ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Effect of Prenatal Isotretinoin Exposure on Neuronal Population of Prefrontal Cortex in Rats

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ABSTRACT

The isotretinoin, a 13-cis-retinoic acid is used in the most severe acne. In humans, isotretinoin treatment is known to cause psychiatric side effects like depression. It is also known to be teratogenic resulting in reduced IQ scores in children who have exposed to isotretinoin during prenatal development. In animal model studies it is known to cause craniofacial deformities including cleft palate. Isotretinoin is known to affect the adult neurogenesis, but there are no studies indicating the teratogenic effect of isotretinoin on intra-uterine neurogenesis. Hence in the present study we investigate teratogenic effect on neuronal population of prefrontal cortex.Pregnant *Wistar* rats were exposed to either 8 or 16mg/kg dose of body weight of isotretinoin during early or mid-gestationalperiod of pregnancy. Pups were sacrificed at postnatal day 7 or 21; brains were removed and processed for histological studies using cresyl violet staining. Neuronal population of the prefrontal cortex was quantified. Isotretinoin treatment resulted in 10% mortality at birth in day 6 to 10 treatment schedule.The results of neuronal assay clearly demonstrate that teratogenic effect of isotretinoin is more when administered during early gestational period in rats& the neurotoxic effects were not dependent on the dose of isotretinoin.

Keywords: Isotretinoin, Retinoic acid, Prenatal exposure & Prefrontal cortex

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ISSN: 0975-8585

INTRODUCTION

The isotretinoin, a 13-cis-retinoic acid is used in the most severe acne. It is a retinoid, meaning it derives from vitamin A.Isotretinoin is a synthetic retinoid that inhibits the differentiation of sebaceous glands, corrects the keratinisation defect in the follicle, and has also some anti-inflammatory activity. It is also used for a number of other dermatological diseases such as psoriasis, ichthyasis, dermatological lesions in systemic lupus erythematosus, in the prevention of various types of skin cancers, or even as adjunctive therapy of acute promyelocytic leukemia. It is sometimes used in prevention of certain skin cancers. In spite of its wide usage, there are growing concerns about its side-effects; specially its teratogenicity [1].

Isotretinoin is a teratogen and is likely to cause birth defects which includes serious craniofacial, cardiovascular, thymic and central nervous system malformations[2].It is known to delay the elevation of palatine shelf and causing cleft palate [3&4].A review paper by O'Donnell [5] shows very threatening information about the teratogenicity of isotretinoin. Since its introduction in September 1982, an estimated 160,000 women of child bearing age have consumed this drug. Between 1982and 1987, an approximately 900-1,300 malformed children,700 - 1000 spontaneous abortion and 5000 –7000 elective abortions were due to isotretinoin. A study by Bremner [6] on effect of isotretinoin on human brain function before and after four months of treatment using PET scans showed a decreased brain metabolism in the orbito-frontal cortex, a brain area known to mediate symptoms of depression. A data generated by FDA in the USA where 4,992 cases of various psychiatric side effects were reported with isotretinoin during the period from 1982 till August 2004. Isotretinoin does tend to increase depression-related behaviour in adolescent mice, at levels proportionate to those given to human patients [7].

It is now well known that fibroblast growth factor and retinoic acid receptors play a major role in differentiation and patterning of skeletal elements of the pharyngeal arches. Experimental manipulation of retinoic status during development alters normal transforming growth factor beta (TGF-beta) status. The critical exposure time is between 3 - 5 weeks of pregnancy, often before the woman knows she is pregnant. It is evident that experimental manipulation of retinoic status during development alters normal transforming growth factor beta (TGF-beta) status. Hence isotretinoin induced craniofacial anomalies were attributed to its effect on retinoic acid receptors of neural crest cells contributing to the craniofacial regions. Retinoid signaling plays a well-established role in neuronal differentiation, neurite outgrowth, and the patterning of the antero-posterior axis of the developing neural tube [8]. However, there is increasing evidence that nutritional vitamin A status and retinoid signaling play an important role in the function of the adult brain.

The studies regarding its teratogenic effects are mainly focused on anomalies of palate. There are no studies addressing the teratogenic effect of isotretinoin on development of brain and neurogenesis. Cases of IQ scores less than 85 with or without other abnormalities in children whose mothers are treated isotretinoin during pregnancy is also reported and this is of serious concern.

Though isotretinoin is a known teratogenic agent, it is still being prescribed without proper physician surveillance. Unplanned pregnancy during isotretinoin treatment can also cause serious effects. Nevertheless, isotretinoin is currently the most broadly prescribed teratogenic drug in the USA and Canada [9].

