanding VA metabolism in the neonatal period. In view of the involvement of VA in a wide variety of physiologic and metabolic systems, newborns and neonates may have a need for a higher rate of retinol oxidation and RA formation to support their rapid growth and the differentiation of tissues. However, the actual biological requirements for VA in this age group are not known. Recently, we conducted studies of retinol kinetics, using a mathematical modeling approach in neonatal rats, from which it was possible to calculate basic parameters such as the fractional disposal rate of retinol, the number of times retinol recycles in plasma before it is irreversibly degraded, and the mean residence time retinol spends in tissues before irreversibly leaving [39]. This study compared two groups of neonatal rats, an oil-treated placebo group nursed by dams fed VA-marginal diet, and thus representing
infants of mothers with low VA status, and neonates that were supplemented with VARA, a combination of VA given at a dose per body weight that is similar to VA given as a supplement to young children [51, 117], and a small portion, 10 mol-% of RA, which we previously showed increases, by approximately 5-fold, the uptake of retinol and the storage of retinyl ester in neonatal rat lung [53, 54]. While these studies have provided insight into whole-body trafficking of the retinol molecule, there still remain many questions regarding retinoid homeostatic gene system during ontogeny, and if micronutrient supplementation with VARA may alter gene expression.

Although our previous studies on VARA supplementation focused on the lung [45, 53, 54, 118, 131], there currently is no integrated view of retinoid homeostatic gene expression that includes the plasma, liver, lung, and kidney throughout the neonatal period. The roles of these tissues may differ, or overlap. Plasma is important for communicating between all other tissues through retinol that is transported in plasma by retinol-binding protein (RBP). The liver esterifies retinol using the enzyme lecithin:retinol acyltransferase (LRAT), thus storing VA as retinyl ester, oxidizes retinoids, and synthesizes RBP for secretion, with bound retinol, into plasma. The lung is a prototypical extrahepatic VA target tissue that not only stores VA as retinyl ester but also is capable of producing RA and catabolizing retinoids. The kidney is the major site of RBP uptake by megalin, a multi-ligand receptor that functions to conserve and recycle retinol [36, 38]. Therefore, our objectives in the present study were to quantify retinol concentrations and retinoid homeostatic gene expression during the period of postnatal development (normal ontogeny, from birth to adult age) in each of these tissues, and to understand which components of this homeostatic system are responsive to VARA supplementation. We also wanted to learn whether treatment of neonates with VARA has any long-term impact (“carry over effect”) at adult age. Thus, our study was designed to address three questions: 1) Is there a natural ontogenic
progression of tissue VA and retinoid homeostatic gene expression in the neonates of mothers fed VA-marginal diet, and in adults fed VA-marginal diet from weaning? 2) Does VARA supplementation given at intervals during the neonatal period affect the expression of retinoid homeostatic genes, either transiently or in a cumulative manner? 3) Does VARA supplementation of neonates have a long-term consequence on retinoid homeostatic gene expression in adults? Together, our data demonstrate significant developmental changes in the levels of some retinoid homeostatic genes, but not others, and reveal that neonatal VARA supplementation results in both short-term changes, and, for certain genes, a prolonged effect that is evident in the adult animal.

3.3 Hypothesis and Aims

_Hypothesis 1._ There is a developmental pattern of expression of retinoid homeostatic genes during postnatal development.

_Hypothesis 2._ Ontogenic expressions of retinoid homeostatic genes will be responsive to VARA supplementation from birth to adult age in neonatal rats.

_Hypothesis 3._ Neonatal supplementation of VARA will display a long-term effect on retinoid gene expression at adult age.

_Aim 1. Quantify retinol concentrations and retinoid homeostatic gene expression during the period of postnatal development_

In this study, we determined the expression of retinoid homeostatic genes _Stra6, Lrat, Rbp4, Cyp26A1/B1, megalin and Raldh -1, -2, and -3_ in principal vitamin A storage organs lung, liver, and kidney by qPCR from birth to adult age (P1, P4, P10, and adult). We then determined liver, lung, and plasma total retinol contents by UPLC at the ages mentioned.
Aim 2. Quantify which components that regulate VA homeostatic system are responsive to VARA supplementation.

We examined expression levels of the genes listed in Aim 1 after oral VARA supplementation to test their response to treatment.

Aim 3. Determine whether treatment of neonates with VARA has any long-term impact (“carry over effect”) at adult age.

Our adult “VARA carry over” received incremental doses of VARA only as neonates up to P10 (P1, P4, P7, and P10) and not as adults. To determine possible long-term affect of early supplementation, we determined the expression of retinoid homeostatic genes in the adult liver, lung and kidney. At the same time, we determined the total retinol concentrations in the plasma, liver, and lung.

3.4 Methods and Materials

Animals and experimental design

Refer to Chapter 2: Methodology section 2.1.1 and 2.1.2, as well as Figures 2.1 and 2.2 and Table 3.1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sequences</th>
<th>Mode of Action</th>
</tr>
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<tbody>
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<td>Cytochrome P450 family 26, subfamily A, polypeptide 1</td>
<td>NM_130408.2</td>
<td>5' - GCACTCATGGGAGAGAGGAG 5' - GGGGGCTTGCTTTGTTA</td>
<td>Terminal oxidation of retinoic acid to polar metabolites [28]</td>
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<tr>
<td>(CYP26A1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450 family 26, subfamily B, polypeptide 1</td>
<td>NM_181087</td>
<td>5' - TTGAGGGCTTGGAAGTTG 5' - AACGTTGCCATAC TTCTCGC</td>
<td>Terminal oxidation of retinoic acid to polar metabolites [28]</td>
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<tr>
<td>(CYP26B1)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lecithin retinol acyltransferase (LRAT)</td>
<td>NM_022280.2</td>
<td>5' - CGGACCCATTTTATACCACTA 5' - CAGACTGCAGGAA GGGTCAT</td>
<td>Esterification of retinol necessary for storage [29, 30]</td>
</tr>
<tr>
<td>Megalin</td>
<td>NM_030827.1</td>
<td>5' - ACCGCGCGCAATGC CGCTGACT 5' - TGCCCAATGCCCTAGGTAACGA</td>
<td>Recycling of retinol binding protein - retinol complex [36, 38]</td>
</tr>
<tr>
<td>Stimulated by retinoic acid 6 (STRA6)</td>
<td>NM_0010029924.1</td>
<td>5' - CCGATCCCTGGACACTTCTGA 5' - CCACCTGGTAAGTGCGTGTT</td>
<td>Cellular uptake and efflux of retinol [31]</td>
</tr>
<tr>
<td>Gene</td>
<td>NM</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
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</tr>
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<td>Retinol-binding protein 4 (RBP4)</td>
<td>0131 62.1</td>
<td>5’- CTGTGGACGAGAA GGGTCTCAT 5’- GGAATACGTGAGAAGGGAGAG</td>
<td>Retinol trafficking through circulation [132]</td>
</tr>
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<td>Retinal dehydrogenase 1 (RALDH-1)</td>
<td>0224 07.3</td>
<td>5’- AATCAAGGAGCT GCAGGAAGAA 5’- CACCCAGTTCTCG TCCATT</td>
<td>Catalyzes oxidation of retinaldehyde to retinoic acid [133]</td>
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<tr>
<td>Retinal dehydrogenase 2 (RALDH-2)</td>
<td>0538 96.2</td>
<td>5’- AGAAGGATGGAC GCTTCTGTA 5’- GTCAGAGTCAGCA TCTGCAA</td>
<td>Catalyzes oxidation of retinaldehyde to retinoic acid [133]</td>
</tr>
<tr>
<td>Retinal dehydrogenase 3 (RALDH-3)</td>
<td>1533 00.1</td>
<td>5’- CGACCTGGAGGCC TGTATTA 5’- CTCTCTTTGCGAG ACTCCAC</td>
<td>Catalyzes oxidation of retinaldehyde to retinoic acid [133]</td>
</tr>
</tbody>
</table>
**Dose preparation**

Refer to Chapter 2: Methodology section 2.2.

**Tissue Collection**

Refer to Chapter 2: Methodology section 2.3 introduction.

**Retinoid Analysis**

Refer to Chapter 2: Methodology section 2.4

**Gene mRNA level determination**

Refer to Chapter 2: Methodology sections 2.5.1 and 2.5.2 and Table 3.1

**Statistical analysis**

Refer to Chapter 2: Methodology section 2.5.3.
3.5 RESULTS

VARA supplementation increased total retinol in plasma, lung and liver of rats fed VA diet

Without VARA supplementation, the plasma retinol of neonatal rats and dams fed VAM diet ranged from 0.6-1 µmol/L (Fig. 3.1B; black bars), consistent with values that are associated with VA-marginal or VA-deficient status in humans [11]. VA concentrations in major storage organs, liver (Fig. 3.1C) and lung (Fig. 3.1D), equaled 50-70 nmol/g and <10 nmol/g, respectively. During neonatal ontogeny, plasma and liver retinol concentrations did not differ significantly, but plasma and lung retinol levels were higher in adults compared to neonates.

VARA supplementation resulted in a significant increase in plasma retinol at all ages (Fig. 3.1B, blue bars). Adults that had been treated with VARA only as neonates (carry over group, red bars) did not differ from adults treated only with oil.

VARA supplementation also increased liver retinol significantly at all ages; this increase was cumulative with each dose up to P10. However, there was only a small carry over effect in the adult, indicating a return to nearly the same tissue total retinol concentration as in adults fed VAM diet without VARA supplementation.

For lung, total retinol concentrations were lower in neonates than in oil-treated adults, and concentrations increased after VARA supplementation at each age (Fig. 3.1D; blue bars). There was no significant carry over effect of neonatal VARA treatment at adult age.

Thus, plasma, liver and lung all responded significantly to VARA treatment (liver > lung > plasma). VARA supplementation of neonates without further treatment at adult age increased VA storage only in the liver.
Figure 3.1. Experimental design and total retinol concentrations in plasma, liver, and lung of control (oil-treated) neonates nursed by dams fed vitamin A-marginal (VAM) diet, and treated with oil (placebo) or supplemented with VARA at postnatal day (P)1, P4, P10, and at adult age.
Design and treatment schedule showing generation of SD offspring by mating female and male rats fed VAM diet, which was maintained throughout gestation and fed to animals that continued in the study after weaning until adult age. Pups were randomly assigned to receive oil or VARA treatments at five ages, as shown by X. The adult group marked 0 received VARA on neonatal days P1 and again on P4, P7 and P10, but not when as adults, and thus was used to assess any carryover effect of neonatal VARA supplementation at adult age. For all ages, the final treatment with oil or VARA was administered 6 h before euthanasia and tissue collection. Total retinol levels increased in lungs with age (ontogeny) and after VARA supplementation in plasma [B], liver [C], and lung [D]. Data are shown as mean ± SEM, n ≥ 5/group. One-way ANOVA with posthoc analysis was used to determine differences between oil-treated groups at different ages, indicative of developmental changes with ontogeny; means without a common letter differed significantly, P < 0.05. For each age, VARA versus Oil groups were compared by unpaired t-test; * indicates P < 0.05. In C, linear trends with age were determined for the Oil and VARA groups separately.
Retinoid homeostatic gene expression is differentially regulated in neonatal and adult liver by VARA supplementation

The level of transcripts for several retinoid homeostatic genes were quantified by qRT-PCR in the liver of rats during normal ontogeny (oil group) and after VARA supplementation, as described in Fig. 3.1A. For liver, these genes included retinol-binding protein (Rbp)4, responsible for the release of retinol from liver into the circulation; lecithin:retinol acyltransferase (Lrat), responsible for VA storage as retinyl esters; and cytochrome P450, family 26, (Cyp26) subfamily A (A1), responsible for the oxidation of retinoic acid (Table 3.1).

Rbp4 transcripts displayed a nearly linear increase during ontogeny (Fig. 3.2A, black bars, P < 0.001). VARA supplementation significantly reduced Rbp4 mRNA at each neonatal age (Fig. 3.2A, blue bars, all P < 0.05). By adult age compared to neonatal age, Rbp4 mRNA was higher for both oil and VARA groups. Additionally, we observed a significant reduction in Rbp4 mRNA in adult rats that had been treated with VARA as neonates, but not as adults.

