ORIGINAL ARTICLE

Histological Changes in Developing Lungs of Albino Mouse on Treatment with Retinoic Acid

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ABSTRACT

Aim: To assess the impact of excessive use of retinoic acid on fetal lung.
Methods: Female albino mice were mated to get conceived and were divided into two groups (A and B) of 6 each; group A was a control and those in group B was treated with retinoic acid (RA) 60mg/kg/day on 7.0, 8.0 and 9.0 days of pregnancy. Mice were sacrificed on 19th day of gestation, the fetuses were dissected; their lungs were removed for gross and microscopic study.
Results: The comparison of dead and alive fetuses was found to be significant between the groups (p value <0.05). In addition, the histological examination of lungs in group B showed severe hypoplasia with underdeveloped bronchial passages, reduced size of the sacculi, and increased thickness of their wall; when compared to controls, the difference between the groups was statistically significant.
Conclusion: The result is suggestive of retinoic acid being responsible to induce morphological changes in the lung architecture when taken during early pregnancy to the extent of even resulting in neonatal deaths. It is therefore pointed out that it may be used with caution in early pregnancy.
Key words*:* Retinoic acid, lung hypoplasia, sacculi, bronchial passageways

INTRODUCTION

Metabolic product of vitamin A, Retinoic acid (RA), is needed for normal embryonic development[1]. Retinoids are required to ensure normal pattern formation in a number of organs and tissues including lungs[2]. Major embryonic defects are produced on account of deficiency and excess both during development[3]. Prior studies also suggested that addition of retinol to the diet of Vitamin A deficient diet dams during pregnancy reversed nearly all the malformations indicating that it is needed at several distinct stages of development[4]. They exert multiple effects upon vertebrate development through binding to two families of nuclear receptors; retinoic acid receptor (RAR) and retinoic acid receptor X (RXR). Activated and dimerized receptors control gene expression by binding to retinoic acid response elements[5]. Mesenchyme of the embryonic mouse lung contains three isoforms of RAR (α,β and γ)[2].

The retinoid revolutionaries, using molecular biologic and genetic approaches have shown that nuclear RA receptors (RAR) are major participants in embryology and organogenesis of lungs[6]. The importance of RA signaling for prenatal lung development was first established with the finding that vitamin A deficient rat fetuses displayed severe bilateral lung hypoplasia, left lung agenesis and agenesis of oesophagotracheal septum[7].

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Marked lung dysmorphogenesis of embryo is produced by teratogenic doses of retinoids as well as its deficiency[7,8] The abnormalities are similar to defects seen in compound knockout mouse mutants for the RA receptors (RAR) possibly because the same molecular targets are altered by deficiency of RA signaling[9].

Lung bud initiation and establishment of a distal-proximal pattern requires optimum amount RA. Mammalian lung development follows a highly regulated morphogentic program beginning near mid gestation and continuing through postnatal life. It proceeds through stages described morphologically as the pseudoglandular, canalicular, saccular and alveolar stages in mice[10].

In rats, it was found that the newly born have large, thick-walled, gas-exchange structures termed, at that stage, sacculi rather than alveoli. On the fourth to fifth postnatal day the sacculi begin to be subdivided into smaller compartments by the outgrowth of septae from their walls. The bulk of the subdivision is completed by the 14th postnatal day[11,12].

In various malignant diseases there had been an increasing use of all -transretinoic acid and its derivatives. Recently it has also been recognized as beneficial against atherosclerotic vascular disorders. [13,14] It has been shown by extensive investigations using cell culture that the active forms of vitamin A regulate the expression of genes during vertebrate development[9]. It is suggested that vitamin A supplements or its metabolites may be sparingly used for various diseases during pregnancy.

MATERIAL METHODS

We used sixteen albino mice (Twelve females and four males) 6-8 weeks old, weighing 25-30 gm. National Institute of Health, Islamabad provided the experimental animals, which were kept in University of Health Sciences, Lahore using its animal house; the temp of the environment was controlled at (22±0.5ºC) and humidity (50±10%). Light and dark periods were set for 12 hours each. They were kept on mice chow and water was readily accessible. The experimental animals were randomly divided into two groups, each containing eight animals, six female and two males. Breeding was accomplished by placing one male with three females overnight for 12 hours. Gestational day 0 (zero) was confirmed upon seeing the vaginal plug[15].

