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Research Article

EFFECT OF PRENATAL ISOTRETINOIN ON POSTNATAL DEVELOPMENT OF RAT RETINA AND EXPRESSION OF DOUBLECORTIN POSITIVE CELLS: A MORPHOMETRIC AND HISTOPATHOLOGICAL ANALYSIS

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ABSTRACT

Objective: Isotretinoin is a 13-cis-retinoic acid (RA), a vitamin A derivative required for normal development of central nervous system (CNS) including the retina. However it is also known to be a teratogen specially affecting the craniofacial region and frontal cortex neurons. Since neuronal proliferation and apoptotic cell death occur continuously during early postnatal retinal development, we investigated the effect of prenatal isotretinoin on postnatal development of retina.

Methods: Pregnant rat received isotretinoin during early or mid-gestational period and the control received an equal volume of vegetable oil instead. The histopathological and morphometric analysis of the retina was performed on postnatal day 7, 14 and 30 using haematoxylin and eosin staining. To evaluate the freshly formed neurons retinal sections were also stained with doublecortin (DCX) on day 14 and day 30.

Results: A significant loss of neurons in inner nuclear and ganglionic cell layers was evident in both treatment regimes. The expression of DCX positive cells were confined to the inner nuclear layers in control group as well as in rats treated with isotretinoin during early gestation on postnatal 14. Postnatal day 30 did not showed any DCX positive cells.

Conclusion: The present study clearly demonstrates that prenatal isotretinoin reduce the neuronal population in retina apart from usual apoptosis. It also confirms that the formation of new neurons occur more specifically in inner nuclear layer of the retina around second week of postnatal development.

Keywords: Doublecortin, Isotretinoin, Postnatal retinal development, Rat retina, Vitamin A

INTRODUCTION

Isotretinoin, a Vitamin A derivative is a drug that is indicated as a treatment of severe acne. It is known to affect the neurogenesis of the developing brain causing reduction in number of neurons in the frontal cortex of the growing brain when treated prenatally [1]. It is also known to cause craniofacial abnormalities malformations by delaying elevation of the palatine shelf resulting in 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-β) status [3]. Interestingly, little is known about the effect of isotretinoin on the developing retina. Normally, retinal cell proliferation increases rapidly throughout the embryonic period. By the 12th day the development of all layers of retina appears to be nearly complete [4]. However, neuronal proliferation and apoptotic cell death is known to occur simultaneously during early postnatal retinal development [5].

During retinal differentiation, cellular retinoic acid-binding proteins were detected during prenatal and postnatal development [6]. Isotretinoin a 13-cis-retinoic acid, has adversely affected the neuronal population in the developing brain. We believe that excess of isotretinoin at a critical point of development could exert similar toxic effect in retina. Hence we tested prenatal isotreinoin effect on development of retina during early (P, 1-7) as well as mid-gestational period (P, 12-18). Evaluating the neuronal population at different time interval during postnatal development would provide an idea about the terminal mitotic activities of various cell layers. Doublecortin (DCX) is a microtubule-associated phosphoprotein required for neuronal migration and differentiation in various regions of the developing central nervous system [7]. This neuronal specific marker is often used to phenotype the newly born neurons [8]. This would also test whether prenatal isotretinoin would affect the formation of new neurons which is known to occur during postnatal development along with known apoptotic cell death.

MATERIALS AND METHODS

Animals and housing conditions

In-house bred male and female albino *Wistar* rats (3-4 months old) of weight 200-230gm were selected for the study. The rats were maintained in 12 hours light and dark cycle in temperature & humidity controlled environment. The rats were fed with standard food pellet and water *ad libitum*. Institutional Animal Ethics Committee approval was obtained before the conduct of the study (IAEC letter dated 09/05/2012).

Mating of rats and animal groups:

Female rats were allowed to mate with fertile sexually active male rat (proportion of two females for every male rat). At the end of 48 hours, female rats were separated and vaginal smears taken to detect the presence of sperm for the confirmation of pregnancy and the rats were designated as day 0 of pregnancy. The pregnant rats were housed individually in separate cages. All dams were allowed to undergo normal delivery. The litter size and body weight were recorded in each group and were left in the cages with their biological mothers. One male and one female pup from each mother were sacrificed on day 7 with overdose of ether and processed for histopathological studies. The remaining pups of the litter were left in their cages with their biological mother. At postnatal day 14 another set of male and female pups from each group were sacrificed for histopathological studies. The remaining pups of the litter was again left in their biological mothers until weaning (21 days after birth) and there after sacrificed on day 30 for histopathological studies. A total number of 8 pups (four male and four female pups) for each group was considered for the entire studies.