There are inadequate or no studies focusing on similar effect on neuroepithelial cells of the developing brain. Hence the present study is focused on neuronal population of prefrontal cortex. In our earlier completed study we found that prenatal isotretinoin has affected the neuronal population of hippocampus (unpublished data). Hippocampus is an area where there will be an active proliferation during postnatal life, but neuronal proliferation in cortical area is limited. Prefrontal cortex is also an integral part of the cognitive function, hence teratogenic effect of isotretinoin on prefrontal cortex would throw more light in this regard. Hence in the present study we quantified prefrontal cortex neurons during postnatal day 7 and 21. The objectives of the study also involve comparison of teratogenic effects at early & mid-gestational treatment.

MATERIALS & 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. Breeding and maintenance of the animals were 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).

Mating of rats and animal groups

Female rats (n=4) were allowed to mate with one fertile sexually active male rat. At the end of 4 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. One male and one female pups from each mother were considered for histological studies (n=8; four male and four female pups). The offspring were raised by their biological mothers until weaning (21 days after birth).

Group 1: Control – The pups belonging to the pregnant rats who received anequivalent volume of vegetable oil instead of isotretinoin.
Group 2: The pups belonging to the pregnant rats who received isotretinoin (8mg/kg body weight dose) during early gestational period (from gestation day 1 to 5)
Group 3: The pups belonging to the pregnant rats who received isotretinoin (8mg/kg body weight dose) during mid-gestational period (from gestation day 6 to 10)

Group 4: The pups belonging to the pregnant rats who received isotretinoin (16mg/kg body weight dose) during early gestational period (from gestation day 1 to 5)
Group 5: The pups belonging to the pregnant rats who received isotretinoin (16mg/kg body weight dose) during mid-gestational period (from gestation day 6 to 10)
Isotretinoin was administered orally using metallic oropharyngeal cannula. The human dose of the isotretinoin is converted to the rat dose.

Study parameters

1. Gestational length & mortality: The gestational length, the number of still born pups (mortality at birth) and also total number of pups born (litter size) to each pregnant rat was recorded at birth. During postnatal period (till 21 days) number of death was also counted to get the post natal mortality rate.

2. Weight of pups was recorded at birth and also during postnatal day 7, 14 and 21 (for weight gain).

3. Pinna detachment: The day on which external ear is 1.0 mm (length) x 0.5 mm (breadth) was defined as day of pinna detachment, and that day will be noted for each pup.

4. Eruption of upper & lower incisors: Pups were observed daily during early postnatal period to witness the eruption of upper and lower incisor teeth. The day on which the teeth were 0.5 mm was considered as day of teeth eruption.

5. Eye opening: A visible opening (2 mm length) of palpebral fissure was defined as eyes are opened. The day on which eyes are opened was noted for each pup.

6. Neuronal assay of the frontal cortex: On 7th and 21st postnatal day, pups were sacrificed for histological studies. Each rat was deeply anesthetized with ether and perfused trans-cardinally with 0.9% saline and 10% formalin. The rat was decapitated and the brain was removed and kept in 10% formalin for 48h. Paraffin blocks were made and coronal sections of 3-5-µm thickness were cut in the dorsal hippocampus using a rotary microtome. Twelve sections from each animal were mounted serially on air dried gelatinized slides.

The sections were stained with cresyl violet stain (100 mg of cresyl violet is dissolved in 100 ml of distilled water + 0.5 ml of 10% acetic acid and filtered before use& stained at 60°C for 30 min).

In each coronal prefrontal cortex section, 300X300 square micron area was selected using oculomicometer. The medial frontal cortex area was selected in every section. The numbers of viable neurons were counted using light microscope (40X). 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 unit square area of the field [10].

ISSN: 0975-8585

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

Isotretinoin in the present study at both doses produced no maternal toxicity. The maternal weight gains among the treated groups were consistent with the control. Isotretinoin treatment during early or late gestational period did not had any significant effect on gestational length, litter size, and early physical developments (day of ear pinna detachment, day of eruption of upper & lower incisors and day of eye opening). There was no gross morphological change in any of the pups who received prenatal isotretinoin.

Isotretinoin treatment (at both dose-8 &16mg) during day 6 to 9 of pregnancy had 10% mortality at birth and 5% during preweaning period (8mg dose on day 6 to 9 treatment). There was no sexually dimorphic effect was observed in assessed parameters, hence mean values for both male and female were collapsed together.

