

## Effect of Prenatal Isotretinoin on Postnatal Development of Cornea and Lens in Albino Wistar Rat: A Morphometric and Histopathological Analysis

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**ABSTRACT:** Isotretinoin is a known teratogen affecting the neuronal differentiation in developing brain. Apart from its effects on brain and other craniofacial malformations, it is also known to cause failure of detachment of the lens from the surface ectoderm. Interestingly very little is known about the teratogenic effect of isotretinoin on the development of eye. Hence in the present study we evaluated the morphometric and histopathological changes in developing cornea and lens in rats during postnatal development. The pregnant rats received 16mg/kg dose of isotretinoin during gestation day 1 to 7 or 12 to 18. The control group received equivalent volume of vegetable oil instead of isotretinoin. The pups were sacrificed on day 7 or 14 or 30 for histopathological and morphometric analysis using haematoxylin and eosin staining. The results of the study clearly demonstrate that prenatal isotretinoin severely affects the development and growth of the cornea in both treatment regimes. There was also a considerable damage to the morphology of the lens. This preliminary animal study clearly demonstrates the teratogenic effect of isotretinoin on developing cornea and lens.

**KEY WORDS:** Developing rat cornea, Developing rat lens, Prenatal isotretinoin, Rat eye development

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### I. INTRODUCTION

Acne is a common complaint of adolescents throughout the world. 13-cis-retinoic acid, isotretinoin is a drug used in the treatment of severe acne. It is a retinoid, derived from vitamin A. Isotretinoin is a synthetic retinoid that inhibits the differentiation of sebaceous glands and corrects the keratinization defects in the follicle. It is also known for its anti-inflammatory activity. It is also being advised for other dermatological diseases such as psoriasis, ichthyosis, dermatological lesions of systemic lupus erythematosus, in the prevention of various types of skin cancers, or even as adjunctive therapy of acute promyelocytic leukemia. There is a growing concern with respect to the teratogenicity of isotretinoin in spite of its wide usage [1]. It is known to cause craniofacial malformations by delaying elevation of the palatine shelf, thereby causing cleft palate [2]. These malformations were attributed to the effect of isotretinoin on the retinoic acid receptors of neural crest cells, by altering the normal transforming growth factor beta (TGF-beta) status [3].

A study by Cook et al demonstrates the teratogenic effect of isotretinoin by preventing the lens detachment and causing corneal opacities in mice [4]. But further studies regarding the postnatal effect on lens was not addressed. The purpose of this study was to ascertain micro-anatomical alteration at three key points of eye development (postnatal day 7, 14 and 30). Though isotretinoin is a known teratogenic agent, it is still being prescribed without proper physician's guidance. Unplanned pregnancy during isotretinoin treatment can lead to serious complications. Hence in the present study we evaluated the teratogenic effect of isotretinoin on cornea and lens. The objectives of the study also involve comparison of teratogenic effects at early and late-gestational treatment.

## II. METHODOLOGY

### 2.1 Animals and housing conditions

In-house bred male and female albino *Wistar* rats (3-4 months old and weight 200-230g) were selected for the study. Breeding and maintenance of the animals was done as per the guidelines of Government of India for use of Laboratory animals as published in *Ind. Journal of Pharmacology* (31:92-95, 1999). Institutional Animal Ethics Committee approval was obtained before the conduct of the study (IAEC letter dated 09/05/2012).

### 2.2 Mating of rats and animal groups

Female rats were allowed to mate with fertile sexually active male rats (proportion of two females for every male rat). After 48 hours, vaginal smears were taken in order to verify the presence of sperms and confirm pregnancy. The rats were then designated as day 0 of pregnancy and were housed individually. All dams were allowed to undergo normal delivery. The litter size, body weight and crown-rump lengths were recorded in each group and the pups were with their biological mothers. Four pregnant rats were used in each group. From each mother one male and one female pup were sacrificed with a high dose of ether and used for postnatal studies on day 7. Hence a total number of 8 pups for each group were considered for the entire studies. The remaining pups were kept with their biological mothers. At postnatal day 14 another set of one male and one female pups from each group was sacrificed for histopathological studies (n=8). The remaining pups of the litter were again left in their cages with their biological mother still weaning (21 days after birth) and there after sacrificed on day 30 (n=8).

**Group 1:** Control – Pups belonging to pregnant rats who received an equivalent volume of vegetable oil instead of isotretinoin.

**Group 2:** Pups belonging to pregnant rats who received isotretinoin (16mg/kg body weight dose) during gestational period 1 to 7 (P, 1-7).

**Group 3:** Pups belonging to pregnant rats who received isotretinoin (16mg/kg body weight dose) during gestational period 12 to 18 (P, 12-18).