Lrat transcripts increased with age in oil-treated rats (Fig. 3.2B, black bars), with a significant linear trend during ontogeny. VARA supplementation (Fig. 3.2B, blue bars) increased Lrat transcript level on average at all ages, which was statistically significant for P4 and adult rats. Adult rats that had been treated with VARA as neonates had significantly lower Lrat expression levels (Fig. 3.2B, red bar) compared both to oil-treated controls and VARA-supplemented adult rats.

Cyp26A1 transcripts increased slightly with age in oil-treated rats (Fig. 3.2C, black bars), with a significant increase in adults. VARA supplementation (Fig. 3.2C, blue bars) increased Lrat transcript level on average at all ages, which was statistically significant for P10 and adult rats. By adult age, VARA induced Cyp26A1 expression to a much greater degree than was observed in
neonates. There was no carry over effect of neonatal VARA supplementation on *Cyp26A1* expression levels in adult liver.

Thus, for liver, VARA supplementation of neonates attenuated the expression of *Rbp4* in liver at adult age. *Lrat* and *Cyp26A1* responded to VARA supplementation more strongly in adult rat liver, compared to in neonates; however, neither of these genes exhibited a carry over effect of neonatal VARA supplementation at adult age.
Figure 3.2. Relative levels of mRNA transcripts of retinoid homeostatic genes in liver.
Oil or VARA treatments were administered at the times shown in Fig. 3.1A, with the last administration 6 h prior to euthanasia and tissue collection. Developmental expression patterns (black bars) of genes Rbp4 [A], Lrat [B], and Cyp26A1 [C] displayed a significant increase in adult liver. After VARA supplementation (blue bars), Lrat (B) and Cyp26A1 [C] expression increased but Rbp4 decreased significantly in neonates. The group labeled “VARA Carryover” (red bars) received VARA on P1, P4, P7, and P10, but were not treated with VARA as adults. In the adult carryover group Rbp4 and Cyp26A1 transcript levels were lower than in the Oil group that had not received VARA as neonates.
**VARA supplementation increased expression of retinoid homeostatic genes in rat lung**

The transcripts for Stimulated by retinoic acid 6 (Stra6), Lrat, and CYP26, subfamily B (Cyp26B1) were determined by qPCR in lung tissue from rats during postnatal development, without and with VARA supplementation, and in adults.

For *Stra6*, transcript levels were similar in oil-treated animals at each age (Fig. 3.3A; black bars), indicating no developmental pattern. After VARA supplementation, *Stra6* expression increased significantly at each age (Fig. 3.3A; blue bars); however, the magnitude of increase decreased linear trend with age (negative linear trend, \( P < 0.0001 \) for VARA). There was no carry over effect of neonatal-age VARA treatment in the adult lung.

For *Lrat*, there was a significant developmental linear trend in the oil-treated rats (\( P < 0.001 \)), driven by higher expression in the adult lung (Fig. 3.3B; black bars). After VARA supplementation, *Lrat* expression increased significantly at each age (Fig. 3.3B; blue bars). There was a significant effect of neonatal VARA supplementation on adult *Lrat* transcript levels, which were significantly lower than in either the oil-treated or adult VARA-treated groups (Fig. 3.3B, red bars, \( P < 0.05 \)).

*Cyp26B1* transcripts were very low in oil-treated rats at each age (Fig. 3.3C; black bars). After VARA supplementation *Cyp26B1* expression increased strongly in neonatal lung, but the increase was not significant in adults (Fig. 3.3C; blue bars). Similar to *Lrat*, there was a significant effect of neonatal VARA supplementation on adult *Cyp26B1* transcript levels, which were significantly lower than in either the oil-treated or adult VARA-treated groups (Fig. 3.3C, red bars, \( P < 0.05 \)).
Figure 3.3. Relative levels of mRNA transcripts of Stra6, Lrat, and Cyp26B1 in lung
Developmental expression patterns (black bars) of genes for retinoid homeostatic genes in lung, \textit{Stra6} [A], \textit{Lrat} [B], and \textit{Cyp26B1} [C]. \textit{Stra6} and \textit{Cyp26B1} in the oil-treated group did not differ throughout development; however, \textit{Lrat} (B) was higher in the adult lung. After VARA supplementation (blue bars), at postnatal day (P)1, P4, and P10, and at adult age, there was a significant increase in expression of all three genes. Prior neonatal VARA supplementation without treatment as adults (VARA carryover) resulted in a significant decrease of \textit{Cyp26B1} and \textit{Lrat} expression in the adult lung (red bars).
**Retinaldehyde dehydrogenases in lung exhibited distinct expression patterns during ontogeny and VARA supplementation**

The transcript levels for three retinaldehyde dehydrogenase (*Raldh*) genes, *Raldh-1*, *Raldh-2*, and *Raldh-3*, were determined by qPCR in neonatal and adult lung. Based on cycle threshold numbers, the relative expression levels among these three genes were *Raldh-1 > Raldh-3 > Raldh-2*. *Raldh-1* expression displayed a linear increase (*P < 0.001*) in the control group, driven by higher expression in adults (Fig. 3.4A; black bars). VARA supplementation altered expression only in adults (Fig. 3.4A; blue bars), and there was no carry over effect of neonatal VARA supplementation in adults (Fig. 3.4A; red bars). *Raldh-2* exhibited similar patterns to *Raldh-1*, except that neonatal VARA supplementation resulted in a significant suppression of *Raldh-2* expression in adults (Fig. 3.4B; red bar). *Raldh-3* transcript levels were lower in neonates than adults (Fig. 3.4C, black bars), resembling *Raldh-1* in this respect. However, VARA supplementation did not alter *Raldh-3* transcript levels at any of the ages we examined.
Figure 3.4. Relative levels of mRNA transcripts of Raldh-1, Raldh-2, and Raldh-3 in lung.
Developmental expression patterns (black bars) of genes for *Raldh-1* [A], *Raldh-2* [B], and *Raldh-3* [C] are shown. *Raldh-1* and *Raldh-3* increased with development with a significant increase in adult rats. There was no change in neonatal expression of *Raldh-1*, *Raldh-2*, or *Raldh-3* after VARA supplementation; however, VARA significantly decreased *Raldh-1* and *Raldh-2* in adult lung. The decrease in *Raldh-2* after neonatal VARA supplementation remained in the VARA carryover group (red bar). For comparison, the average value for the control group (P1, oil) was set to 1.00 for each experiment and fold change for each gene is shown as relative to this value. Data are shown as mean ± SEM, n ≥ 5/group. One-way ANOVA with post-hoc analysis was used to determine differences between oil-treated groups at different ages, indicative of differences due to ontogeny. Means without a common letter differ significantly, P < 0.05. For each age, VARA versus Oil groups were compared by t-test; * indicates P < 0.05.
Neonatal megalin expression in kidneys is not regulated by VARA supplementation

The transcript level for megalin (Lrp2), responsible for reuptake of plasma retinol-RBP in proximal tubules, was determined in neonatal and adult kidney. There were no differences during ontogeny or after VARA treatment in neonates (Fig. 3.5). However, VARA supplementation at neonatal age increased megalin mRNA approximate 10-fold (P < 0.01) in the adult kidney (blue bar). There was no effect of neonatal VARA treatment on megalin expression in adult rats (red bar).
There was no developmental difference in megalin mRNA. Treatment with VARA significantly increased megalin transcript expression in adult rats. Conditions were as described in Fig. 3.1A. Asterisks (*) denote differences between oil and VARA groups in adult kidney, $P < 0.05$. Data are mean ± SEM, $n \geq 7$/group.

Figure 3.5. Relative levels of mRNA transcripts for megalin in kidney
3.6 Discussion

The results of study provide insight into VA homeostasis during the period of postnatal development, and suggest that supplementation with VARA, when administered to animals with marginal VA status, may influence their retinoid homeostatic gene system acutely, and also may have longer-term effects, which can be observed as differences in the level of gene expression in adult animals that received VARA only as neonates. As my intention was to compare results of different ages and in responses to VARA supplementation, on both retinol concentrations and several genes in several tissues, numerous comparisons are of interest and, therefore, for convenience, I have summarized the main findings in Table 3.2. Several inferences or conclusions can be drawn from these results. First, based on the results of plasma, lung, and liver total retinol concentrations, the marginal VA status of the neonatal and adult rats in this study was confirmed (Fig. 3.1). Interestingly, of these 3 tissues and although a VAM diet was fed to all rats after weaning, lung total retinol concentration increased (denoted as “higher” in Table 3.2) approximately 4-fold between neonatal (P10) and adult age. However, at the same time, the liver retinol concentration did not increase. This suggests that the lung has some priority for the uptake of retinol from plasma, as compared to liver. It is well known that VA is necessary for lung development [105]. The low levels of VA in neonatal lung tissue could be due to increased utilization of retinoids for growth and postnatal alveolarization processes [93].
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<th>Ontogenic changes without VARA (OIL group)</th>
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<td>Differences in neonatal stages, P1 to P10</td>
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<td>Kidney (Megalin)</td>
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Secondly, there were significant developmental patterns in the expression of retinoid homeostatic genes. Our objective was to examine the patterns of expression of retinoid homeostatic genes in major VA storage/homeostatic organs (lung, liver, and kidney) throughout postnatal development in rats nursed by dams fed a VAM diet. As mentioned, rats fed VAM diet were chosen for this study because their VA status resembles that of human population at risk for VA deficiency [115]. Our results indicate that each of the genes we examined – *Lrat* (VA storage in liver and lung), *Stra6* (VA uptake in lung), *Rbp4* (VA trafficking from liver to plasma), megalin (VA recycling from plasma), *Raldh*-1, -2, and -3 (RA production from retinal), and *Cyp26A1/B1* (RA terminal oxidation and degradation) – is expressed throughout the neonatal period. However, none of these changed significantly during the neonatal period (P1 to P10). However, interestingly, *Rbp4* in liver, *Lrat* in both the liver and lungs, in addition to *Stra6*, and *Raldh*-1 and *Raldh*-2 in lungs, all displayed a developmental pattern of expression with a significant increase in adult rats compared to neonates. Therefore, retinol trafficking, storage, uptake, and retinoic acid production are not only dependent on dietary status but also on age. Neonates are constantly growing and therefore do not maintain a metabolic steady state; as a result, VA among other nutrients is in constant demand for proper development [43]. Even though the VA status of the neonatal and adult rats in our study was marginal, the expression of *Rbp4* and *Lrat* mRNAs in liver and of *Lrat* mRNA in lung increased from neonatal to adult age. These results suggest that VA tissue accumulation through *Lrat* is dependent on both diet and age [43]. They may also suggest that adults, who have a lower basal metabolic rate than neonates, are able to conserve dietary VA more efficiently and thereby are able to accumulate retinol in tissues, such as the lungs, even when the intake of dietary VA is below normal levels. This may suggest that the amount of VA in the VAM diet actually meets the biological requirement for VA in the adult animal, and that any excess VA is stored.

Thirdly, VARA supplementation significantly increased the concentration of retinol in
plasma, liver, and lung. These results were anticipated based on previous findings in neonatal rats [39, 53] and mice [134, 135], but no previous studies had addressed the effect of neonatal VARA supplementation on retinoid homeostasis in the adult animal. Here, we examined the possible effect of VARA supplementation on retinoid homeostatic gene expression at several times during the neonatal period, and in adults that had received VARA supplementation either acutely (6 h before tissue collection) or solely as neonates. Liver total retinol was higher, which was not unexpected as the amount of VA in VARA was based on an amount of VA given to children as a supplement that has been shown to reduce the rates of morbidity and mortality for 4-6 months [117]. VARA may thus be anticipated to result in significant storage of VA in the liver. Rbp4 expression in the liver of neonates was significantly decreased after oral VARA supplementation. Previous studies of acute treatment with RA, or synthetic retinoids, have shown a reduction in RBP4 and retinol in plasma [136, 137]. Lrat and Cyp26A1 expression levels were directly correlated with VA and RA availability through VARA supplementation in both neonatal and adult liver (Fig. 3.2). Rbp4 expression in the liver of neonates was significantly decreased after oral VARA supplementation (Fig. 3.2A and Table 3.2). Previous studies have shown that oral treatment with RA increased Lrat and Cyp26A1 expression in adult rat liver [112]. Our results show similar effects of VARA supplementation on Lrat and Cyp26A1 expression in both adult and neonatal liver, with Cyp26A1 expression exhibiting the greatest increase (denoted as “markedly higher” with ≥100 fold increase in Table 3.2). Although the secretion of Rbp4 is tightly regulated by the availability of retinol [138], our results suggest a significant decrease in neonatal Rbp4 expression levels after VARA supplementation. These results support previous findings of a significant reduction in the level of RBP protein in liver after treatment of adult rats with a synthetic retinoid, N- (4 hydroxyphenyl) retinamide (HPR) [136], or RA [112]. Collectively, these results may support the idea that the liver interprets the increase in available retinoids after VARA supplementation as a signal of the body’s VA adequacy and senses this
increase as extrahepatic tissues having sufficient VA [139]. Consequently, the liver may reduce the production and flow of RBP. To maintain normal VA homeostasis, *Lrat* and *Cyp26A1* may be up-regulated to store the excess VA for later use and to remove excess RA, respectively. This collaborative regulation of *Lrat* and *Cyp26A1* via VA availability has been suggested based on observation in several other studies in adult rat tissues [139]. Our work confirms these same observations during neonatal development.

