Marginal Vitamin A Deficiency Affects Lung Maturation in Rats from Prenatal to Adult Stage

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Summary  Mild or marginal vitamin A deficiency (MVAD) is still a serious and widespread public health problem in pregnant women and children in developing countries. This study investigated rat lung maturation from prenatal to adult stage during pregnancy and postnatal MVAD and the recovery after postnatal vitamin A supplementation (VAS). Adult female rats and their offspring were randomized into three groups. 1. Control: the mothers and offspring received a normal diet. 2. MVAD: The mothers and offspring received a MVAD diet. 3. VAS: the mothers received MVAD diet till parturition, and then received the normal diet. The offspring of the VAS group were given low-dose vitamin A from postnatal day 1 to day 7 and received the normal diet after weaning. The lung development, structure, and collagen and elastic fiber of offspring were monitored by morphometric analysis at age 1 d, 2 and 8 wk, respectively. Lower body weight, lung weight, reduced numbers of alveoli and total alveolar surface area as well as increased alveoli septa thickness was observed in MVAD compared to that in the control animals. Increased collagen deposits and decreasing elastic fiber were found in MVAD rats. However, all of these were significantly improved in VAS-treated animals. These data suggest that the rat lung is sensitive to MVAD during the developing stage. Early postnatal vitamin A supplementation can partially restore the normal lung structure.

Key Words  vitamin A, lung development, alveolar, extracellular matrix, rat

Mild or marginal vitamin A deficiency (MVAD) is still a serious and widespread public health problem in pregnant women and children (1, 2), although severe VAD has been controlled effectively (3). Rwanda’s previous study (4) indicated that the occurrence of MVAD was 34.8% in preschool children in a suburb of Chongqing, China. The impact of MVAD is often ignored since it usually happens without severe clinical signs.

It has been reported that vitamin A and its derivatives, retinoic acid (RA), have a profound influence on alveolar development, maintenance and regeneration (5). Lung morphogenesis is a highly regulated process including prenatal and postnatal stages. Alveologenesis arises is multiple interactions between the fibroblastic, epithelial, and microvascular, and with the extracellular matrix (ECM) (6). In the alveolus, the fibroblast is a major contributor to the formation of the extracellular matrix (7). The lipid-laden interstitial fibroblast (LIF) contains retinyl esters. During late gestation, the lungs of rats contain retinyl esters, but their concentration decreases considerably at the time of birth (8), which is accompanied with an increase in the retinol and RA contents of these cells (9). The majority of alveoli are formed between day 4 and day 14 in the rat and mouse (10, 11) and probably up to 18 mo in humans (12). These data collectively implicate a potential role for vitamin A in alveologenesis and the extracellular matrix.

Previous reports showed that vitamin A deprivation during pregnancy (13, 14) or double knockouts of RA nuclear receptors (15) in the mouse disturbed lung organogenesis. In many studies rats become VAD until after the period of maximal alveolar formation, which is completed by 3 wk of age (16). These studies indicated that VAD in rats is associated with a decrease in gas-exchange surface area, parenchymal elastin and collagen fibers (17, 18). A recent study also suggests that exogenous RA can induce alveolar regeneration in the mouse or rat model of experimental emphysema. Mastro and Massaro presented evidence that RA partially restores elastase-induced emphysema in rats (19, 20). Some health-related scores were improved in chronic obstructive pulmonary disease (COPD) patients who were treated with all-trans retinoic acid (ATRA) in a time- and dose-dependent manner (21). However, studies of the effect of VAD on lung development were limited to fetal, newborn or adult animals, separately.

Recently, it has been hypothesized that adult COPD originates in the fetal stage (22). Previous studies showed that alveoli structure and all components of the lung (collagen, elastic fibers, proteoglycans) have been potentially altered in COPD. However little is known about the effect of MVAD on the lung structure and
ECM from pregnancy to the adult period. We addressed the hypothesis that durative pregnancy and postnatal MVAD could impact alveoli structure and the ECM, and postnatal administration of RA might partially abrogate the changes on the alveoli structure, collagen and elastic fibers induced by MVAD.