Isotretinoin was administered orally using a metallic oropharyngeal cannula. The human dose of the isotretinoin was converted to the rat dose. In our earlier study 16mg/kg dose had proved to be teratogenic on the developing brain [5] hence it was decided to use the same dosage in order to avoid using unnecessary number of animals.

### 2.3 Histopathological studies

On 7<sup>th</sup>, 14<sup>th</sup> and 30<sup>th</sup> postnatal day, pups were sacrificed for histological studies. Each rat was deeply anaesthetized with ether and followed by trans-cardiac perfusion (with 0.9% saline and 10% formalin). The rat was decapitated and the eye globes were removed and kept in 10% formalin for 48h. Paraffin blocks were made and sections (either transverse or sagittal) of 3-5- $\mu$ m thickness were cut using a rotary microtome at the level of optic disc. Twelve sections from each animal were mounted serially on air dried gelatinized slides. The sections were stained with haematoxylin and eosin. In each section of eye, the thickness of the cornea at the center point was measured using ocular micrometer. The percentage of growth of corneal thickness was also evaluated. A qualitative evaluation of corneal epithelium, arrangement of the stromal fibres, structure of descemet membrane and endothelial layers were made.

In each section the morphology of the lens, including anterior lens capsule, cortical and nuclear lens fibres and posterior lens capsule were observed.

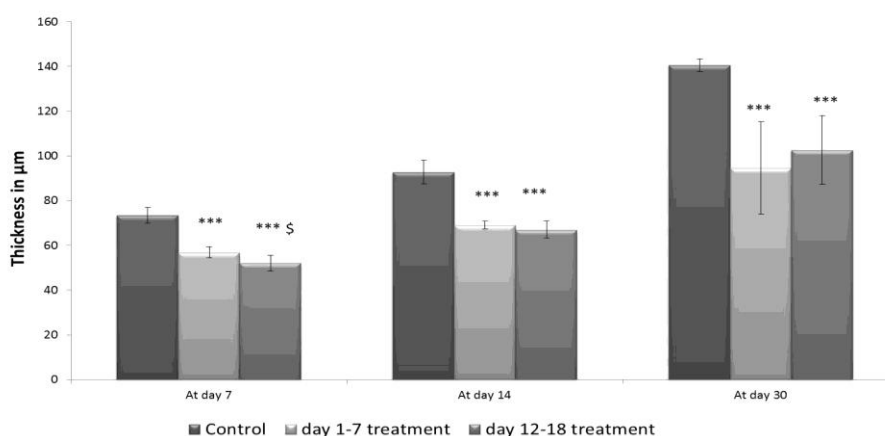
## III. STATISTICAL ANALYSIS

The thickness of the corneal values was expressed as mean  $\pm$ SD. The significance of differences among the groups was assessed using one way analysis of Variance (ANOVA) test followed by Bonferroni's multiple comparison test. P values < 0.05 were considered as significant.

#### IV. RESULTS

There was a highly significant ( $P < .001$ ) decline in the litter size among both the treatment regimes as compared to the control. Though there was a decline in the birth weight at P, 12-18, the weight gain improved by postnatal day 7. Subsequently there was no statistically significant difference in the body weight at each point of time weighed. There was no significant difference in CR length among the groups. At postnatal day 7, the mean thickness of the cornea differed significantly ( $P < .001$ ) between control and both treatment regimes. The P, 12-18 regimen showed a significant difference ( $P < .05$ ) when compared to the P, 1-7 regimen.

Evaluation of corneal thickness at postnatal day 14 and 30 also showed a highly significant decrease for both treatment regimes. Interestingly the comparison of corneal thickness between P, 1-7 with P, 12-18 regime did not show any statistically significant difference on both postnatal day 14 as well as day 30 (Fig.1).



**Fig 1** Thickness ( $\mu\text{m}$ ) of the rat cornea measured at the central region at different postnatal days of development ( $n=8$ ). Values expressed as mean & the error bar indicates  $\pm$ SD. Comparison between control and treated groups. \*\*\* =  $P < .001$ , comparison between treated groups \$ =  $P < .05$

Percentage comparison: The growth pattern of thickness in the rat retinal cornea between postnatal day 7 and 14 (at 7 days of growth) was 25.85% in control group. Similar comparison on day 1-7 of prenatal treatment was 21.54% and 28.69% with day 12-18. The growth pattern between days 14 to 30 (16 days of growth) in control group was 52%, in day 1-7 of treatment, 36.71% and in day 12-18 was 53.22%.