Olive oil was given orally to the animals of group A; the quantity used was 0.1ml during 7th, 8th and 9th days of pregnancy, whereas Group B received retinoic acid, 60mg/kg/day dissolved in 0.1ml of olive oil orally on the same days of pregnancy. The experiment came to an end on day 19 when the animals were sacrificed and dissected to obtain the fetuses. The fetuses were dissected using stereo microscope and their lungs were removed for histological preparation; these were fixed in 10% formalin for 48 hours. The lungs were processed, embedded in paraffin wax and 5µm sections were stained with haematoxylin and eosin and examined by light microscopy.

The size of the alveoli and alveolar wall thickness were separately calculated by randomly selecting 8 different places in the sections, using ocular and stage linear micrometer at X20 objective.

Statistical analysis: The statistical analysis was carried out using computer software IBM SPSS Statistics version 22. The arithmetic mean, standard deviation and the significance between two groups was calculated by Mann-Whitney test and Fisher's exact test. The difference was regarded statistically significant if the 'p' value was < 0.05.

RESULTS

The litter size was 50 in group A and 42 in group B. There were 10 dead and 32 alive fetuses in group B. On comparison, the difference between dead and alive fetuses was significant when compared among groups. The fetuses who had severely compromised lungs failed to survive. The lungs were bilaterally present and normal in shape in all fetuses of both groups.

In control group, the terminal saccular stage of development of lung was achieved on day 19; the size of air spaces had increased relative to interstitial tissue. There was characteristic formation of bronchial passageways. Saccular development is well advanced. Blood vessels were integrated within the walls of sacculi (Fig. 1).

In group B, lungs Sacculi were smaller in size with thick walls of mesenchymal tissue consistent with an immature appearance (Fig. 2). The decrease in size of sacculi in treated group B compared with size of sacculi in group A was significant ($p<0.05$) (Table)

The saccular wall was thicker in group B, possible due to increased proliferation of mesenchymal cells induced by retinoic acid. The difference in the thickness of saccular wall in group B compared to group A was statistically significant ($p<0.05$; Table below). In addition, the morphological pattern of branching of bronchial passages also looked underdeveloped in the treated group B. Terminal bronchioles were lined by simple cuboidal epithelium and showing lack of differentiation of smooth muscles in their walls (Fig. 3).

Table: Comparison between litter size & parameters of fetal lungs from groups A & B.

Parameter	**Control Group(n=45) Mean(±S.D)**	**Treated Group(n=42) Mean(± S.D)**	***p*-value**
Dead fetuses n (%)	0 (0%)	10 (23.8%)	<0.001*
Size of sacculi(µm)	54.70± 9.22	34.69 ± 6.88	< 0.001*
Saccular wall thickness(µm)	14.09± 3.28	21.16 ± 5.38	<0.001*

**p≤ 0.05 is statistically significant*

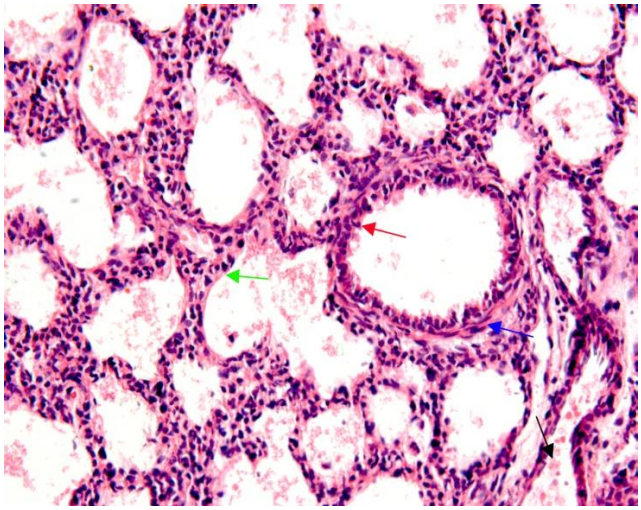

Fig 1: Photomicrograph of fetal lung from group A: showing well developed Sacculi (green arrow), and terminal Bronchole(red arrow)lined by simple columnar epithelium surrounded by a layer of smooth muscles(blue arrow). Branches of pulmonary artery are also seen (black arrow).H & E stain X 200.