MATERIALS AND METHODS

Animals and diets. The study protocol was approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. All animals were housed in individual cages at a controlled temperature (21 ± 20°C), at a relative humidity of 50–70% and with a 12-h light–dark cycle. Animals were allowed free access to feed and water, but the amount of diet per day we gave to the three groups was same. Adult female Wistar rats were obtained from the Laboratory Animal Center of the Chongqing Medical University (Chongqing, China). After 1 wk of acclimatization, the animals were randomly assigned to one of the three groups (six per group), control, MVAD and vitamin A supplementation (VAS) group.

The control animals were fed with 6,500 IU retinol/kg diet (normal diet) per day for 3 wk, then throughout pregnancy, and then on; the pups were fed the corresponding dam diet until 8 wk. The animals in the MVAD group were fed with 400 IU retinol/kg diet (MVAD diet) per day for 3 wk, then throughout pregnancy, and then on; the pups were fed the MVAD diet until 8 wk. The animals in the VAS group were fed with 400 IU retinol/kg diet (MVAD diet) per day till parturition, and then received the normal diet; their offspring were given intragastric administration of low-dose vitamin A (50 IU vitamin A daily) (23) from postnatal day 1 to day 7 (our pre-experiment confirmed that the plasma retinol levels of MVAD rats could resume to normal level at day 7) and after weaning followed with a normal diet until pups were 8 wk of age. All experimental diets were formulated on the basis of a modified AIN-76 diet and previous studies (24, 25).

Table 1. Composition of diet.

| Ingredient          | g/kg |
|---------------------|------|
| Casein              | 200  |
| Sugar               | 680  |
| Soybean oil         | 50   |
| Vitamin mixture     | 10   |
| Mineral mixture     | 60   |
| Total               | 1,000|

1 All experimental diets were formulated on the basis of a modified AIN-76 diet and previous studies (24, 25).

2 Provided the following in milligrams per kilogram of diet: cyanocobalamin, 0.2; biotin, 0.5; folic acid, 0.5; menadione, 0.60; thiamin, 5.0; riboflavin, 5.0; pyridoxine, 5.0; α-tocopherol acetate, 45.5; Ca pantothenate, 50.0; niacin, 50.0; i-inositol, 100.0; choline chloride, 1,000.0; and ergocalciferol, 0.01. The normal diet (control) contained vitamin A 6,500 IU/kg diet. The marginal vitamin A deficiency (MVAD) diet contained vitamin A 400 IU/kg diet.

3 Provided the following in grams per kilogram of diet: CaCO$_3$, 18; K$_2$HPO$_4$, 19.5; CaHPO$_4$, 3.6; NaCl, 10.08; FeSO$_4$·7H$_2$O, 1.5; MgSO$_4$·7H$_2$O, 7.5; KI, 0.015; ZnSO$_4$·7H$_2$O, 0.252; CuSO$_4$·5H$_2$O, 0.018; and MnSO$_4$·H$_2$O, 0.138.

Serum retinol assay. Pups from each group sacrificed at 1 d, 2, and 8 wk by intraperitoneal injection of sodium pentobarbital (25 mg/kg). Blood was obtained by cardiac puncture from day 1 pups and from the saphenous vein of rats at 2 and 8 wk. Plasma retinol was determined (n=13). Vitamin A was extracted from serum with hexane and assayed by high-performance liquid chromatography (HPLC) (Waters 1525 Binary HPLC Pump, Waters Breeze, USA) according to the method of the previous study (26). The flow rate of mobile phase was 1 mL/min. Concentration of retinol was determined by spectrophotometer (Waters 2487 Dual λ Absorbance Dector, USA) at 315 nm. Three control samples with low (0.70 µmol/L), medium (1.40 µmol/L) and high (2.79 µmol/L) concentrations of retinol were supplemented with retinol standard solution (Sigma, USA) in the pooled serum. All retinol analyses were performed by the same individual. All extraction procedures were conducted under lower light in order to prevent oxidation of the compounds.