Group 1: Control: The pups belonging to the pregnant rats who received an equivalent volume of vegetable oil instead of isotretinoin.

Group 2: P 1-7: The pups belonging to the pregnant rats who received isotretinoin (16mg/kg body weight dose) during gestational day 1 to 7

Group 3: P 12-18: The pups belonging to the pregnant rats who received isotretinoin (16mg/kg body weight dose) during gestational period 12 to 18

Isotretinoin was administered orally using metallic oro-pharyngeal cannula. The human dose of the isotretinoin is converted to the rat dose.

Histopathological studies

On 7th, 14th and 30th postnatal day, pups were sacrificed for histological studies. Each rat was deeply anesthetized with ether and trans-cardiac perfusion with 0.9% saline and 10% formalin was carried out. The rat was decapitated and the eye globes were removed and kept in 10% formalin for 48h. Paraffin blocks were made and sections of 4-5µm thickness were cut using a rotary microtome at the level of optic disc either transverse or sagittal. Each section cut contained the entire retina extending from the ora serrata in the temporal hemisphere to the ora serrata in the nasal hemisphere while passing through the optic nerve head. Therefore, only the sections with optic nerve were stained.

Twelve sections from each animal were mounted serially on air dried gelatinized slides. The sections were stained with haematoxylin & eosin stain. In each section of eye, 200 micron area was selected using oculomicrometer for normal and necrotic cell count. The numbers of viable cells were counted using light microscope (40X). The quantification of cells in various layers of the retina was performed at three defined retinal locations in central retina and at the equator (on both sides). Slides from different groups of rats were decoded to avoid manual bias while counting the cells. The cell counts were expressed as the number of cells per 200µm length of the field. The absolute cell count of the inner nuclear layer and the absolute cell count of the ganglionic cell layer were calculated.

Morphometric analysis: Total retinal thickness for each eye, assessed as the distance from the retinal pigment epithelium to the inner limiting membrane using oculomicrometer.

Immunohistochemical Assay for Doublecortin (DCX) in the developing retina

On postnatal day 14 or 30 rats were used for immunohistochemical assay. Animals were sacrificed by cardiac perfusion with 4% paraformaldehyde under ether anaesthesia. The globe of the eye was removed, postfixed in the same fixative 48 hours. Paraffin blocks were made in an embedding bath and blocked. Coronal sections of 6 µm thick were taken near the equator of the globe of the eye using a rotary microtome (Jung Biocutt 2035, Leica, Germany) and sections were mounted on poly-L-lysine-coated glass slides. Sections were washed in phosphate buffer solution, contained 3.0% H_2O_2 to remove the endogenous peroxidase activity. Sections were blocked in 10% Normal Horse serum, with 0.01% triton x-100 for 30 minutes. These sections were incubated with anti doublecortin antibody (1:250; Santa Cruz, CA, sc-8066) 4 hour at room temperature. These sections were washed and incubated in biotinylated anti goat IgG as secondary antibody (1:200; Vector Lab, BA-9500) for 1 hour, followed by avidin biotin complex (1:50;Vector Lab, SK-3600) for one hour. Sections developed colour using diaminobenzedine / vector grey (Vector Lab, SK-5671) as chromogen. All sections were observed under a light microscope at 40X magnification [8]. The numbers of newly born neurons, i.e. DCX positive neurons were qualitatively estimated in different layers of the developing retina.

Statistical analysis

All the values were expressed as mean ±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. Comparison of data between male and female group was assessed by unpaired "t" test. P values < 0.05 were considered as significant.

RESULTS

There was no sexually dimorphic effect was observed in all the assessed parameters, hence mean values for both male and female were collapsed together.

Neonatal parameters

The mean gestational length in control group was 21.8±0.44, in day 1-7 treatment regimes was 21.2±0.44 and in day 12-18 regime was 21±0.7. There was a highly significant (p<0.001) decline in litter size in both the treatment regimes compared to control group.