Fourth, the lung is also a VA storage organ, as well as a tissue that depends on adequate retinol and RA for proper alveolar septation in the neonatal period [12, 80, 93, 96, 105, 140]. In our study, the expression in the lung of genes involved in VA uptake (*Stra6*) and the removal of excess RA (*Cyp26B1*) remained constant during the postnatal period; however, their expression was strongly up-regulated 6 h after VARA supplementation. On the other hand, *Lrat* expression in the lung appeared to be dependent on age and on VA availability. Previously, neonatal VARA supplementation was shown to increase the expression of *Stra6*, *Lrat*, and *Cyp26B1* in P7 rat lung [34, 131]. The regulatory effect of VARA supplementation on these genes was transient, as mRNA levels were significantly up-regulated 6 h after dose administration but had returned to near baseline levels 12 hours after VARA supplementation. Our current study that examined the response to VARA across the neonatal period indicates that VARA supplementation induces the expression of all three genes as early as P1. Interestingly, *Stra6* displayed highest expression after oral VARA supplementation of newborn rats (P1), with a decrease in magnitude with age. It should also be noted that by P10, neonates had received VARA supplementation on 4 occasions (Fig. 3.1A), so the attenuation of the expression of *Stra6* to VARA supplementation with age could be the result of sufficient retinoid accumulation, resulting in a feedback effect on retinol uptake. For *Cyp26B1* in the lung, expression was increased after VARA supplementation at all ages, similar to that for *Stra6*, with a decrease in the magnitude of *Cyp26B1* response with age, especially in adults. This contrasts to the response of *Cyp26A1* in liver, in which the greatest
increase was observed in adult VARA-supplemented rats. Our results suggest that excess retinol availability due to accumulative VARA supplementation results in decreased uptake of VA in lung through Stra6 and less RA production through down-regulated expression of Raldh. Despite the significant increase in Lrat expression after VARA supplementation, neonates at P4 (the beginning of alveolar septation in the rat) and P10 (middle of the septation period) [63, 80] had the smallest increase in Lrat expression compared to P1 and adult rats. This may imply that esterification and storage of retinol in response to the VARA dose was reduced in the middle neonatal period, which could be concomitant with an increased utilization of VA for proper lung septation and alveolarization.

Fifth, the expression of Raldh genes is often considered a surrogate indicator of RA production, as RA itself has a very short half-life and its production per se would be extremely difficult to measure in vivo. In our study, we represented RA production by examining Raldh-1, -2, and -3 expression in the lungs of neonatal and adult rats. RALDHs have been shown to be expressed in rodent organs, including the lung, throughout embryogenesis [133]. In our study, the expression in these three genes in the lung differed during ontogeny, as well as in response to VARA supplementation. Raldh-1 and -3 expression levels increased from neonatal to adult age (Fig. 3.4 and Table 3.2). Previous research confirmed this linear trend in mucosal dendritic cells [141]. Interestingly, VARA supplementation, which acutely provides both VA and RA to the animals (see elevation in plasma retinol, Fig. 3.1B), suppressed Raldh-1 and -2 expression, but only in adults. Because RA acts as a ligand for nuclear transcription factors and its concentration is tightly regulated [142, 143], this suppression may prevent the accumulation of an excess of endogenously formed RA. It may also be that Raldh-1 and -2 in the lung of neonates, compared to adults, were less regulated by VARA supplementation because the expression of Cyp26B1 after VARA supplementation was greater in neonates than in adults (Fig 3.3 and Table 3.2).
Sixth, the kidney is known to play an important role in retinoid homeostasis, contributing to about 50% of the total circulating pool of the RBP-ROH complex in rats [38]. Christensen et al. [36] have demonstrated the importance of megalin for maintaining VA homeostasis. Approximately 5% of the circulating RBP-ROH complex is not bound to the co-transporter protein transthyretin (TTR), and is thus more readily filtered in the kidney and lost through urinary excretion. Interestingly, megalin is an LDL endocytic receptor protein for a variety of substances and vitamins, including VA and others, and is important for proximal tubular retinol reabsorption. Other researchers have shown that megalin is expressed during embryogenesis as early as day 4 of gestation and in adult kidneys, but have not examined the postnatal period. RA has also been shown to up-regulate the expression of megalin [36, 38, 144]. Our results (Fig. 3.5A) show that VARA supplementation did not alter the expression of megalin during ontogeny in the neonatal kidney, but significantly increased megalin expression in adult kidney. The significant increase in LRAT expression after VARA supplementation in neonates (Fig. 3.5B) may prime the kidneys for increased expression of megalin in adult kidney that in turn contributes to enhanced uptake of retinol from plasma. Although megalin is generally considered part of a process of conservation and tightly regulated in neonates, megalin might also contribute to removal of excess retinol under conditions of VA surplus.

In addition to investigating the direct effect of VARA supplementation on retinol concentration and the ontogenic expression of retinoid homeostatic genes in VA storage tissues, we asked if there is a carryover effect of neonatal VARA supplementation in adult rats. The “VARA adult carry over” rats had been supplemented with VARA as neonates (up to P10) but they were not treated with VARA prior to euthanasia as adults. Our results demonstrated a carry over effect on tissue retinol concentration only in liver. Recent studies support our findings of longer maintenance of retinol levels in liver in comparison to extrahepatic tissues such as the lung [39]. It is interesting to note that the VARA adult carryover rats displayed a significant decrease
(denoted as “negative”) in liver Rbp4, liver and lung Lrat, and lung Cyp26B1 (Table 3.2), in comparison to both control (oil-treated) rats and adult rats that received VARA 6 h prior to euthanasia. While VARA can be said to push excess VA towards esterification and oxidation to maintain homeostasis, it may be that by adulthood gene expression for this purpose is no longer needed. In addition, the lack of a carryover effect of neonatal VARA supplementation on adulthood megalin expression suggests that this would likely be a short-term regulatory mechanism. At present, we do not have an explanation for these results, however further studies are needed to characterize these changes as they have implications for the use of retinoid therapy in neonates.

In conclusion, our results show a postnatal developmental pattern of total retinol concentration and expression of genes that regulate retinoid homeostasis in plasma and VA storage organs such as the lung, liver, and kidney in rats. In addition, we shown that oral supplementation of VA in conjunction with 10% RA can alter total retinol availability and gene expression. Although we are unsure whether the regulation of these genes in neonates is beneficial, we have gained insight on how neonatal VA supplementation regulates recycling (megalin), trafficking (Rbp4), storage (Lrat), and oxidation of retinoids (Rallds and Cyp26s). It has been of great interest to determine whether neonatal treatment with VA or RA has any long-term benefit, or at least no adverse effect, in those that have received supplementation at birth. Therefore, we have provided data on the possible long-term effects of neonatal oral supplementation of VARA. The evidence from this study expands on the comprehensive analysis on the effects of VA supplementation in neonates and provides insights into VA homeostasis by the expression of genes that participate in this regulation. Understanding of the neonatal retinoid metabolism is highly relevant clinically; as the VARA supplementation for vitamin A deficiency is normally performed during the neonatal stage. Our findings may help inform the clinical treatment of VA deficiency diseases and the development of nutritional recommendations for neonates.
Higher denotes a statistically higher value in VAM-fed adults vs. neonates

Column denotes a statistically significant change 6 h after last VARA treatment in neonates. If only at certain times, these are noted in parentheses.

Column denotes a statistically significant change 6 h after last VARA treatment in adults. If >100 fold, these are noted as “Markedly increased”.

Column denotes a statistically significant difference in adult levels, without VARA 6 h previously, as compared to adult levels in OIL group adult rats. Positive indicates prior VARA treatment as neonates resulted in higher levels in the adults. Negative indicated prior VARA treatment resulted in lower levels in the adults.
Chapter 4

VARA SUPPLEMENTATION AND LUNG MATURATION

4.1 Abstract

Vitamin A (VA) is necessary for proper lung development in early life. Previously, the combination of VA and 10% all-trans-retinoic acid (VARA) synergistically increased total retinol uptake and storage in neonatal rat lung. In this study, we investigated whether periodic VARA supplementation during postnatal development and in adulthood alters the expression of genes essential for proper lung alveolarization (proliferation) and septation. Rat dams and their post-weaning offspring were fed a VA-marginal diet, while pups received an oral dose of oil (placebo) or VARA on post-natal day (P) P1, P4, P7, P10, and 2 months (adult). Tissues were collected 6 h after the final dose and qPCR was used to determine gene expression. Ontogenic expression of PDGFRα (secondary septation) increased linearly with significant attenuation by VARA. VARA decreased EGFR (epithelial maturation) and surfactant protein expression of SP-A and SP-C while increasing VEGF (vascularization) expression in adult lung. Interestingly, VARA significantly increased SP-B expression at P1. Ontogenic expression of αSMA and elastin was highest in neonatal lung; however, VARA reduced αSMA expression pre-alveolarization at P1. Elastin expression was not effected by VARA. Our results advance understanding of the ontogenic pattern of proliferative genes during neonatal lung development and show that expression is also regulated by VA supplementation.
4.2 Introduction

Vitamin A is essential for normal lung development and maturation, and VA inadequacy may contribute to the impairment of lung development noted in various chronic lung diseases observed in preterm infants. Adequate VA nutritional status maternally and postnatally represents the situation in most developed countries, while supplementation of the neonate of mothers whose VA status is likely to be low represents the scenario being tested in infant supplementation studies in developing countries. Whether the maternal and post-natal diet were normal or VA deficient, we have shown how effective VA and retinoic acid (RA), known as VARA, work synergistically (1:10 molar ratio) at promoting VA uptake and retinol esterification in lungs, with an increase in total retinol in the lungs after a single dose of VARA [34, 45, 53] and throughout post-natal development after multiple doses (Study 1). Esterified retinol (retinyl ester; RE) is an important mobilizable precursor for RA production. In studies by Massaro and others [65, 92, 93, 118], retinoic acid (RA) induced lung maturation and reversed the failure of alveolar septation that results from treatments with dexamethasone and/or high oxygen therapy, and in calorie-restricted neonatal rats and mice. These findings suggest that VA has protective or reparative properties in infants with structurally immature lungs.

The lung undergoes four stages of development: pseudoglandular stage, canalicular stage, saccular stage, and finally the alveolar stage. The alveolar stage involves the process of alveolarization, the formation of definitive alveoli by secondary septation of the primitive saccules, with major occurrence within the first 6 months after birth in humans and by post-natal (P) day 14 in rodents [57, 58, 63, 145, 146]. This stage in development is important for expanding the gas-exchange surface area in order to supply the organism with sufficient oxygen and to remove carbon dioxide. Alveolarization involves the cooperative interactions between interstitial, epithelial, and vascular components of the lung. The lung tissue is composed of over
40 different cell types, including the most common cell types alveolar epithelial type I, type II cells, endothelial cells, macrophages, and fibroblasts [63, 147]. Each cell serves various roles in lung alveolarization and vascularization. Investigators have shown that the alveolar wall is rich in fibroblasts that contain lipid storage granules, also referred to as lipid interstitial cells (LICs), which are also rich in VA [97, 107, 148]. As a result, it has been speculated that VA metabolism mainly takes place in LICs of the lung.