#### 3.1 Histopathological evaluation of cornea

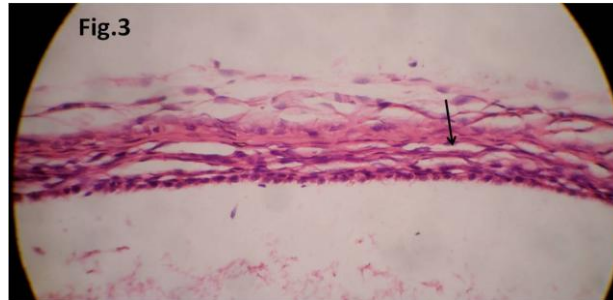
In the control group of pups, histopathological evaluation of cornea showed a normal corneal epithelium, regular arrangement of the collagen fibres with numerous fibroblasts on postnatal day 7 (Fig.2).



**Fig 2** Histomicrographic picture showing normal corneal features in control group of rats on postnatal day 7 (Haematoxylin and Eosin staining, under 40 X magnification).

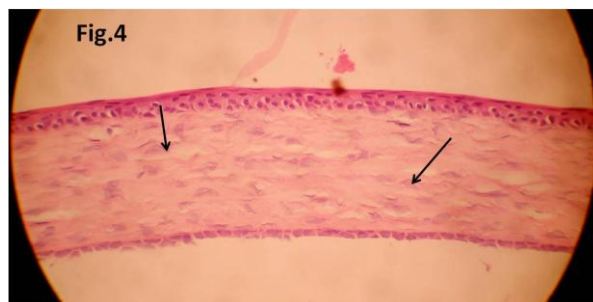
The descemet membrane and a single layer of corneal epithelium were evident amongst other normal features on postnatal day 30.

Histopathological evaluation in both treatment regimes showed an irregular arrangement of stromal fibres in the cornea (Fig.3).



**Fig 3** Histomicrographic picture showing irregular arrangement of the stromal fibres in rat cornea on postnatal day 7 at P, 1-7 regime (H and E, under 40 X).

There was an increase in corneal stromal mitotic activity, characterized by increased fibroblast cell nuclei in the substantia propria, which was observed in few slides among the treated groups, but this feature was not consistent with all the groups tested (Fig.4).



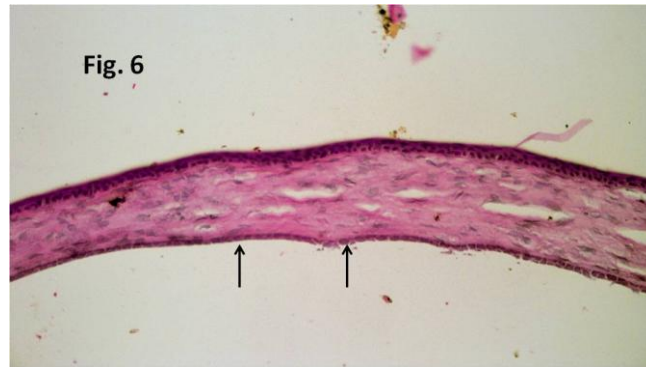
**Fig 4** Histomicrographic picture showing enhanced stromal mitotic activity characterized by more fibroblast cell nuclei in substantia propria of rat cornea on postnatal day 7 at P, 1-7 regime (H and E staining, under 40 X).

Slides of P, 12-18 regime showed a few focal morphological changes and corneal swellings with irregular and widely separated wastage of stromal fibres when examined on day 14 (Fig.5).



**Fig 5** Histomicrographic picture showing corneal swellings with irregular and widely separated wastage of stromal fibres of rat cornea on postnatal day 14 at P, 12-18 regime (H and E, under 40 X).

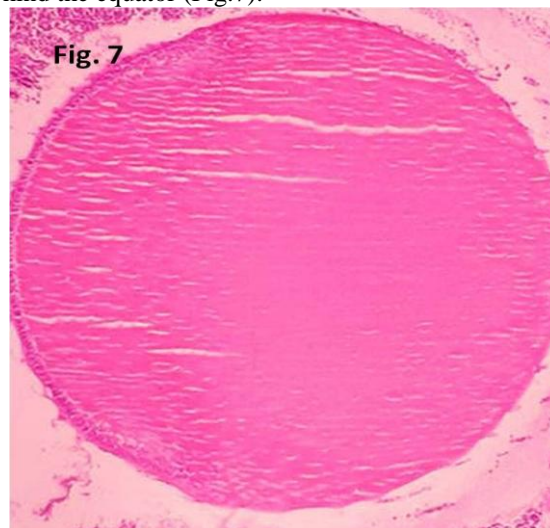
Corneal endothelial cells agenesis and disruption was evident in treated group when examined at day 7, 14 and 30. These features are almost consistent with all the treated groups. In addition irregularly oriented, vacuolated and polymorphic endothelial cells were also observed (Fig.6).