Though mortality rate was nil during birth, there was 25% mortality in pups received isotretinoin during P 12-18 treatment (Table-1).

Table 1: Neonatal parameters studied (n=12), values are expressed as mean±SD

	Gestational length	Litter size	Mortality at birth	Mortality during preweaning period	Day of eye opening
Control	21.8±0.44	10.2±0.83	Nil	Nil	11
16mg(1-7)	21.2±0.44	6.6±0.89	Nil	15%	15
16mg(12-18)	21±0.70	7.4±0.54	Nil	25%	15

Birth weight and postnatal weight gain

Comparison of birth weight showed that P 1-7 treatment has not affected (p>0.05) birth weight in comparison with control group. P 12-18 treatment showed a marginally significant (p<0.05) decline in birth weight compared to control group. However P 12-18 treatment showed a highly significant (P<0.001) weight loss compared to P 1-7 treatment. Comparison of body weight on day 7, 14 and day 30 did not showed any significant difference (p>0.05) between the groups (Fig.1).

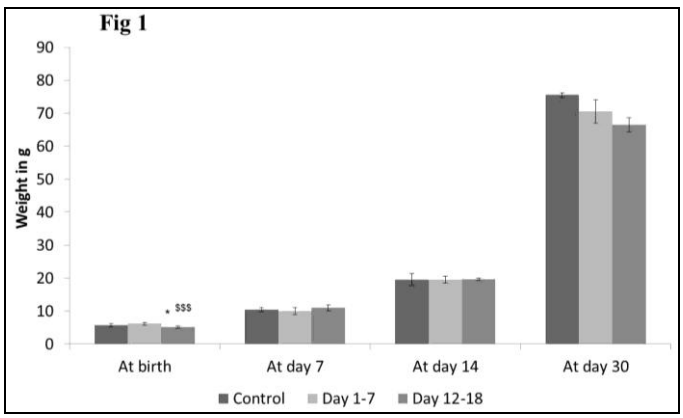

Fig. 1: Birth weight & postnatal weight gain in rats. Values are expressed as mean & error bar indicates +SD. Comparison between control and treated groups *=p<0.05, comparison between the treated groups $$$=p<p.pp1

Absolute cell count in the inner nuclear layer

Quantitative analysis of cellular population in inner nuclear layer on postnatal day 7 showed a highly significant ($p<0.001$) reduction in cell number in both treatment regimes compared to control (Fig.2). There was also a marginal significant ($p<0.05$) difference between P 1-7 and P- 12-18 treatment regimes. On postnatal day 14 and day 30 similar results were observed except for no difference between ($p>0.05$) comparison between two treatment regimes (Fig.2, 3 & 4).

Absolute cell count in the ganglionic cell layer

Quantitative analysis of cellular population in the ganglionic cell layer on postnatal day 7 showed a highly significant ($p<0.001$) reduction in cell number in both treatment regimes compared to control (Fig.2). Similar results were obtained on day 14 and day 30 quantitative evaluation. There was no significant difference between the two treatments regimes at day 7 and day 14. On day 30 there was a significant difference with p value < 0.05 between the two treatment regimes (Fig.2, 3 & 5).

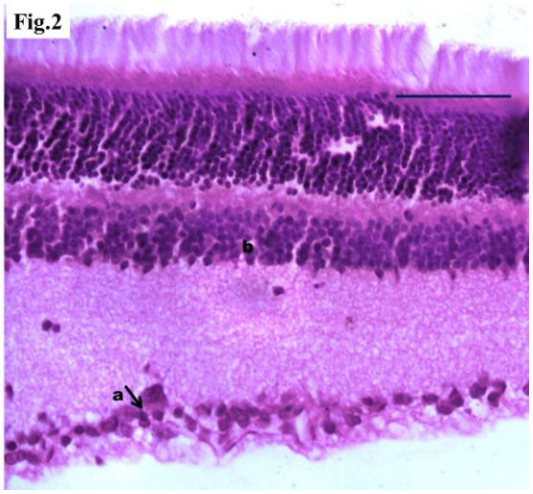

Fig. 2: Histo-micrographic picture showing control group rat retina on day 30 (H & E staining under 40X) a- ganglionic cells, b- inner nuclear cells. Scale bar indicates 20µm.