The late stages of lung development are influenced by a wide variety of signaling molecules (Fig. 4.1). RA activates growth factor signaling pathways, regulates transcription factors important in expression of lung-specific genes necessary for secondary septation, and enhances the expression of homeobox genes, which influence axial patterning and lung bud formation critical for alveologenesis [80]. Platelet-derived growth factor A (PDGFA), produced by epithelial cells, is needed for the migration of myofibroblasts to the peripheral sites of the lung, where elastin deposition takes place [149]. Extracellular matrix components α-smooth muscle actin (αSMA) and elastin [149] are synthesized and secreted by mature fibroblast and deposited in the wall of terminal alveoli in order to carry out secondary septation. Similar to PDGFA, Vascular endothelial growth factor (VEGF) is also secreted by type II cells of the alveolar epithelium. VEGF is important for vascularization and angiogenesis necessary for mature and efficient development of air-blood interface of the lung for gas exchange [149]. The Epidermal growth factor receptor (EGFR), expressed also by airway epithelium, is important for surfactant protein biosynthesis. Surfactant proteins decrease airway tension and prevent collapse of alveoli. All together, these components are necessary for complete and proper development and expansion of the lung airways in postnatal life.
Figure 4.1 Principal cell-cell and cell-matrix interactions during secondary septation

Figure adapted and modified by Bourbon, J. Pediatr Res. 2005;57(Pt 2):38R-46R.
Little is known about the regulation of alveolarization at a compromised VA nutrition status. In this study, we investigated whether periodic VARA supplementation during postnatal development and in adulthood alters the expression of genes essential for proper lung alveolarization (proliferation) and septation. At the same time, we investigated morphological differences in the lungs supplemented with VARA in comparison to non-supplemented lungs. Our results show that VARA supplementation does modify expression of αSMA, growth factor and receptors, and surfactant proteins during post-natal lung development outside of the lung septation window. However, VARA supplementation has little effect on lung morphology.

4.3 Hypothesis and Aims

Hypothesis 1: A combination of VA and its active metabolite RA (VARA) will alter expression of genes essential for proper lung maturation and alveologenesis in the offspring of VA marginal fed dams.

Hypothesis 2: VARA supplementation will promote lung maturation through the alteration of lung morphology throughout postnatal development.

Aim 1: To determine whether VARA supplementation, compared to placebo (oil), will promote proper lung maturation throughout development (newborn to adulthood) through the alteration of proliferative retinoid-regulated gene expression and their proteins involved in lung maturation and septation.

In this study we addressed the question: How does VA nutritional status and VARA supplementation promote proper lung maturation throughout development? A VA-deficient diet can lead to premature birth, low-birth weight, and incomplete development of
organs such as the lungs, resulting in respiratory complications [17, 110, 150]. Therefore, we explored the effect of VARA supplementation on the mRNA expression of proliferative retinoid-regulated genes involved in lung maturation and septation. To examine extracellular matrix components important for secondary septation, we determined the expressions of elastin and αSMA. To examine proper microvascular development, fibroblast maturation necessary for secondary septation initiation, and surfactant protein biosynthesis, we determined the expression of growth factors and receptors VEGF, PDGFRα, and EGFR respectively [149]. We then observed the expression of surfactant proteins A, B, and C, which are important for the reduction of lung surface tension and preventing the collapse of airspace and reduction [67, 149].

Aim 2: To investigate morphological changes in neonatal lungs associated with VA marginal nutritional status and VARA supplementation.

A combination of quantitative morphometry and immunohistochemistry techniques were used to investigate lung augmentation in response to VARA. VARA was orally supplemented to neonatal rats of a VA marginal status at crucial times in lung septation: postnatal (P) day 1 (saccular stage; equivalent to 32 weeks gestational age in human infants regarding lung development), P4 (equivalent to a term infant, beginning of lung rodent lung septation), P7, P10 (mid-ending of lung septation), and 2 months (human equivalent to young adult, complete lung development). Hemotoxylin-eosin (H&E) stained sections of these lung tissues allowed for visual morphological changes in lungs after VARA supplementation and for analyses of morphology to determine airspace perimeter, alveolar tissue area, and alveolar wall thickness.
4.4 Methods and Materials

*Animals and experimental design*

Refer to Chapter 2: Methodology section 2.1.1 and 2.1.2.

*Dose preparation*

Refer to Chapter 2: Methodology section 2.1.3

*Tissue Collection*

Refer to Chapter 2: Methodology subset section Lung Inflation and Histological Preparation

*Lung Morphometric Analysis*

Refer to Chapter 2: Methodology section 2.3.2

*Gene mRNA level determination*

Refer to Chapter 2: Methodology section 2.5.1 in reference to Study 2 for mRNA analysis and 2.5.2
4.5 Results

We investigated the expression of extracellular matrix components, growth factors, and growth factor receptors in lung tissue from birth to adult age and whether the expression of these genes are altered after VARA supplementation by using qPCR. We also investigated whether there are any alterations in lung morphology after supplementation by performing H&E staining on lung tissue sections and using MatLab to analyze the results.

*VARA altered gene expression of key players involved in secondary septation*

Extracellular matrix components $\alpha$SMA (Fig. 4.2A) and elastin (Fig. 4.2B), important for differentiation alveolar myofibroblasts and the initiation of secondary septation, were expressed throughout neonatal lung development (black bars), with a decrease in expression in adult lungs, parallel to results seen in various studies, as alveolarization progresses, elastin [146] $\alpha$SMA [151] decline in expression, with minimal expression in adult lungs. VARA administration did not effect elastin expression; however, VARA caused a significant decrease in $\alpha$SMA expression at P1. Interestingly, only adult lungs responded to VARA supplementation with an increase in expression of growth factor VEGF (Fig. 4.2E) and decrease in expression of receptors PDGFR$\alpha$ (Fig. 4.2C) and EGFR (Fig. 4.2D).
Figure 4.2. Relative levels of mRNA transcripts for extracellular matrix components and growth factors in lung.
Developmental expression pattern (black bars) of extracellular matrix components α-sma and elastin (B) was constant in neonatal lung, with attenuation in adult lung. VARA significantly decreased αsma expression at P1 (blue bar); however VARA had no effect on expression at later ages and none at all in elastin expression. Growth factor receptors Pdgfrα (C) and Egfr (D) and growth factor Vegf (E) had no change in developmental pattern of expression (black bars) from birth to adult age. VARA treatment significantly decreased Pdgfrα and Egfr (blue bars) expression but significantly increase Vegf in adult lungs.
VARA does not alter surfactant gene expression during secondary septation

Surfactant proteins are synthesized, stored, and released by lamellar bodies in type II epithelial cells of the lung [57]. Its production correlates with lung maturation and is important to prevent the collapse of airways during respiration. Based on our findings, surfactant protein a (SP-A) increased in expression in adult lung (Fig. 4.3A, black bars) while SP-B (Fig. 4.3B) and SP-C (Fig. 4.3C) did not display a developmental expression pattern (black bars). However, VARA supplementation decreased expression of SP-A and SP-C in adult lungs (gray bars), with a significant increase in SP-B expression in neonatal lungs at P1 (gray bar). Interestingly, response to VARA supplementation in neonatal lung was outside the crucial time periods in which rodent lung septation and alveolarization takes place (P4 – P14) [57, 63, 90, 152] with no change in surfactant protein expression at P4 or at P10.
Figure 4.3. Surfactant protein expression in lungs of neonatal to adult rats fed VAM diet supplemented with or without VARA
SP-A (B) and SP-C (C) displayed constant ontogenic expression from birth to adult age (black bars). SP-A expression remained constant in neonates, with significant increase in expression in adult lungs. VARA supplementation (blue bars) significantly decreased SP-A and SP-C transcript expression in adult lungs, with a significant increase of SP-B in perinatal lung at P1.
Effect of VARA on Lung Morphology

The morphology of non-supplemented rat lungs displayed a natural progression in lung development with age. At P1, the saccules were large and thick-walled, displaying the immaturity of the gas-exchange units. By P4, the beginning of the alveolar phase and the subdivision (septation) of the saccules began displayed by thinner walled alveolar tissue. At P10, areas of projections of the secondary septa were visible. Microvascular maturation takes place at P14-P21 [145]. Therefore, the completion of septation, alveolarization, vascularization and overall maturation was displayed in adult lung tissues with visible deposition of capillary beds, increased number of airspaces, and the complete transition of double to single capillary network of the alveolar wall for optimal respiration. After VARA supplementation, although slight, there were areas of visible alterations in the morphology of the lungs throughout postnatal development in rats. Saccule walls appeared thinner at P1, and there were areas of elongated septa by P4 instead of at P10. By P10, areas of vascularization with the deposition of capillary beds were visible. By adult age, there was a slight increase in airspace abundance with increase presence of red blood cells (Fig. 4.4A).

A morphometric analysis was performed to quantify the cross-sectional area, perimeter, and thickness of the alveolar tissue in each section in order to determine morphological differences between age and treatment as described in Chapter 2: Methodology section 2.3.2. Although visual changes were observed in H&E stained lung sections, morphological measurements confirmed that these changes were not significant with or without VARA supplementation (Fig. 4.4B). Even though there was no significant change based on our morphometric measurements between treatment groups, our results displayed a developmental increase in alveolar perimeter with age (p = 0.0029) and a developmental decrease in alveolar
wall thickness with age ($p = 0.0150$), which resembles the change expected throughout lung development [57, 58, 63].
Figure 4.4 Morphometric analysis of lungs after VARA supplementation

Developmental progression of the post-natal lung beginning at birth to adult age of oil group (placebo; A), P1 representing saccular phase of lung development and alveolar phase beginning at P4 to P10. Arrows point to areas of elongated secondary septa, representing initiation of alveologenesis. Thick-walled primitive airway saccules are visible at P1 with progression of development seen at P4. At P10, the black arrow indicated elongation of secondary septa at P10. Areas of red represent capillary bed deposition visual in adult lung. VARA supplemented group display slight alteration in saccules at P1 with thinner walls. In P4 lungs, there were areas of elongated secondary septa, which was more prominent by P10. In addition, capillary bed visual at P10. Adult lung has increased areas of red blood cells and airspaces. In Panel B, morphometric analysis of perimeter, alveolar area, and alveolar wall thickness are shown from graphs displayed from left to right. Linear increase in perimeter (P = 0.0029) and linear decrease in alveolar wall thickness (P = 0.0150), with no significant difference between treatment groups.
4.6 Discussion

All stages of lung development are crucial for the proper development of the lung. During the saccular and alveolar phase of lung development time period in humans, the accumulated storage of VA in the lung is utilized, concomitant with further expansion and septation of the airways [96, 140, 153]. The development of the lung during the saccular and alveolar phases is essential for independent respiration of the fetus once born. There are several key players during septation and maturation of the lungs, which include extracellular matrix components (αSMA and elastin), growth factor and receptors (VEGF, PDGFRα, and EGFR), and surfactant proteins (SP-A, SP-B, and SP-C). Previous studies have investigated the expression of these genes postnatally in rodent [146, 151, 154, 155] and human [156] lungs. What is unique about our study is that we have explored the ontogenic expression of these extracellular matrix components and growth factors postnatally in rats having a VA-marginal status, thus resembling the most common human VAD at-risk population, and we have examined the changes in developmental expression pattern of retinoid homeostatic genes, after administration of a unique form of VA supplementation known as VARA. In addition, we have been able to examine the saccular and alveolar phase postnatally in rats, which in humans takes place during gestation. We concurrently have investigated the change in morphology of these rodent lungs after VARA supplementation. By determining effects of VA nutritional status and VARA supplementation on the ontogenic expression of genes crucial for lung septation, we can better understand the requirements for VA and RA in neonates.