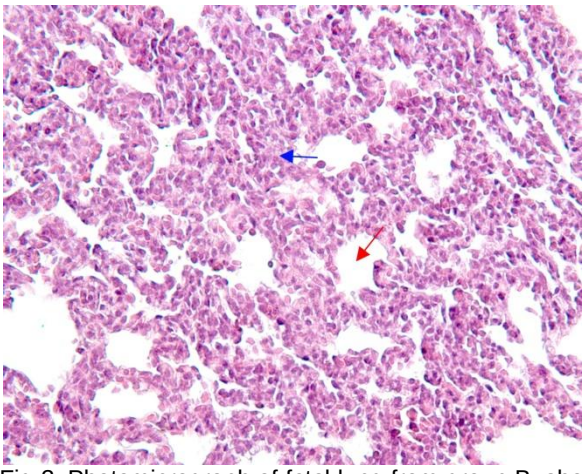

Fig 2: Photomicrograph of fetal lung from group B: showing altered morphology ; sacculi are smaller in size and less advanced in development (red arrow). There is significant increase in the saccular wall thickness (blue arrow). H & E stain X 200.

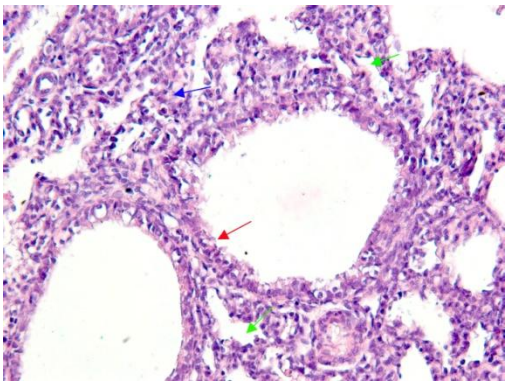

Fig 3: Photomicrograph of fetal lung from group B: showing terminal bronchioles (red arrow) lined by simple cuboidal epithelium separated by thick mesenchymal tissue (blue arrow) indicating lung hypoplasia. Branches of pulmonary artery (green arrow) are also seen. H & E stain X 400.

DISCUSSION

In the present study we focused on the effects of RA on histological structure of fetal lung. The RA was given on 7th, 8th & 9th days of gestation, considered to be critical for developing lung. The lung bud arises as a ventral diverticulum from the foregut endoderm at 9 days after fertilization in mouse and undergoes numerous rounds of dichotomous branching to form the bronchial tree[11].

The importance of RA signaling for prenatal lung development was first established with the finding that vitamin A deficient rat fetuses display among other malformations severe lung hypoplasia , left lung agenesis and oesophagotracheal septum[8].

Examination of the histological sections of lungs from control group showed normal looking well developed organ in terminal saccular stage, whereas lung development was retarded in fetuses from treated group (Fig.2). Similar findings were reported earlier in which disruption of RA resulted in numerous teratogenic effects including lung dysmorphogenesis[16].

Normally the terminal saccular stage lasts from E17.4 to postnatal day 5 in mouse. There is substantial thinning of interstitium of developing lung which appears to be due to process of apoptosis during ongoing differentiation of mesenchymal cells. [17]I t was also noticeable that size of sacculi was smaller and their walls were thicker than normal in fetal lungs from group B, suggesting that saccular development was inhibited or delayed. Thickening of saccular walls could possibly be explained on basis of the ability of retinoids to induce proliferation of mesenchymal cells by a mechanism involving epithelial mesenchymal interactions. Schuger found that retinoids can induce mitogenic effect in mesenchymal population on exposing lung monocultures of isolated epithelial and mesenchymal cells to RA; the effect in part produced by stimulation of EGFR expression. [18] Our results also support earlier studies that demonstrated abnormal alveolar formation due to disruption of RA signaling by mutating the RA receptors γ[19].

In the current study, histological examination of lungs obtained from embryos of group B appeared to have rudimentary bronchial passageways; lined by simple cuboidal epithelium and there is lack of differentiation of smooth muscles in their walls (Fig. 3). Our findings are consistent with an earlier study in which Felicia Chen and colleagues determined that RA has key role in restricting the airway smooth muscle differentiation program during airway formation [20].

Our findings are further supported by another study in which it was proposed that RA acts to maintain high levels of expression of Hox and Sonic hedgehog genes in a fashion that is characteristic of immature lung[21].

The role of vitamin A in the process of maturation of lung tissue has not been fully illustrated. However, the interaction of retinoic acid with its receptors is responsible to produce ultimate changes in the expression of structural genes[22].

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