Histology. Pups from each group sacrificed at 1 d, 2, and 8 wk by intraperitoneal injection of sodium pentobarbital (25 mg/kg). The body weights were determined (n=8). The entire lung was weighed after being rinsed in saline and blotted on tissue (n=8).

The histology of lung was studied (n=5). The trachea was cannulated and infused with 4% paraformaldehyde (PFA) at a pressure of 20 cmH$_2$O. The lungs were removed from the thorax and immersed in 4% PF A for 24 h. Left lung lobe volume was measured by means of water displacement (27). The lungs were then dehydrated through a graded series of alcohol solutions and xylene and embedded in paraffin. All morphometric assessments were performed on the region of the left lung lobe which was cut into 5-µm serial slices. Ten slices were randomly chosen for morphometric examination by hematoxylin-eosin (HE) staining. Another ten slices were randomly chosen for elastin fibers by aldehyde-fuchs in and collagen examination by Masson trichrome staining (28).

Morphometry. Morphometric analysis was performed by the same investigator, blinded, using an optical light microscope (Nikon 108) and image analysis software (Image-Pro® 6.0, USA). Measurements were performed on ten H&E-stained sections per lobe and 5 animals in each group. Fifty nonoverlapping images were selected for each animal and the images (HE, ×100) were captured through image analysis software. The intercept counting of alveoli walls was calculated and the length of each straight line was measured in the image analysis software.

Total alveolar surface area equaled SV×FLV×PF. SV was the surface area of alveoli and alveolar ducts per
Body weight and lung weight

None of the rats died before the termination of the study. No visible clinical manifestations of vitamin A deficiency were detected in any MVAD rats. Lower body weight was observed in the MVAD group at the ages of 1 d and 2 wk (p<0.05). But no difference in body weight was found at the age of 8 wk in the MVAD group compared to the control group. Lower lung weight was observed in the MVAD group at the ages of 1 d, 2 wk

Table 2. The body weight and lung weight of offspring (mean values and SD, n=8 per group per date).

| Variable      | Age | Control mean±SD | MVAD mean±SD | VAS mean±SD |
|---------------|-----|-----------------|--------------|-------------|
| Body weight (g) | 1 d | 7.97±0.42       | 6.22±0.40*   | 6.20±0.77*  |
|               | 2 wk| 34.43±3.51      | 22.97±0.91*  | 22.90±3.41* |
|               | 8 wk| 229.48±26.11    | 198.72±19.03 | 221.54±34.86 |
| Lung weight (g) | 1 d | 0.15±0.02       | 0.12±0.02*   | 0.12±0.03*  |
|               | 2 wk| 0.52±0.07       | 0.39±0.04*   | 0.36±0.04*  |
|               | 8 wk| 1.10±0.10       | 0.99±0.06*   | 1.21±0.15#  |

MVAD: marginal vitamin A deficiency; VAS: vitamin A supplementation.
* represents statistical difference versus control animals (p<0.05) at the same age.
# represents statistical difference versus MVAD animals (p<0.05) at the same age.

Table 3. Plasma retinol of pups at postnatal 1 d, 2 and 8 wk (Mean values and SD, n=13 per group per date).

| Group    | Plasma retinol (μmol/L) |
|----------|-------------------------|
|          | 1 d mean±SD | 2 wk mean±SD | 8 wk mean±SD |
| Control  | 1.46±0.248 | 1.25±0.119 | 1.58±0.468 |
| MVAD     | 0.84±0.095* | 0.86±0.093* | 0.85±0.101* |
| VAS      | 0.82±0.141* | 1.14±0.373# | 1.73±0.566# |

MVAD: marginal vitamin A deficiency; VAS: vitamin A supplementation.
* represents statistical difference versus control animals (p<0.05) at the same age.
# represents statistical difference versus MVAD animals (p<0.05) at the same age.