Based on our findings, we show that even with a VA marginal status, the expression patterns of extracellular matrix components αSMA (Fig. 2A) and elastin (Fig. 2B) in lungs resembled those observed in well-nourished lungs [151, 156, 157], with a decrease in expression in adult compared to neonatal tissue. This suggests the necessity of extracellular matrix
components during secondary septation (P4-P14) of the lung, which, however, may no longer be necessary by adult age. We also observed that the steady expression pattern of growth factor and receptor PGDFRα (Fig. 2C) VEGF (Fig. 2E) expression in our rats resembled that reported for well-nourished lungs [155, 158]. On the other hand, various studies have shown that EGFR expression increases throughout postnatal lung development [159, 160]. Meanwhile, our findings suggest that there is no developmental pattern of expression of EGFR in the lungs of VA marginal rats. Growth factors EGF and transforming growth factor (TGF) both bind to EGFR [62]. Activation of EGFR stimulates biosynthesis of surfactant precursors, induces fetal branching morphogenesis, accelerates fetal lung maturation, and is crucial for lung septation and maturation of the lung [62, 149]. Expression of EGFR is indirectly dependent on the bioavailability of RA [96], thus affecting production levels of surfactant. This may explain the stagnant ontogenic expression of EGFR in our rat lungs. The developmental increase of SP-A driven by the increase in adult lung transcript levels seen in our results was also observed in a previous study by Schmiedl et al [161].

Interestingly, VARA supplementation altered the level of transcript expression of genes for extracellular matrices, growth factors and receptors, and surfactant proteins at times outside of the secondary septation window (P4 – P14) with a significant decrease in αSMA and significant increase of SP-B at P1, as well as a significant decrease in PDGFRα, EGFR, SP-A, and SP-C genes and a significant increase in VEGF expression in adult lung tissues. Our findings therefore suggest that secondary septation is a tightly regulated process. The response to VARA was surfactant type specific. Although the specific functions of each surfactant protein have not been fully elucidated, transcript levels of surfactant proteins examined in our study was in increasing order of SP-C > SP-A > SP-B. VARA altered perinatal expression of SP-B in neonatal lungs with a significant increase in expression, thus displaying early sensitivity to increase VA supply; whereas, VARA significantly decreased SP-A and SP-C expression in adult tissue where these
proteins may not be necessary in a fully developed lung. The same can be speculated for the significant decrease in growth factors PDGFRα and EGFR expression in adult lung in response to supplementation.

Although there were slight changes in lung morphology after oral administration of VARA (Fig. 4A), these changes were not found to be significant based on our morphometric analysis of perimeter, cross-sectional area, and wall thickness of alveolar tissue in H&E stained lung tissue sections (Fig. 4B). Despite these findings, there is a possibility that even a slight change in lung structure may result in functional changes. VA deficiency can result in pulmonary hypoplasia [91, 106] and is associated with a variety of lung disorders [17, 150, 162], especially in neonates. We are not able to say whether the VA-marginal dietary standing in our rat model has resulted in compromised respiratory functions, but slight alterations in development such as earlier signs of thinner primitive saccule walls at P1, secondary septation at P4 instead of at P10, vascular deposition at P10, and increased vascularization in adult tissue, which may be a result of increased VEGF expression (Fig. 2E) after VARA supplementation, may serve as beneficial [61]. Further studies are needed to understand the functional benefits of VARA in the neonatal lung.

In summary, the late stages of lung development that are necessary for the expansion of the airways and maturation are influenced by a wide variety of signaling molecules which includes transcription of growth factors, extracellular matrix components, and surfactant proteins. Although the expression of these molecules has been explored extensively during fetal lung organogenesis, there has not been a study that explores all of these genes collectively throughout post-natal lung development, especially at a VA marginal status. We were able to determine the transcript expression pattern of these genes postnatally in rat lung in animals with a VA marginal nutritional status and the alterations of expression of these genes after VARA supplementation. At the same time, we explored morphological differences in supplemented versus non-supplemented lungs. Identification of the effects of VARA on lung alveolarization and
mechanisms of action may eventually help to optimize therapy for preterm infants as well as term infants with marginal VA stores.
Chapter 5

BLEOMYCIN INDUCED BRONCHOPULMONARY DYSPLASIA STUDY

5.1 Abstract

Bronchopulmonary dysplasia (BPD) is a chronic lung disorder common in preterm infants, characterized by acute inflammation with signs of early fibrosis in the lung, which can lead to arrest of normal maturation. Clinically, treatment with VA has been shown to reduce BPD and improve outcomes in very low birth-weight infants (VLBW) infants. Experimentally, RA is remarkable in its ability to promote alveolar septation, as shown in models of septation failure caused by dexamethasone and oxygen treatments. In the present study, we used a chemotherapeutic antibiotic, bleomycin (BLM) sulfate, to induce chronic lung injury resembling BPD, in neonatal rats. We hypothesized that VARA supplementation will attenuate inflammation and repair lung morphology of BLM-induced BPD in neonatal rats. Rat dams and their post-weaning offspring were fed a VA-marginal diet, while pups received a daily i.p. injection of BLM sulfate (0.4µl/µg adjusted to 5µl/g body weight) one day after birth for 5 days (P1-P5) and then were orally supplemented with VA and retinoic acid (VARA) or placebo (canola oil) on alternating days from P6 – P14. At P5 and P15, pups were euthanized for lung tissue collection. BLM treated neonates had a significantly reduction in total retinol concentration in plasma at P15, which was restored after VARA supplementation. BLM also led to increased expression of interleukin-6 (IL-6) at P5 and increased tumor necrosis factor (TNF)-α at P15 in lungs; however, VARA did not alter the expression of these cytokines. We observed increased immune cell
infiltration and hemorrhaging in hemotoxylin and eosin-stained lung tissue after BLM treatment, which was reversed after VARA supplementation. There was improvement in alveolar wall integrity. BLM treated neonates had increased deposition of collagen, a marker of the early presence of fibrosis, as well as disrupted secondary septation, represented by the abnormal deposition of elastin in lung tissues. VARA reversed fibrosis and restored elastin deposition at the secondary septal crest. Proliferative damage of lungs due to BLM treatment resulted in decreased expression of Ki67 in lung tissue, while oral supplementation with VARA significantly increased Ki67 expression. Our findings suggest the potential for therapeutic benefits of VARA in treatment of lung injury resembling BPD in neonatal rats.

5.2 Introduction

Vitamin A (VA) is important for a variety of physiological events and functions including lung differentiation, growth, and health [52, 96]. VA has implications of prevention and repair of lung injury in newborns [65, 94, 95, 100, 110, 163, 164]. Vitamin A deficiency (VAD) results in pulmonary hypoplasia and is associated with a higher incidence of BPD [162]. BPD is chronic lung injury that commonly affects low-birth weight preterm infants within the first four weeks of birth [103], characterized by impaired alveologenesis resulting from the impact of inflammation, oxygen toxicity, infection, followed by pulmonary fibrosis due to poor recovery of the immature lung [149]. As a result, independent respiration of neonates becomes difficult, making BDP the main cause of morbidity in preterm infants.

We have shown that a unique combination of the nutrient vitamin A (retinol) and its active metabolite retinoic acid (RA) increases cellular uptake and storage of VA (VARA) [34, 45, 53-55, 118], promote alveolar septation as shown in models of septation failure caused by dexamethasone[118] oxygen [134] treatments, and attenuate inflammation [134]. The promotion
of cellular storage of VA, referred to as retinyl esters (RE), may be important in for usage in times of VA deprivation of the lung tissues. Our previous results suggest that VA may serve as a substrate in promoting RE accumulation as well as further production of RA. Although not a substrate for RE synthesis, RA may serve as the driving force for the metabolism and distribution of VA through changes in gene expression [39]. In our present study, we proposed that this combination of VA and RA (VARA), will attenuate inflammation, repair or reverse inflammatory induced injury in lung, and increase RE levels that may be beneficial in improving lung maturation and septation in neonates with compromised lung development due to a deficient VA dietary status and induced inflammation. We used a chemotherapeutic antibiotic, BLM sulfate, to induce chronic lung injury to reflect a clinically relevant BPD model [39] in neonatal rats. Our overall rationale is that further investigation on elucidating beneficial effects of VARA supplementation on the lung may provide insight on possible therapeutic interventions for treatment of chronic lung injury in neonates.

5.3 Hypothesis and Aims

**Hypothesis:** VARA supplementation will attenuate inflammation and repair lung morphology of BLM-induced BPD in neonatal rats.

**Aim:** To determine whether VARA will alter cytokine expression, repair morphological damage of lungs, promote cell proliferation, and restore secondary septation in bleomycin-induced BPD neonatal rats compared to non-supplemented neonatal rats.

In this study, we measured total retinol concentration in plasma and lungs of neonates using UPLC and mRNA expression of a variety of pro-inflammatory cytokines using qPCR in lung tissues to determine inflammatory response of BLM sulfate and after oral supplementation
of VARA. BLM sulfate, derived from bacterium *Streptomyces verticillus*, has been used clinically as a chemotherapeutic antibiotic [165]. Its mode of action causes single and double stranded DNA breaks by disrupting cell cycle through the overproduction of reactive oxygen species [166, 167], which is inhibited by BLM hydrolase activity [168]. The lung has low activity of BLM hydrolase, making the lung susceptible to BLM-induced damage [167, 169]. To determine morphological damage, elastin deposition for secondary septation, and fibrosis, we stained lung tissue sections (5 µM) with hemotoxylin and eosin (H&E), Hart’s Elastin Staining, and Masson’s Trichrome Staining, respectively. At the same time, we measured change in proliferation by immunohistochemical staining of nuclear marker Ki67 in lungs after BLM treatment and oral VARA supplementation.

5.4 Materials and Methods

*Animals and experimental design*

Refer to Chapter 2: Methodology section 2.1.1 and 2.1.2. Upon birth, pups were randomly assigned to 5 different treatment groups: Saline (Control) and BLM groups euthanized at P5, Saline + PBS (Placebo), BLM, and BLM + VARA groups euthanized at P15 (*Table 5.1*).
Table 5.1. Experimental Groups

Animals were maintained a VA marginal diet maternally and postnatally throughout experimental process (5.4 Methods and Materials: Animal and experimental design). At birth, pups were randomly assigned to 5 treatment groups: a control (saline) and BLM treated group at P5; control (saline + Oil), BLM + Oil, and BLM + VARA. The numbers of pups per group (n) were as listed in Table 5.1.

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Treatment

Intraperitoneal injection of bleomycin

Neonatal pups received a single intraperitoneal (i.p.) injection of BLM daily beginning one day after birth (P1) until P5 at a concentration of 0.4 µg/µl (5µl/g adjusted to body weight) (Fig. 5.1). Bleomycin sulfate (Cayman Chemical, Ann Arbor, MI) was reconstituted in Dulbecco's Phosphate Buffered Saline (DPBS) 1X (Corning Inc., Manassas, VA) to adjust for dose concentration. Controls received i.p injection of DPBS.
BLM was administered to rat pups through intraperitoneal injection beginning one day after birth (P1) until P5 at a concentration of 0.4 µg/µl adjusted to body weight (5 µl/g bw). Control group received 1X DPBS. Supplementation of VARA (or placebo) began at P6 every other day up to P14. Animals were euthanized for collection of plasma and lung tissues at P15 for analysis.

Figure 5.1. Animal experimental design
VARA Dose preparation

Refer to Chapter 2: Methodology section 2.2. The times of dosing are described in Fig.

5.1.