**Fig 6** Histomicrographic picture showing disrupted endothelial cells of the rat cornea (H and E, under 20 X).

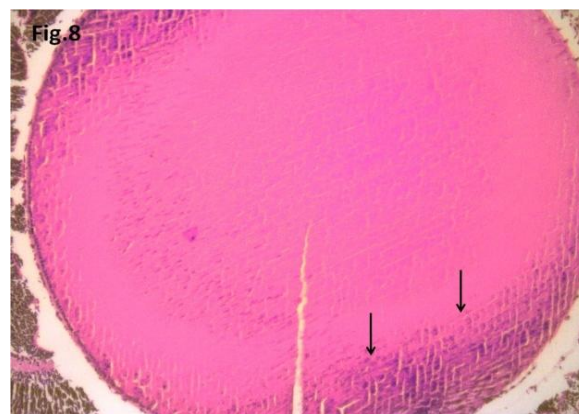
### 3.2 Histopathological evaluation of lens

The lens belonging to the control group showed normal findings with single layer of anterior cuboidal epithelial cells, regular arrangement of cortical and nuclear lens fibres. The posterior lens capsule was clear, without any epithelial cells behind the equator (Fig.7).



**Fig 7** Histomicrographic picture showing normal lens with a single layer of anterior cuboidal epithelium and regular arrangement of the lens fibres (H and E staining, under 40 X).

Prenatal isotretinoin in both treatment regime employed in the study demonstrated a severe damage of the lens structure. The most common finding was irregularly oriented lens fibres. The fibres were swollen at different parts. Prenatal isotretinoin treatment on day 12-18 also showed retention of nuclei (basophilic appearance stained with haematoxylin) in the lens fibres especially behind the equator (Fig.8).



**Fig 8** Histomicrographic picture showing damaged lens (H and E staining, under 40 X).

## V. DISCUSSION

Lens vesicle detachment is known to occur on day 11 in mice [6] and day 35 in the humans. During normal lens development cells of the posterior wall begin to elongate anteriorly and form long fibres that gradually fill the lumen of the vesicle. In human embryos, by the end of the seventh week, the primary lens fibres reach the anterior wall of the lens vesicle. Secondary lens fibres are continuously added to the central core. During this process of anterior migration, their nuclei normally disintegrate so that the central lens area lacks nuclei. In the present study in few slides belonging to the P, 12-18 regime, exhibited numerous nuclei in the posterior segment of the lens. Congenital cataract occurring in rubella syndrome is characterized by persistent appearance of the lens epithelial nuclei within the lens fibres [7]. By correlating these findings, it can be confirmed that lens programming may be damaged by toxic insult of isotretinoin if a mother consumes it during pregnancy. In histological examination, the lens has an anterior subcapsular single layer of epithelial cells that normally terminates at the lens equator in HandE staining preparation [8]. The posterior lens capsule does not have this layer and appears smooth and thin. The migration of the lens epithelial cells over the posterior lens capsule behind the equator is responsible for the formation of posterior subcapsular cataract [9]. In the present study prenatal isotretinoin treatment during day 12-18 showed similar findings in many rats. Degeneration of corticallensfibres can result in cortical cataract formation [10]. In the present study we also observed degeneration of lens fibres, their irregular arrangement and also swollen fibres at certain area of lens. These observations were consistent with the histopathological findings of cortical cataract.

A study by Cook and Sulik [4] demonstrated a delay in lens detachment on gestation day 17, when isotretinoin was administered prenatally on gestation day 7. Though the present study does not involve the observation of lens detachment, its postnatal growth and structure was assessed. In the present study gestational day 12-18 treatment proved to be a sensitive period for teratogenic effect on lens on day 7 but not on day 14 or 30. Hence from our findings it cannot be concluded whether early (1-7) or late (12-18) gestation treatment is more sensitive in inducing teratogenic effect on lens.

Vitamin A is an essential dietary nutrient and precursor of retinoic acid. Retinoic acid (RA) signaling plays multiple roles during embryonic eye development. Cvek revealed the importance of RA signaling for reciprocal interactions between the invaginating lens placode and the optic cup vesicle. It also promotes the normal development of the retina and the optic nerve through its activities in the neural crest cell-derived periocular mesenchyme [11]. Though retinoic acid is required for normal development of eye and many other structures, it is proven to be teratogenic at a higher dose and during a sensitive period.

The corneal epithelial layer is derived from the surface ectoderm; the mesenchymal tissue in front of the developing eye forms the substantia propria. Abnormal lens detachment can result in mechanical interference with neural crest migration (between the surface ectoderm and the lens) which is destined to form the corneal stroma and endothelium, and even stroma of the iris<sup>[4]</sup>. Hence in the present study the histopathological changes observed in the cornea may be due to defective migration of neural crest cells, which is a consequence of delayed lens detachment. An immunohistochemical method labeling the neural crest cells in cornea and lens during, different points of prenatal and postnatal development would provide further insight into isotretinoin induced morphological defects in lens and cornea.

## VI. CONCLUSION

Prenatal isotertinoin treatment in rats during any gestation period is known to deform the lens structure as well as cornea.

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