RESULTS

Body weight and lung weight

The proportion of elastin fibers and collagen deposits in the pulmonary parenchyma were determined as the ratio between positive area and negative area by using a counting grid with 50 lines and 100 points (x400) (30). Twenty fields per animal were counted. Elastin and collagen fibers that were associated with airways or blood vessels were excluded from the analysis.

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Statistical analysis. Data are expressed as mean±SD. A one-way ANOVA followed by Bonferroni tests for multiple comparisons was used to compare the three groups. Serum vitamin A level of mother rats was analyzed using Student’s t-test. Significance was defined as p<0.05.

The proportions of elastin fibers and collagen deposits in the pulmonary parenchyma were determined as the ratio between positive area and negative area by using a counting grid with 50 lines and 100 points (x400) (30). Twenty fields per animal were counted. Elastin and collagen fibers that were associated with airways or blood vessels were excluded from the analysis.
similar to that in the MVAD at 2 wk, but alveoli became more organized at 8 wk, which was similar to that in the normal control (Fig. 1G, H, I). Statistic analysis indicated that MVAD reduced numbers of alveoli and total alveolar surface area, and increased alveoli septa thickness ($p < 0.05$). VAS could partially prevent the changes which were induced by MVAD. In the VAS group, numbers of alveoli and alveoli septa thickness can match the control level at 8 wk of age. In spite of that, the total alveolar surface area wasn’t restored to the control level, but still obviously increased (Fig. 2).

**Collagen and elastin fiber**

The alveoli morphological changes were accompanied by expression of both elastin and collagen. The reductions of elastin fiber deposit were detected at 2 and 8 wk in the MVAD group ($p < 0.05$). MVAD reduced collagen deposits at postnatal day 1 ($p < 0.05$), but increased deposits at 2 and 8 wk ($p < 0.05$). VAS treatment increased elastin fiber deposits and reduced collagen deposits at 2 and 8 wk compared to that in the MVAD group ($p < 0.05$) (Fig. 3).

**DISCUSSION**

There is increasing evidence that vitamin A has a profound effect on lung development. The present study elucidated the effect of MVAD on alveologenesis, and elastic and collagen fibers in the alveoli and alveolar ducts from the prenatal to adult period. To ensure the animal model success, we measured the vitamin A level of mothers before mating and their offspring at postnatal day 1, and confirmed the MVAD status during pregnancy in the MVAD group. We also measured the vitamin A level in pups at postnatal weeks 2 and 8, confirmed the MVAD status from early age to adulthood. The plasma retinol concentrations of mothers and offspring were reduced by approximately 40% in the MVAD group; however both were higher than 0.7 μmol/L. There were no visible clinical manifestations of vitamin A deficiency in the MVAD mothers or offspring. Our study might be helpful to clarify whether, in such status, alveoli structure and elastic and collagen fibers are impacted by mild vitamin A deficiency.

**MVAD from prenatal stage reduces body weight and lung weight**

Previous studies have found that no reduction in food intake and body weight was observed in adult VAD rats (31). So in our study, the rats were fed ad libitum, but the amount of diet per day we gave to the control and MVAD group was same. There was no difference in body weight between the two groups during the pre-mating period.
Our results show that maternal MV AD decreased body weight and lung weight of the newborn, but body weight showed no difference compared to the control at 8 wk of age. Other researches also have established that maternal VAD in the rat has marked effects on the fetus and neonate including impaired fetal lung development (13, 14, 32). It indicated that MV AD affects fetal and early postnatal lung viability, and can last to adulthood. These adverse effects might be easy to detect during infancy and childhood by decreasing body weight. But adult MV AD status may be ignored for lack of typical clinic manifestation.