Tissue Collection

Refer to Chapter 2: Methodology section 2.3 introduction and subset section Lung

Inflation and Histological Preparation

Immunohistochemistry

Refer to Chapter 2: Methodology section 2.3.1

Lung Morphometric Analysis

Refer to Chapter 2: Methodology section 2.3.2

Gene mRNA level determination

Refer to Chapter 2: Methodology section 2.5.1 in reference to Study 3 for mRNA analysis and 2.5.2
Statistical analysis

Refer to Chapter 2: Methodology section 2.5.3

5.5 RESULTS

VARA restored plasma retinol in BLM treated neonates

To determine effect of BLM injection and intervention of VARA supplementation we measured total retinol concentration of plasma and lungs of neonatal rats using UPLC. Our results show that there was no difference in total retinol concentration after BLM treatment in lungs (Fig. 5.2C) and plasma (Fig. 5.2A) of P5 pups. However, at P15, BLM treatment significantly decreased plasma retinol levels (Fig. 5.2B). After VARA supplementation, plasma retinol levels were restored. Total retinol concentration in lung tissues at P15 (Fig. 5.2D) did not change after BLM treatment in comparison to control group, yet VARA supplementation significantly increased lung retinol levels by approximately 40 µg/g. These results show the age and tissue specific response to BLM treatment.
Figure 5.2. Total retinol concentration of plasma and lung after BLM treatment and VARA supplementation
Total retinol concentrations in plasma (A) and lung (C) of neonates at P5 did not change after BLM treatment. By P15, plasma retinol concentration (B) significantly decreased in BLM treated neonates, but significantly increased to that comparable to control group after VARA supplementation. BLM did not affect total retinol concentration in lungs at P15 (D); however, VARA supplementation significantly increased total retinol concentration by approximately 40 µg/g). Data are shown as mean ± SEM, n ≥ 3/group. One-way ANOVA with posthoc analysis was used to determine differences at P15 between treatment groups. At P5, PBS vs. BLM groups were compared t-test. Means without a common letter differ significantly (a>b>c>d) indicates P < 0.05.
VARA did not attenuate inflammation in BLM treated neonates

To confirm that inflammation was induced in our neonatal rats, we determined the expression of certain inflammation markers in the lung by real-time PCR. At P5, there was significant elevation of IL-6 expression in lung (Fig. 5.3A), representing an early inflammatory response to BLM treatment. At P15, IL-6 was no longer elevated and displayed no response to BLM treatment. However, there was significant elevation in pro-inflammatory cytokine TNFα (Fig. 5.3B), but remained elevated after VARA supplementation. IFNγ is a cytokine critical for immunity against viral or bacterial infection [170]. Suppression of this cytokine increases susceptibility to infection. In our results, BLM treatment significantly decreased IFNγ at P15 in neonatal lungs and remained attenuated after VARA supplementation. These results demonstrated that neonatal rats that received BLM experienced a state of inflammation. On the other hand, VARA supplementation did not restore alterations in inflammatory to that seen in control group.
Figure 5.3. Cytokine expression in neonatal lung after BLM treatment and VARA supplementation
BLM significantly increase IL-6 (A) expression at P5 in neonatal lungs. By P15, TNFα (B) expression significantly increased while IFNγ (B) expression significantly decreased in response to BLM treatment. VARA supplementation did not restore expression of these cytokines levels comparable to control group. Data are shown as mean ± SEM, n ≥ 3/group. One-way ANOVA with multiple t-test posthoc analysis was used to determine differences at P15 between treatment groups. At P5, PBS vs. BLM groups were compared using multiple t-test. Means without a common letter differ significantly (a>b>c>d) indicates P < 0.05.
BLM increased hemorrhaging and immune cell infiltration in neonatal lungs

To explore changes in morphology between treatment groups in response to BLM and possible restoration after VARA supplementation, we examined H&E stained neonatal lung tissue of pups from experimental groups (Fig. 5.4A). At P5 we see a slight increase in airspace perimeter due to the thinning of the alveolar walls, infiltration of neutrophils and macrophages in airspace lumen, which correspond to the elevation of cytokine expression (IL-6) seen in qPCR results (Fig. 5.3A), and areas of hemorrhage in the airspaces. At P15, there was a further increase in immune infiltration and an increase in hemorrhage. Morphometric analysis of perimeter, cross-sectional area, and thickness of alveolar tissues did not demonstrate significant differences between BLM vs. PBS (control) at P5 (Fig. 5.4B-D). At P15, VARA supplementation restored loss in alveolar wall integrity (Fig. 5.4G).
Figure 5.4. Alterations of lung morphology in response to BLM treatment and VARA supplementation
BLM treatment resulted in infiltration of immune cells and hemorrhaging into airway lumen at P5 (A; Control vs. BLM). At P15, BLM caused increased hemorrhaging and immune cell infiltration, with no major change in overall structure of lung tissue. VARA supplementation reversed areas of hemorrhage and immune cell infiltration of the airspace in neonatal lungs. Morphometric calculations of perimeter (B), cross-sectional area (C), and thickness (D) of alveolar tissue showed no significant difference at P5. In P15 neonatal lungs, neither BLM nor VARA altered perimeter (E) or cross-sectional area (F) of alveolar tissue. However, slight decrease in alveolar wall thickness was restored after VARA supplementation (G). H&E stained image magnification 20X.
VARA supplementation reverse early onset of fibrosis in neonatal lungs

To determine further damage caused by BLM and therapeutic benefits of VARA, lung tissue sections of neonates were stained for collagen deposition using Masson’s Trichrome Staining method for the presence of fibrosis, and imaged at 40X magnification. Collagen is ubiquitously expressed in lung tissues (Fig. 5.5A, C) and important for branching morphogenesis, alveologenesis, and maintenance of lung integrity [171]. However, increased deposition of collagen is indicative of a fibrotic activity [167, 171, 172]. Based on our results, BLM treatment increased collagen deposition (blue stain) in neonatal lung tissues at P5 (Fig. 5.5B) with a more dense deposition at P15 (Fig. 5.5D). The appearance of collagen decreased after supplementation (Fig. 5.5E) suggesting that VARA may reverse early presence of fibrosis in neonatal lung injury.
To determine presence of fibrosis in response to BLM treatment, we stained for the presence of collagen using Masson’s Trichrome Staining protocol (see Chapter 2: Methodology section 2.3.1). Image labeled Positive Control that of mouse bone serves a staining reference and positive control. The colors of stained tissues is as follows: collagen – blue; nuclei – black; Muscle, cytoplasm, and keratin – red. At P5 (A) and P15 (C), collagen (blue) is ubiquitously expressed in neonatal lung tissues. BLM treatment increased expression of collagen within alveolar epithelium (B) with increased staining intensity at P15 (D). VARA supplementation (E) decreased collagen expression in BLM treated neonates to seen in control lungs (C). 40X magnification of image.

**Figure 5.5. Masson’s Trichrome Staining for collagen deposition in BLM treated lungs**
Elastin deposition at the secondary septal crest is necessary for secondary septation, a process completed by P14 in rodents, for the formation of alveoli and complete maturation of the lung (Fig. 4.2). Formation of the alveoli results in expansion of lung surface area, necessary for optimal respiration. Various studies confirm that BPD pathogenesis is linked to structural lung immaturity and an arrest of development during the transition from a saccular to alveolar phase [103, 149]. In the present study, we stained neonatal lung sections for the presence of elastin using Hart’s Elastin Staining protocol (See Chapter 2: Methodology section 2.3.1) for presence of elastin deposition in response to BLM treatment. In control group (A), neonatal rats that did not receive any treatment of BLM or VARA displayed a normal distribution of elastin deposition localized in the periphery of lung tissue septa. Based on our findings, BLM administration caused the abnormal distribution of elastin expression in several areas lacking secondary crests within the alveoli (Fig. 5.6B), which is then restored after VARA supplementation (Fig. 5.6C). Therefore suggesting restoration properties of VARA supplementation in BLM-induced BPD in neonatal rats.
In reference to control group (A) with elastin deposition at septal crest (black arrows), BLM caused abnormal distribution of elastin in areas outside of the secondary septal crests (red arrows; B). VARA supplementation (C) restored the normal distribution of elastin (black arrows), with appeared increase in deposition of elastin in secondary septal crests. Images taken at 40X magnification.

Figure 5.6 Lung elastin expression after BLM treatment and VARA supplementation.
VARA supplementation promoted cellular proliferation after BLM-induced lung injury

To further investigate the repair properties of VARA, we performed an immunohistochemical staining for the presence of ki67 antigen (brown) in lung tissues (See Chapter 2: Methodology section 2.3.1). Ki67 protein is highly expressed in proliferating cells and serves as a marker for proliferation [173]. Neonatal chronic lung injury results in pulmonary hypoplasia, therefore we determined whether possible loss of proliferation in the presence of BLM would be restored after VARA supplementation. In our control pups at P5 (Fig. 5.7A) and at P15 (Fig. 5.7C), Ki67 is highly expressed in alveolar tissue. BLM treatment significantly decreased expression of Ki67 in neonatal lung tissue (Fig. 5.7B,D), which was then restored at P15 after VARA supplementation (Fig. 5.7E). Our results suggested that loss of cellular proliferation in BLM-induced lung injury of neonatal rats is restored after VARA supplementation, which suggest proliferative properties of VARA.
5.7. **Immunohistochemical staining of Ki67 in neonatal lung tissues**

Immunohistochemical staining of nuclear proliferative marker, Ki67 (brown), was expressed throughout alveolar tissue of control group (A, C). BLM treatment significantly reduced expression of ki67 (P = 0.0176) at P5 (B) and at P15 (D; P=0.0174) confirmed by Ilastik morphometric analysis (F, G). After VARA supplementation at P15 (E), there was a significant increase (P=0.0003) in ki67 expression in alveolar tissue. Data are shown as mean ± SEM, n ≥ 3/group. One-way ANOVA with multiple t-test posthoc analysis was used to determine differences at P15 between treatment groups. At P5, PBS vs. BLM groups were compared using multiple t-test. Means without a common letter differ significantly (a>b>c>d) indicates P < 0.05.
5.6 Discussion

VA storage is apparent in the fetal lung in the last trimester; it undergoes a rapid depletion right before birth and throughout early post-natal lung development [140], which is believed to be utilized to supply the VA demand by the lung, necessary for rapid growth and differentiation of the lung tissue. Infants born prematurely often miss the gestational period in which adequate VA accumulates. As a result, preterm infants, who are commonly born at a low birth weight, begin life with significantly less VA than term infants, resulting in a state of VA deficiency (VAD). Consequently, VAD is often observed in premature infants and associated with a higher incidence of susceptibility to respiratory complications such as BPD. BPD is the most common cause of death in preterm infants. With VA serving as a substrate for regulatory genes that produce RA, and RA serving as the driving force for increased storage and distribution of retinoids, VARA (VA and RA combined; 1:10 molar ratio) works synergistically to increase VA storage and uptake in neonatal lungs [53-55, 134]. Studies have shown that VARA significantly increased LRAT (VA storage) and STRA6 (VA uptake). In other studies, retinoid supplementation in both human [21, 50, 108, 164] and rodents [65, 92, 134] has been shown induced lung maturation, reversed the failure of alveolar, and reduced mortality in neonates with chronic lung injury. Our present study sought to determine whether treatment with VARA might provide similar benefits, involving molecular mechanisms critical for postnatal lung development, using a BLM-induced BPD neonatal rat as our model. Our results showed that VARA preserved alveoli formation, reversed early fibrosis, promoted proliferation, and maintained alveolar wall integrity. To our knowledge, this is the first report describing a beneficial effect of VARA on preventing alveolar hypoplasia caused by BLM-induced BPD during neonatal lung development.

Alveolar formation is a crucial stage involving the transition from a rudimentary lung bud to a mature gas-exchange system. An adequate supply of VA is necessary for proper formation
of the lung. Our findings show that BLM treatment further suppressed plasma retinol levels. Although pups were fed and maintained a marginal diet maternally and weaned (average plasma retinol concentration 0.961 µmol/L), BLM did not alter plasma or lung retinol concentrations at early onset of lung injury (P5). BLM has been shown to induce acute lung injury results in a switch from acute inflammation to fibrotic development within a 9-day period [166]. Even though we seen an elevation in $\text{Il-6}$ expression in BLM treated lungs at P5, the response to BLM induced inflammation may take longer than 5 days to cause an attenuation in total retinol concentrations. In a previous study by Wu et al [131], LPS-induced inflammation in neonatal rats did not alter total retinol concentration in the lungs. The same was observed in this present study in both P5 and P15 BLM-treated rat lungs. However, VARA restored plasma retinol concentration of BLM treated neonates (P15), and as previously seen in the presence of inflammation [131], VARA significantly increased total retinol concentration in lung tissue. Therefore, VARA improved VA status in neonatal lungs in the presence of lung injury.

We explored a variety of pro-inflammatory cytokines that have been shown to be elevated in BPD, clinically or in animal models [103, 167, 174, 175]. However, $\text{Il-6}$ was the only cytokine highly expressed after BLM treatment in P5 lungs. At P15, TNF\(\alpha\) significantly increased and $\text{Ifny}$ significantly decreased in expression in P15 lungs. However, VARA supplementation did not impact cytokine expression after BLM treatment. Interestingly, infiltration of immune cells and increased hemorrhaging in response to BLM-induced lung injury was reversed after VARA supplementation, seen in our H&E stained lung sections. Although alveolar wall structure was not significantly altered in the presence of BLM, VARA was able to restore the slight decrease in alveolar wall thickness of BLM-injured lungs. Perhaps the impact of VARA on inflammation depends on the lung injury model and when VA or RA exerts pro-or anti-inflammatory effects. While various studies have showed that treatment with VARA of neonatal rodents has reduced and prevented increases in gene expression of several pro-
inflammatory cytokines such as *Il-1β*, *Il-5*, and *Tnfα* [118, 134], other studies have shown that maternal VA supplementation increased levels of oxidative products and induction of antioxidant-defensive enzymes in offspring [176]. Further research is needed to elucidate the effects of VARA supplementation in neonates.