Weight of the pups

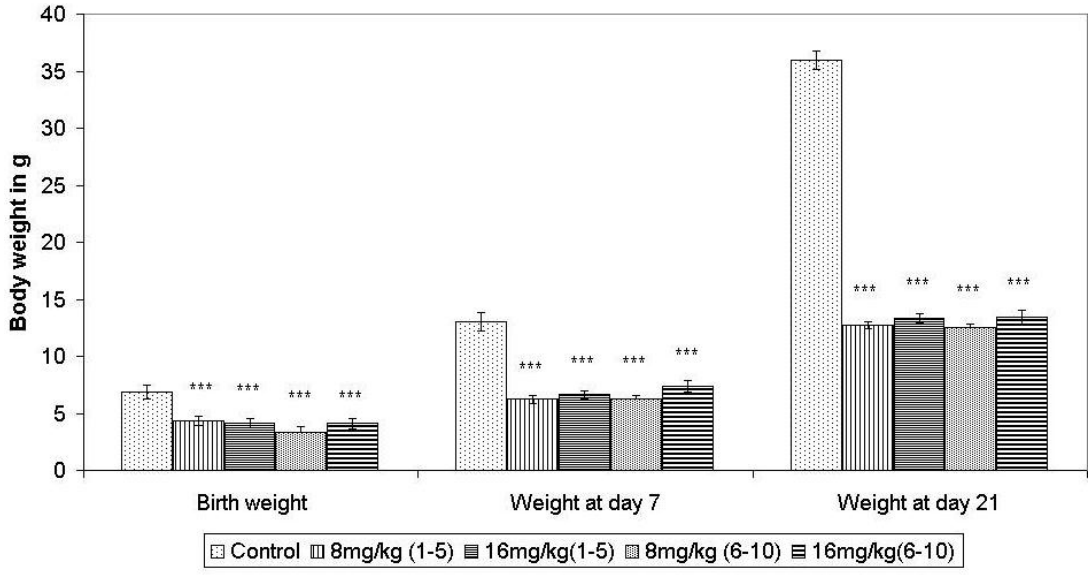

Figure-1: Comparison of body weight of the rat pups at birth and during pre-weaning period. Values are expressed as mean g±SD (n=12). Comparison between Control Vs other groups *** = p<0.001, (For birth weight F=42.73; for day 7 F=90.89 and for day 21 F=1332.3)

Isotretinoin has severely affected (p<0.001) the birth weight at both early & mid-gestational treatment at both the doses studied. Comparison between the two doses used in

ISSN: 0975-8585

this study has not shown any significant (p>0.05) difference in birth weight. Similar observations were made with the weight analyzed during day 7 as well as day 21. The body weight of the control pups showed a normal gain during day 21, but treated group failed gain the weight in the same manner (Fig.1)