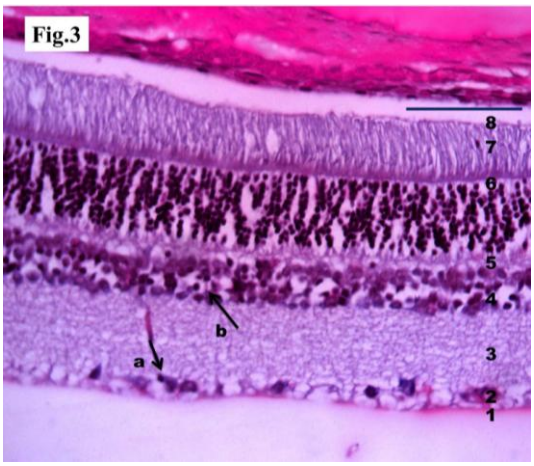

Fig. 3: Histo-micrographic picture showing P, 12 - 18 day 30 retina (H & E staining, under 40X)

a-affected ganglionic cells, b-affected inner nuclear cells. Scale bar indicates 20µm. 1. Nerve fibers 2. Ganglionic cells 3. Inner plexiform 4. Inner nuclear cells 5. Outer plexiform 6. Outer nuclear cells 7. Rods and cones 8. Retinal pigment epithelium

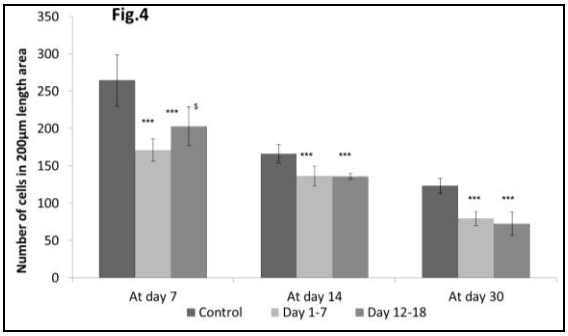

Fig. 4: Absolute cell count in inner nuclear layer of retina for 200µ length area near equator (n=12). Values are expressed as mean + SD. Comparison between control and treated groups, ***=p<0.001, comparison between treated groups $=p<0.05

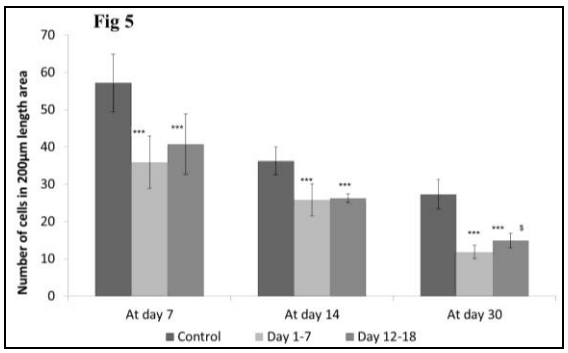

Fig. 5: Absolute cell count in the ganglionic cell layer of retina for 200µ length area near equator (n=12). Values are expressed as mean + SD. Comparison between control and treated groups, ***=p<0.001, comparison between treated groups $=p<0.05

Morphometric analysis of thickness of retina: The morphometric analysis showed significant reduction in retinal thickness in P, 1-7 regime compared to control on postnatal day 7 and day 30 with p value <0.001 but not during day 14. On day 30 P, 12-18 showed a significant difference with p value <0.01. There was no significance between the two treatment regimes at all 3 days of treatment (Fig.6)

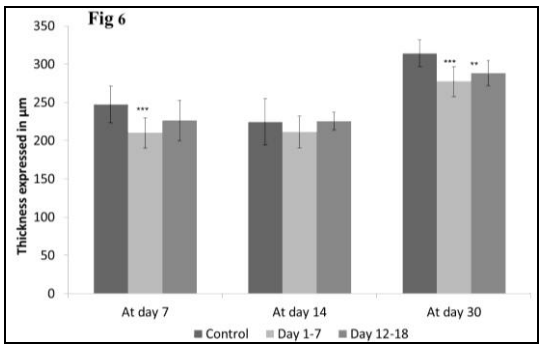

Fig. 6: Thickness of the retina at the equator. Values are expressed as mean + SD (n=12) Comparison between control with treated groups ***= p<0.001 and **=p<0.01

Expression of DCX positive neurons: A distinctive layer of DCX positive cells towards outer aspect of the inner nuclear layer was observed in control rats on day 14 (Fig.7). On same day, isotretinoin treated P. 1-7 showed a distinctive DCX positive cell layer towards inner aspect of the nuclear layer (Fig.8). However these sections also showed DCX positive cells in outer aspect of inner nuclear layer and also layers of rods and cones. The formation of new neurons is distinctive during 14th day of postnatal development while at day 30, it was almost nil.