Despite the fact that VARA did not attenuate cytokine expression in neonatal lung in pups treated with BLM, we were able to demonstrate other therapeutic properties of VARA supplementation in our BPD rodent model. Our histopathological findings show that BLM increased collagen deposition, which serves as a marked for the presence of fibrosis. VARA remarkably reversed collagen deposition in alveolar tissue. We understand that correct deposition of elastin is required for secondary septation of the lungs. BLM caused an abnormal distribution elastin, which was then restored towards a normal distribution after VARA supplementation. Repair properties of VARA were further solidified in our Ki67-stained lung tissues. Although BLM treatment caused cell death and attenuation of Ki67 expression, VARA promoted cell proliferation exemplified by a significant increase in Ki67 even in the presence of BLM. We speculate that the increased total retinol concentration (RE + retinol) after supplementation shown by our UPLC results may have promoted the proliferative and transcriptional properties of RA. In turn this could have allowed for restored secondary septation of damaged lungs.

A few limitations of our study merit consideration. There are various routes of administering BLM, which, in various studies, has been given intravenously, intratracheally, intraperitoneally, or subcutaneously [167]. We decided to use the intraperitoneal (i.p.) route of administration because it has been shown to be effective in initiating lung injury in neonatal rats [174]. In addition, we administered BLM beginning 1 day after birth; therefore, this route of administration was the safest and provided the highest rate of survival. Although intratracheal instillation has been shown to be the best method to resemble the histopathological fibrosis of the lungs in BPD, the i.p. injection model reflects more of the systemic damage. As a result, our
morphometric measurements may not adequately reflect the damage we would expect if BLM were administered intratracheally. Secondly, there was considerable variation in the severity of BLM-induced lung injury in neonates. This variation may account for the lack of immune response we expected to observe in our BPD animal model. As a result, anti-inflammatory properties of VARA may not have been adequately reflected in our findings. A larger sample size might have allowed for a better test of our hypothesis and representation of results. Finally, the response to BLM is age dependent. As noted above, BLM hydrolase activity inhibits BLM’s mode of action. Umezewa and coworkers [177] showed that BLM hydrolase activity is greater in the lungs of newborn mice than in the lungs of mature mice, reflecting age-related resistance. We used daily exposure to BLM up to 5 days after birth to make up for possible resistance to BLM treatment. Possibly by increasing the days of BLM treatment we might have observed more profound effects; however, survival rates would have been compromised (data not shown).

In conclusion, the results of the present study provide evidence to suggest that even in a state of moderate inflammation, VARA could still be an effective therapeutic strategy for improving VA status in the neonatal lung. Currently, the treatment for BPD includes oxygen supplementation, ventilators, steroids and nutrient administration [103]. In this study, I have shown that i.p. injection of BLM in neonatal rats results in decreased plasma retinol concentration, increased inflammation, and histopathological damage, that results in arrested secondary septation and may serve as an ideal rodent surrogate model for studying BPD. Furthermore, I have shown that oral treatment with VARA can prevent alveolar hypoplasia by restoring normal deposition of elastin, reversing signs of early fibrosis, and promoting proliferation. These findings provide further insight to VARA as an additional or alternative mode of therapy for BPD, or similar chronic lung diseases in preterm/term infants.
Chapter 6

DISCUSSION

6.1 Developmental expression pattern of retinoid homeostatic genes from birth to adult age

Although studies on the expression of genes participating in retinoic acid biosynthesis and degradation during embryogenesis, and in adults, have been conducted in various models, yet little is known about their pattern of expression during intense growth in neonates. Therefore, there are significant gaps in fully understanding VA metabolism during early development, especially during postnatal development. We presented here a study on retinoid homeostatic gene expression in principal VA storage organs -- lung, liver, and kidney -- of VAM neonatal rats, examining tissues at time periods that are considered crucial for secondary septation and alveolarization (P1, P4, P10,) of the lung, and in adult rats. In addition, we examined total retinol content (sum of retinol + RE) in the plasma, lung, and liver of neonatal and adult rats. As mentioned earlier, half of the world’s population that is affected by VAD is preschool-age children. Therefore, we reasoned that a sustained VAM status would be of clinical relevance for understanding the overall metabolism of neonates, in a VA-compromised situation that highly represents the VAD population in developing countries.

My findings were quite interesting in that the regulation of VA metabolism was tightly regulated in our neonatal rats, even at a VAM status. The total retinol concentration in the plasma (Fig. 3.1B), liver (Fig. 3.1C), and lungs (Fig. 3.1D) remained at constant levels. It was not until neonates reached adult age that we observed a significant increase in total retinol concentration in
the lung, which may we related to the significant increase in Lrat expression in adult lungs. VA is important for overall development and maintenance of the lung [52, 61, 96]. As rapid lung development takes place after birth, VA is rapidly being utilized, thus increasing the VA demand of the lung. It could be that this demand is even higher in VAM neonatal rats in comparison to their VAS counterparts. As a result, Lrat expression was low and constant during neonatal development (ontogeny). The increased expression of Lrat may suggest that adult lung tissue, once formed, may not have the same metabolic demand for VA as in neonates, therefore promoting increased deposition and storage of VA for later usage. The expression of Rbp4 (Fig. 3.2A) and Lrat (Fig. 3.2B) mRNAs in liver as well as Raldh-1 and -3 mRNAs in lung was also constant in neonates, with increased expression at adult age. These results suggest that VA accumulation in tissues through Lrat, increased circulation of retinol to extrahepatic tissues through Rbp4, and increased RA production to maintain the demands for RA in adults, through Raldh-1 (Fig. 3.4A) and -2 (Fig. 3.4C), is dependent on both diet/nutritional status and age. They may also suggest that adults, known to have a lower basal metabolic rate than neonates, are able to conserve dietary VA more efficiently and thereby are able to accumulate retinol in tissues, such as the liver and lungs, even when the intake of dietary VA is below normal levels. This may suggest that the amount of VA in the VAM diet actually meets the biological requirement for VA in the adult animal, and that any excess of VA is stored.

In the past 15 years, megalin, a multi-ligand receptor for retinol-RBP complex, has been investigated in other laboratories for its role on the conservation and recycling of retinol. Megalin is expressed in a number of organs [37], but is most highly expressed in the kidneys and responsible for the re-uptake of plasma ROH-RBP in the proximal tubules, therefore making the kidney responsible for approximately 50% of the circulating pool of the retinol [36, 38]. While the kidney is known for conservation and recycling of retinol, it is also the major site for RBP catabolism. In the same respect, the kidney plays a significant role in maintaining VA
homeostasis. The liver, lung, and to a lesser extend, the kidney have the capacity to store excess VA, confirmed by work of Nagy et al [178]. We explored the expression of Lrat in the kidneys, which showed that expression levels are comparable to those in liver tissue (data not shown), thus confirming the storage potential of the kidneys. Unlike the increased expression of adults, compared to neonates, of Lrat in the liver and lung, Lrat expression remained constant from birth to adult age (data not shown). Our main focus in the kidney was on its incredible capability of recycling retinol. Therefore, we determined the developmental expression of megalin in the kidneys, which we showed remained at constant mRNA levels throughout postnatal development (Fig. 3.5). Although not statistically significant, there were slightly higher levels of megalin at P4 and P10, a time period in which secondary septation takes place in the lungs.

In summary, our study in the Oil-treated placebo group provided valuable insights into VA metabolism and homeostasis during ontogeny from birth to adult age in the absence of treatment, in a VAM state. While various studies have confirmed the expression pattern of retinoid homeostatic genes in postnatal tissues at an uncompromised dietary status [30, 35, 36, 42, 114, 132, 133, 141, 179, 180], we are the first to explore the developmental expression pattern of retinoid homeostatic genes at a VAM dietary status in VA storage organs, from birth to adult age. Although we are unsure if the mRNA expression levels match those of VAS populations, our results provide a better understanding of how VA homeostasis is regulated in neonates at risk for VAD.

6.2 VARA regulation on neonatal retinoid homeostasis

Understanding of neonatal retinoid metabolism under conditions of supplementation is highly relevant from a clinical and public health perspective, as VA supplementation is a form of intervention to combat VAD. However, VA supplementation has not displayed consistently
beneficial results in infants and preschool age children in diverse populations [18, 117]. Based on previous analysis of gene expression, retinol concentrations in tissues, and kinetic studies, we have shown benefits of VARA supplementation on increasing VA uptake [34, 39] and RE storage in neonatal tissues. Previous kinetic studies showed that the addition of RA to VA increased the total time VA spends in the liver, lung, kidney as well increased total VA mass in these tissue [39]. In our current study, described in Chapter 3, we administered VARA, orally, at several time points known to be crucial for neonatal lung development. As previously described, expression of retinoid regulated genes necessary for the maintenance of VA homeostasis were found to be nearly constant throughout the neonatal development in the liver, lung, and kidney tissues. However, after VARA supplementation (6 h after last administration), there was a significant increase in expression of the majority of these genes, displayed in patterns that were both age and tissue dependent.

As expected based on previous studies in both VA sufficient and VA compromised rodents [39, 45, 53-55, 118, 134], in our current studies VARA supplementation significantly increased total retinol concentrations in the liver, lung, and plasma of neonatal and adult rats. What was interesting about our results was that an accumulative, approximately linear increase was observed in neonatal liver retinol concentrations (Fig. 3.1C), and a cumulative increase in total retinol concentrations in lung from birth to adult age (Fig. 3.1D). It appeared that the additional doses of VARA after P1 significantly accumulated retinol in these tissues, with the lung having a greater capability of maintaining this increase, observed in adults supplemented with VARA right before tissue collection. Although VA is rapidly utilized in neonates, due to the increased demand for retinoids to accommodate rapid organ development, we show here that VARA has an incredible ability of surpassing the amount that is necessary for these neonates; the results suggest these neonates may be able to rapidly store and then utilize the stored VA in times of VA deprivation, to ensure adequate development.
The body system appears to have communication between the organs, based on the gene expression response to VARA supplementation. For instance, our results showed that there was a significant decrease in Rbp4 transcripts in liver, which may imply a possible decrease in the liver’s RBP4 protein production. The increased plasma retinol concentrations due to VARA may have been a signal for the liver to decrease its production of RBP, and to increase the hepatic storage of RE (increased Lrat expression in adult liver) and the removal of excess RA (increased Cyp26A1 expression in adult liver). As well, the lung may have increased its cellular uptake of retinol from circulation (increased expression of Stra6), resulting in increased storage of RE (increased expression of Lrat), and removal of excess RA (increased expression of Cyp26B1).

It is interesting to note that the adult animal’s response to VARA was tissue specific. While in neonates Rbp4 transcripts in liver were lower after oral supplementation with VARA, the level of expression in the adult did not change. This result may correspond to the fact that the storage (Lrat) and terminal oxidation of RA (Cyp26A1) experienced approximately between a 40 – 300 fold increase in expression in comparison to control and to neonatal responses to VARA. Therefore, Rbp4 expression may be restored in adult tissue to accommodate this change in storage of RE and removal of RA. The lung displayed an intriguing developmental response to VARA. Although uptake of VARA was significantly increased in lung tissues (Fig. 3.3A), the repeated dosing with VARA lessened the effect size with each dose, matching that seen for the removal of RA (Fig. 3.3C) after P4. During the time period of lung secondary septation and alveolarization (P4 and P10), the increased storage of RE (Fig. 3.3B) was less in magnitude, possibly due to increased utilization of VA in the lung. At the same time, VARA significantly decreased the expression of Raldh-1 (and -2 in the adult lung. Raldh expression in neonatal lung was unresponsive to VARA supplementation, possibly related to preventing formation of toxic levels of RA. However, Raldh-1 and -2 expression levels were significantly decreased in adult
tissue, perhaps this could be to accommodate the significant decrease in *Cyp26B1* expression in adult tissue after VARA treatment.