**MVAD results in lung structure changes**

There are accumulative reports on the effect of VAD in alveolar development. Frey et al. showed that 60% VAD deregulates the normal phases of lung growth. Lung surface areas of air spaces were decreased on day 4, while the barrier thickness was increased (33). Our study showed that MVAD begun from pregnancy could also affect the offspring’s lung structure. MVAD resulted in a thicker-walled, smaller alveoli structure that seemed like the structure ahead of alveoli subdivision. Along with the continuous MVAD after birth the number of alveoli and lung surface area significantly decreased compared with the control group. We speculated that such a lung structure was not good for the respiration although it lacked serious respiratory symptoms. This lung structure may easily form chronic lung diseases such as bronchopulmonary dysplasia (BPD) and COPD under adverse environmental factors. Recently, researchers have been interested in the study of the relationship between early lung development and adult lung disease. Population research showed that poor vitamin A status during the first month of life significantly increased the risk of developing BPD and long-term respiratory disability although the mechanism has not yet been clarified (34). Taken together, it is speculated that the impact of even mild vitamin A deficiency on lung development also deserves our attention.

**MVAD results in collagen and elastic fibers changes**

The ECM in the lung plays an important role in regulating alveolarization, tissue compliance, tensile and compressive strength and elasticity, tissue repair and remodeling (35). In this study, MVAD during pregnancy caused an increase in collagen deposits and reduction of
elastin deposits compared to the normal control. Interestingly, the effect of mild vitamin deficiency on collagen occurred earlier than on elastin fibers. It has been reported that elastic protein synthesis occurs mainly in the peak period after birth (36). Therefore MVAD caused a reduction mainly after birth, in agreement with other researches such as McGowan et al. (17), who reported that lung elastin decreased in VAD rats. Collagen was involved in ECM restoration. Maintenance of normal tissue structure requires a balance between newly synthesized collagen and degraded collagen. Therefore the effects of MVAD on collagen may be more complicated. In our study, MVAD during pregnancy led to the reduction of collagen. But lung collagen deposition increased after birth with the continued MVAD. The possibility is that the lung has adapted to the MVAD status, and started the process of restoration. But in MVAD rats, the structure of alveoli has been changed. An excessive amount of collagen deposition in the collapse or dilatation of the alveolar interval leads to imbalance in the surrounding normal alveolar expansion. In human emphysema, increased collagen deposits correlate with lung destruction, and air space size (37, 38). To our knowledge, this study is the first to report that long-time MVAD increases collagen deposits in the alveoli and alveolar ducts which may be a tissue compensational remodeling.

**Postnatal early VAS partly restores lung mal-developments**

We selected low-dose VAS and postnatal day one as the administration time point to better meet with the clinical requirements. Our data showed that lung development and structure was obviously improved in the VAS group, despite the gas exchanging surface area of alveoli not completely resuming normal levels at adulthood. These results also proved that vitamin A plays an important role in lung development. Recent studies suggest that exogenous RA can induce alveolar regeneration in a rat experimental emphysema model (19, 20). But no definitive clinical benefits related to the administration of retinoids were observed in feasibility study of retinoids for the treatment of emphysema (21). Our results remind us that most beneficial to lung development is insurance of enough maternal vitamin A during pregnancy; even early supplementation after birth can not completely replace it.

The supplementation of low-dose vitamin A effects a long improvement process. Lung structure, collagen and elastin have not completely recovered to normal at 2 wk. McGowan et al. (17) found that lung elastin in VAD rats was not restored after 21 d of RA-treatment. It might be due to having missed the important development period of elastin and collagen (39). In our study, early supplementation may restore elastin and collagen to normal levels in adulthood. So we have reason to presume that postnatal early vitamin A supplementation helps to restore elastase and collagen, which can reduce the risk factor for adult chronic lung disease.

In conclusion, this study indicates that the developing rat lung is sensitive to MVAD and the effects of MVAD on lung development, lung structure, elastin and collagen deposits are permanent and could be persistent. Postnatal VAS can restore most lung development but needs a long process. It is suggested that clinical monitoring and supplementation of vitamin A soon after birth can ensure that each fetus and child has the best possible environment in which to develop their lungs and reduce the possibility of adult chronic pulmonary disease.

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