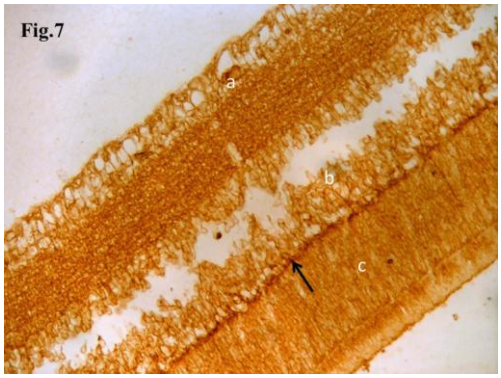

Fig. 7: Photomicrograph (under 40 X) showing section of retina on postnatal day 14 in control group. Arrow indicates DCX positive cells. a-ganglionic cell layer, b-inner nuclear layer, c-layers of rods and cones

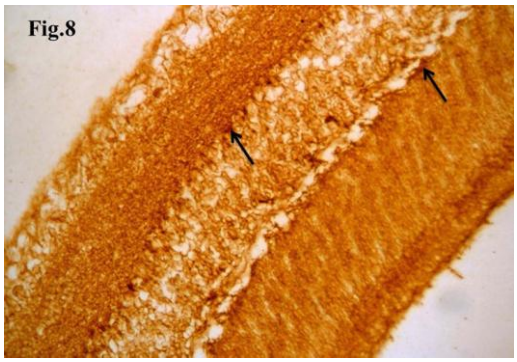

Fig. 8: Photomicrograph (under 40 X) showing section of retina on postnatal day 14 in P, 1-7 treatment group. Arrows indicates DCX positive cells in inner nuclear layer.

DISCUSSION

25% mortality during preweaning period in P, 12-18 treatment and 10% in P, 1-7 demonstrates severe teratogenic effect of isotretinoin. Though the present study focused on the development of retina, isotretinoin has a wide range of toxic effect on various systems. A low birth weight and a failure to gain body weight during preweaning period in P, 12-18 treatment can be correlated with this high mortality rate. Further investigation of teratogenic effect of isotretinoin on other system would likely to throw more light on this result. The loss of birth weight after prenatal isotretinoin was reported [9], but it was depending on time of isotretinoin exposure during gestation, it was not the case in the present study.

In the retina at least two phases of cell death have been reported to occur during development [5]. An early phase takes place concomitant with the processes of neurogenesis, cell migration and cell differentiation. A later phase affecting mainly neurons which occur when connections are established and synapses are formed, resulting in selective elimination of inappropriate connections. However regarding the timings of these two phases the available reports are of different claims. In the present study there was a gradual decline in total cellular population in inner nuclear and ganglionic cell layer in control group. A similar decrease in both cellular populations was observed but the decline in the number was more severe in experimental groups indicating a teratogenic effect of isotretinoin on postnatal development of rat retina.

Previous studies have shown the time interval at which different types of cells undergo apoptosis during postnatal development. The reduction in ganglionic cell apoptosis ceases gradually from the day of birth to postnatal day 6 [10]. The reduction in bipolar neurons begins from postnatal day 4 to 48, while amacrine cell death does not cease until postnatal day 26. The photoreceptor cells undergo apoptosis between days 12 and 72 [11]. The results of the present study with gradual decline in neuronal population are parallel with these findings.

Many of the cellular and molecular mechanisms involved in retinal cell death have been explained in the past. In particular many of the molecular triggers which underlie cell suicide or programmed cell death have been discovered. It is observed that neuronal cell death during late phase of apoptosis seems to be associated with lack of trophic factor. It is also established that retina derived TGFβ signal is responsible for the developmental decline in retinal proliferation [3]. In this regard there is a need to explore the role of retinoic acid receptor on expression of trophic factor as well as TGF.