To summarize, VARA supplementation alters the expression of retinoid homeostatic genes throughout postnatal development. Alterations of these transcript levels are dependent on both age and tissue type. While we are unsure whether the upregulation or downregulation of these genes are beneficial during neonatal development, our study provides valuable insights on the effects of VA supplementation on VA homeostasis in the liver, lung, and kidney at a VAM status.

### 6.3 Long-term effect of neonatal VARA supplementation

In addition to investigating the expression of retinoid homeostatic genes during ontogeny and the direct effect of VARA supplementation on retinol concentration and the expression of retinoid homeostatic genes in VA storage tissues from birth to adult age, we investigated the long-term effect, or “carryover effect,” of neonatal supplementation in adult rats. The “VARA adult carry over” rats had been supplemented with VARA as neonates (up to P10) but they were not treated with VARA prior to euthanasia as adults. Our results demonstrated a carry over effect on tissue retinol concentration only in liver. Recent studies support our findings of longer maintenance of retinol levels in liver in comparison to extrahepatic tissues such as the lung [39]. Opposite of what was seen in both control (oil-treated) rats and adult rats that received VARA 6 h prior to euthanasia, the liver *Rbp4*, liver and lung *Lrat*, and lung *Cyp26B1* significantly decreased in adult tissues that received only neonatal supplementation of VARA. While VARA can be said to “push” excess VA towards esterification and oxidation, to maintain homeostasis, it may be that by adult age the expression of genes for this purpose is no longer needed. In addition, the lack of a carryover effect of neonatal VARA supplementation on adult-age megalin expression suggests
that this would likely be a short-term regulatory mechanism. In addition, as exemplified in the liver and lung, there was a negative effect in carryover expression of Lrat in the kidneys (data not shown).

Overall, early supplementation of VARA does result in a long-term impact on genes that regulate retinol trafficking, storage of RE in the liver and lung, and terminal oxidation of RA in the kidneys and lung. In our study, we expanded on the comprehensive analysis of the effects of VA supplementation when we investigated the carry-over effect of oral VARA treatment. Perhaps these changes would not have been apparent after only one treatment with VARA. To our knowledge, we are the first to provide a comprehensive analysis on genes that participate in regulating VA homeostasis from birth to adult age in neonates fed a VA marginal diet, as well as effects of VARA supplementation and its long-term impact on adult retinol concentration and gene expression in VA storage organs (Table 3.2). Further studies are needed to characterize these changes as they have implications for the use of retinoid therapy in neonates.

6.4 VARA supplementation during postnatal lung development

VA deficiency can lead to premature birth, low-birth weight, and incomplete development of organs such as the lungs, resulting in respiratory complications. We explored the effect of VARA supplementation on the expression of proliferative retinoid-regulated gene expression involved in neonatal lung maturation and secondary septation using lung tissues collected from our study in Chapter 3. The process of secondary septation and alveolarization begins approximately at P4 and ends at approximately P14 in rodents. Binding of RA to RARs activates the transcription of lung-specific genes, including extracellular matrix components αSMA and elastin, growth factor VEGF, growth factor receptors PDGFRα and EGFR, and the
synthesis of surfactant proteins of the SP-A, SP-B, and SP-C families. All of these components are key players in promoting proper lung maturation and septation.

Our results shown that the expression patterns of these genes, despite our using rodents of VAM dietary status, resembled those seen at an uncompromised dietary status [151, 155, 156, 158, 159]. Our results showed that supplementation altered the perinatal expression of α-sma (Fig. 4.2A) and Sp-b (Fig. 4.3B) with a decrease and increase at P1, respectively; a significant increase in expression of Vegf (Fig. 4.2E) in adult lung; and a significant decrease in expression of Egfr (Fig. 4.2D), Pdgrfα (Fig. 4.2C), Sp-a (Fig. 4.3A), and Sp-c (Fig. 4.3C) in adult lungs. Our findings suggest that the lung tissue is either tightly regulated or unresponsive to VARA supplementation in regards to proliferative gene expression during secondary septation. In addition, although not statistically significant, there was a decrease in elastin expression at P4 and P10 after VARA supplementation. Extracellular matrix deposition is necessary for secondary septation initiation. Perhaps a decrease in expression prevents precocious elongation of the secondary septa. Decreased expression of growth factor receptors Egfr and Pdgrfα may be that by adulthood gene expression for this purpose is no longer needed since lung maturation and alveolarization is complete. Although the specific difference between the surfactant proteins are not yet known, we showed that while VARA significantly increased Sp-b expression at P1, while Sp-a and Sp-c expression decreased. Our results confirm that surfactant proteins are differentially regulated in response to VARA supplementation.

We analyzed the morphology of lung tissues in response to VARA supplementation. In our control group, we could visually see the maturation and development of lung tissues beginning at P1 to adult age in our H&E stained lung sections. Our morphometric calculations supported the visual progression of lung maturation in Fig 4.4B, where there was a linear increase in airway perimeter and a decrease in alveolar wall thickness. Although VARA supplementation appeared to cause thinner saccular walls at P1, presence of secondary septal elongation at P4
instead of at P10 (oil group), areas of capillary deposition at P10 instead of adult (oil group), and increased vascularization in adult tissue, however, our calculations for these morphometric measurements were not statistically significant. Increased Vegf expression after VARA supplementation in adult lung supported the significant increase in red blood cells and capillary bed disposition observed in sections of adult tissues (Fig. 4.4A). Although morphological differences were not significant, small changes in lung structure may cause a physiologically significant change in lung function. Further research is necessary to determine functional differences in the lungs after VARA supplementation.

6.5. Therapeutic properties of VARA in neonatal lung injury

Retinoids have been shown to be beneficial in improving lung development in conditions of malnutrition [91, 108, 181] and chronic lung injury [93, 95, 118, 182, 183]. For our final study, we investigated whether VARA would be effective in rescuing morphological damage due to chronic lung injury. Therefore, we developed a rodent model of BLM-induced BPD in neonatal rats. BPD is chronic lung injury prevalent in low-birth weight (LBW) preterm infants often associated with VA deficiency [150, 162]. Neonatal rats of mothers fed VAM diet were treated with an i.p injection of BLM one day after birth and daily for 5 days, then supplemented with VARA every other day beginning on P6 until P14, and finally euthanized on P15 for lung and plasma (Fig. 5.1). In the present study, we examined for early damage due to BLM at P5 and for a possible therapeutic effect of VARA supplementation, given after BLM treated ended, at P15 in the BLM damaged lungs (Table 5.1). We examined plasma and retinol concentration as well as histological differences in the lungs after BLM treatment and VARA supplementation.

Our results showed that BLM only altered plasma retinol concentration at P15 (Fig. 5.2B) with a significant decrease in total retinol concentration. Although there was a significant
increase in the expression of the pro-inflammatory cytokine IL-6 in lung tissues at P5, it may have occurred too early to cause an alteration in plasma retinol concentration. In a previous study, LPS induced inflammation did not alter the concentration of total retinol in neonatal lungs [131]. An increase in TNFα and decrease in IFNγ were not restored after VARA supplementation. Perhaps plasma cytokine expression after BLM treatment and VARA supplementation would have displayed different results and, possibly, improved the BLM-induced lung injury after VARA, as suggested by results in H&E sections (Fig 5.4A). BLM induced immune cell infiltration and slight hemorrhage, observed at P5 and further increased at P15. After VARA supplementation, it appeared that hemorrhage was attenuated and immune infiltration of the airways was reduced. Perhaps due to the animal-to-animal variation in response to BLM treatment in our study, visual differences in lung morphology were not supported in our morphometric analysis, which showed considerable variability.

To further investigate damage to lungs after BLM treatment and possible improvement after VARA supplementation, we examined sections for the presence of fibrosis, the disturbance of secondary septation, and cell death using a Masson’s Trichrome Staining, Hart’s Elastin Staining, and Ki67 immunohistochemical staining, respectively. BLM increased collagen deposition at P5 and at P15, caused an abnormal distribution of elastin outside of the secondary septal crest, and resulted in cell death in neonatal lungs. VARA remarkably reversed early presence of fibrosis, restored elastin deposition at the secondary septal crest, and promoted proliferation in the presence of neonatal lung injury. Our results further confirmed the potential therapeutic benefits of retinoids.

In conclusion, the combination of the nutrient VA and its active metabolite RA worked synergistically to restore plasma RE, improve alveolar wall thickness, reverse the presence of early fibrosis, restore elastin deposition, which is necessary for secondary septation and lung maturation, and promote lung proliferation, all in the presence of BLM-induced neonatal lung
injury. Our results suggest that VARA supplementation could be used as a mode of therapy for BPD or similar chronic lung diseases in preterm/term infants. Together with the information from previous studies, our findings provide insight on the ontogenic expression of retinoid regulated gene expression from birth to adult age and the use of the combination of VA and RA as a supplement and therapeutic intervention in VAM neonates, as well as provide better understanding of retinol metabolism in neonatal VA storage organs (Fig. 6.1).
**Long-Term Effect**

- Liver ROH concentration
- ROH storage and circulation
- RA oxidation in lung
- RA production in lung

**No Effect on Proliferative Gene Expression During Secondary Septation**

- αSMA and SP-B at P1
- PDGFRα and EGFR in adult
- VEGF in adult
- SP-A and SP-C in adult

**Neonatal Retinoid Homeostasis**

- Plasma, lung and liver ROH concentration
- ROH uptake and storage, and oxidation of RA
- Circulation of ROH

**Therapeutic Properties in Lung**

- RE storage
- Improve alveolar integrity and elastin deposition
- Proliferation
- Fibrosis

Figure 6.1. Effects of VARA supplementation from birth to adult age
6.6 Future Directions

Lung septation mainly occurs between P4 to P14 in rodents. Our present studies determined the ontogenic expression of retinoid homeostatic genes in VA storage organs (liver, lung, and kidney) due to their importance for maintaining VA homeostasis at crucial time periods of lung development when the demand for VA is high. These time periods included after birth prior to secondary septation at P1, beginning of secondary septation at P4, near ending of secondary septation at P10, and when the lung is fully developed as an adult. To gain a better understanding on the regulation of VA homeostasis during rapid post-natal lung development at the saccular and alveolar phase, the time period in which alveolarization is complete should be incorporated, which is at approximately P14. In addition, VARA supplementation should be administered at P14 to gain a better understanding on the effects of supplementation at the completion of alveolarization and possible long-term effects in adults, as well as to compare gene regulation at P14 to earlier postnatal ages.

For our second study, I noticed slight alterations in lung morphology after VARA supplementation in our H&E stained sections. The response to VARA was small and our morphometric calculations did not find this response to be significant. However, despite what was concluded from our calculations, it is possible that such slight changes may result in a profound effect on the physiological functioning of the lung. It would be interesting determine the influence of retinoids on lung function and mechanics, as demonstrated by Massaro and coworkers [61, 184] where they measured parameters such as ventilation and the forced expiratory volume (FEV), which measures gas forcefully expired after full inspiration in order to determine elastic recoil of the alveoli and airway. Incorporation of lung tissues from the VARA carry-over adults in study 1 would be deemed potentially beneficial for providing further insight into long-term mechanical effects of early neonatal VA supplementation in adult lungs.
In my last study, I investigated possible therapeutic benefits of VARA supplementation in BLM-induced BPD in neonatal rats. Our results showed that VARA was effective in reversing inflammatory induced damage and promoting further maturation of the lung. However, I experienced a large variation in inflammatory response to BLM, given by i.p injection, to neonates. Although this route provides a more systemic response to inflammation, it would be of great interest to administer BLM through intratracheal instillation, which would provide a more direct form of injury, specifically to the lungs, with only a single dose [166, 167, 169]. In addition, histopathology would more closely resemble that seen in humans. Although characteristics of BPD were identified based on histological findings (i.e., attenuation in plasma retinol, disorganized elastin deposition, early fibrosis, immune cell infiltration, hemorrhaging, and cell death), our results were not consistent among neonates that received BLM treatment. Future use of isolated cells from neonatal lung for flow cytometry as well as serum cytokine levels measured using ELISA would possibly provide more insight on the inflammatory response to BLM, which may not be well represented on the mRNA level, and the response of BLM treatment to VARA supplementation.
References


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