Though there is a decline of cellular population in retina during postnatal development there is also a production of new neurons. We have investigated the expression and cellular localization of doublecortin in the developing rat retina using immunohistochemistry. On postnatal day 14, a distinct DCX positive cell layer was observed in control group inner nuclear layer towards its outer aspect which is close to the photoreceptor layers. This would indicate that there will be a production of new neurons in this layer. This may be in response to the loss of neurons which triggers the formation of new neurons. On day 14 in P, 1-7 regime similar distinct layer of positive DCX layer was observed on the inner as well as outer layers of inner nuclear layer and also ganglionic cell layer. This would further prove that during postnatal day 14, there will be formation of new neurons irrespective of prenatal isotretinoin treatment or not. It was also observed that formation of new neurons was distinct with early gestational isotretinoin treatment. It was further noted that the reduction of neurons was severe in early gestational treatment. The quantification of cells in this layer in H& E staining showed a profound loss of neurons. The expression of DCX positive cells in this particular cell layer could be a compensatory mechanism for the loss of neurons. On postnatal day 30 no DCX positive cell layers were observed indicating that the neuronal proliferation and migration must have terminated by this point of time. The involvement of inner nuclear layer, in specific, in this production of new neurons could be a compensatory mechanism for severe loss of neurons in this area and also expresses that the retinoic acid receptors differs in different layers.

During the first 2 weeks of postnatal development, the retina undergoes a considerable neural differentiation from immature neuroblastic layering to adult appearance. Although at postnatal day 11 retina appeared similar to that of an adult, a tremendous loss of neurons and formation of new neurons in restricted areas is seen as was found in this study. In the retina, doublecortin is detectable as early as embryonic day 15 (E15), is highly expressed between E18 and E20, and is poorly expressed postnatally. A study by Wakabayashi et al [12], showed the expression of DCX positive cells in horizontal cells of retina in adulthood. These results suggest that either horizontal cell have the capacity to continuously remodel their neurites or doublecortin has a different function in horizontal cells from the control of neuronal plasticity that it is known to modulate other neurites. Our present also shows that during postnatal day 14, there will a DCX positive cells and also further confirms even in altered retinoic acid receptors; there will be a DCX positive cell expression.

Embryonic neurogenesis is closely regulated by retinoic acid at various time points and regions [13]. The function of retinoid is mediated via a retinoid signaling system which includes enzymes, RA receptors and RA binding proteins. In order for retinoic acid to be present and to have a function in the brain, this sophisticated molecular machinery must be present. The presence of these retinoid receptors and proteins has been found in the retina [14]. A study by De Leeuw et al [6] suggests that retinoic acid, the natural ligand of cellular retinoic acid-binding protein, may be involved in neuronal differentiation in the inner retina. Retinoic acid is also known to cause selective rods apoptosis in retina [15]. Hence the loss of neurons in outer nuclear and ganglionic layers of retina can be attributed to the presence of RA receptors in more number and the neuronolysis is a consequence of it.

The results of morphometric analysis of retina measuring thickness of retina at equator has showed a decline in thickness during postnatal day 14, but which is increased during postnatal day 30. On the other hand the cellular population of retina investigated in the present study, the inner nuclear layer and ganglionic cell layer showed a gradual decline in their number. It is the enhancement of synaptic connectivity in a larger scale as evidenced by the pictures would be the primary cause for increase in the thickness of the rat retina. The qualitative observation of retinal section showed an increase in the thickness of both inner outer plexiform (synaptic) layers.

To quantify the apoptotic cell death during postnatal retinal development TUNEL method would have been more specific which we need to look into in the future. Further dendritic morphological study using Golgi staining would provide more insight into postnatal synaptogenesis at different intervals. But the present study clearly confirms the teratogenic effect of isotretinoin on postnatal development of rat retina.

CONCLUSION

The present study clearly demonstrates that prenatal isotretinoin reduces the neuronal population in retina apart from usual apoptosis. It also confirms that the formation of new neurons occur more specifically in inner nuclear layer of the retina around second week of postnatal development. Though isotretinoin is a known teratogenic agent, it is still being prescribed without proper physician surveillance. Unplanned pregnancy during isotretinoin treatment can also lead to various harmful effects on developing brain and also the retina as proved in the present study.

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