Neuronal population of the prefrontal cortex on day 7

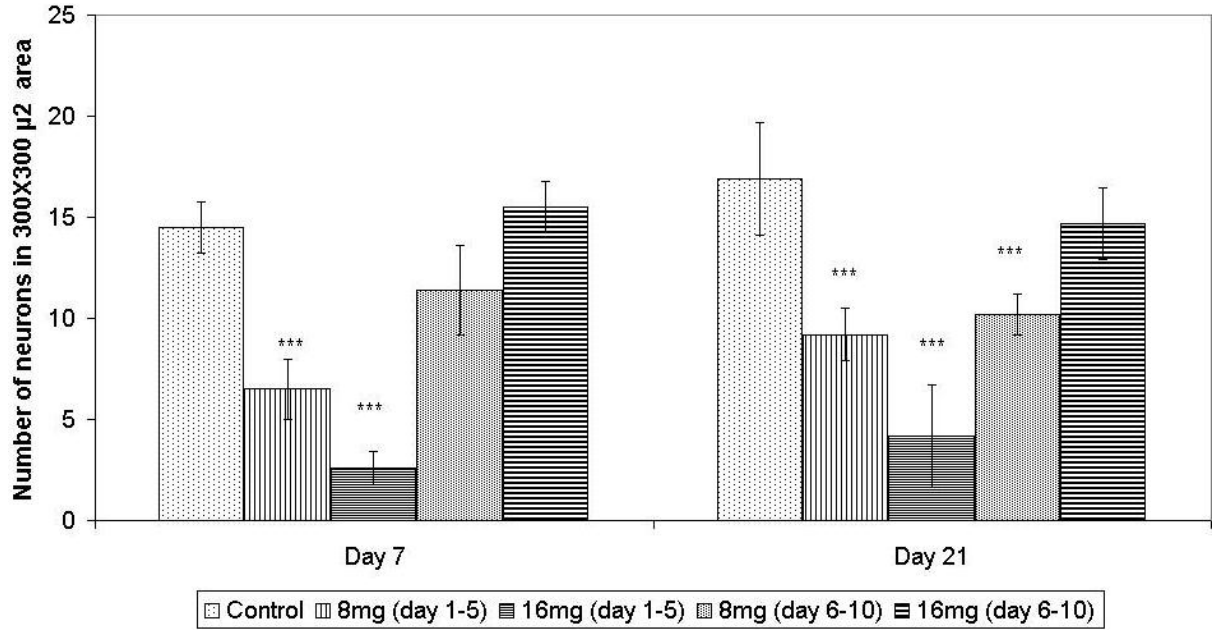

Figure-2: Neuronal population of the prefrontal cortex of the rats who received prenatal isotretinoin. Values are expressed as mean±SD. Error bar indicates SD (n=12). Comparison between Control Vs other groups at day 7 *** = p<0.001. Comparison between Control Vs other groups at day 21 c = P<0.001, F=133.95 (for day 7) & F=61.02 (for day 21)

Isotretinoin at 8mg/kg dose in early gestational treatment has significantly (p<0.001) affected the neuronal populations in prefrontal cortex when compared to control group. 16mg/kg dose of isotretinoin at early gestation treatment also showed a highly significant (p<0.001) reduction in normal neuronal population when compared to control. However the same dose at mid-gestational treatment did not differed (p>0.05) in prefrontal cortex neuronal expression from control group (Fig.2).

Comparison between early and mid-gestational treatment at 8mg/kg dose showed a significant (p<0.001) difference. The neuronal loss was severe in early gestational treatment. Similar results were obtained at 16mg/kg dose also.These results clearly demonstrate that teratogenic effect of isotretinoin is more when administered during early gestational period in rats.

Comparison between 8 and 16mg/kg dose at early gestation treatment showed a decline in neuronal population of prefrontal cortex (p<0)at 16mg/kg dose group. Similar comparison at mid-gestation treatment showed a decline in neuronal number at 8mg/kg

ISSN: 0975-8585

dose.From these results it cannot be concluded that, isotretinoin-induced neurotoxic effect is dose dependent (Fig.3a, 3b &3c).

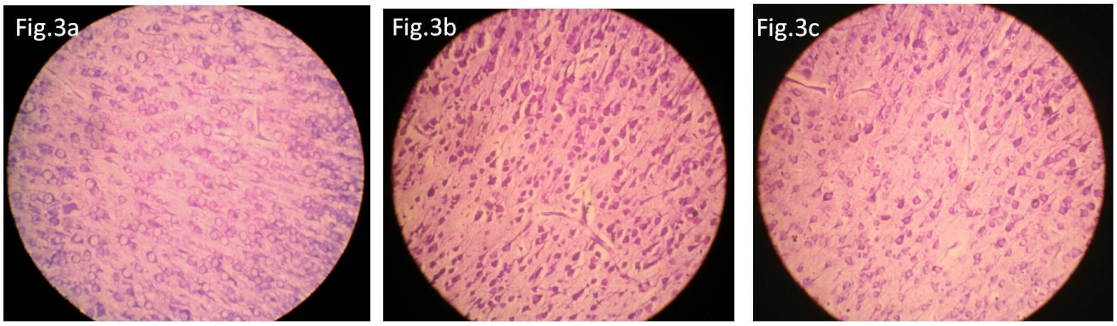

Figure-3: Histomicrograph of rat prefrontal cortex at day-7, stained with cresyl violet under 40X. Fig.3a-Control, Fig.3b-8mg kg/dose (day 1-5 treatment), Fig.3c-16mg/kg dose (day 1-5 treatment)

Neuronal population of frontal cortex on day 21

Isotretinoin at 8mg/kg dose has significantly affected the neuronal population of prefrontal cortex ($p<0.001$) in early as well as mid-gestational treatment compared to the control group (Fig.2). Isotretinoin treatment at early gestation at 16mg/kg dose has significantly ($p<0.001$) reduced the frontal cortex neurons compared to control, but not at mid-gestational treatment ($p>0.05$).

From these results it is clear that prenatal isotretinoin will decline the neuronal population even at postnatal day 21 at 8mg/kg dose, but interestingly not at 16mg/kg dose at mid-gestation.

Comparison between early and mid-gestational treatment at 8mg/kg dose did not showed any significant ($p>0.05$) loss of neurons in prefrontal cortex. Similar comparison at 16mg/kg dose showed a significant decline in neuronal number in frontal cortex ($p<0.001$) of rats who received early gestational treatment. These results indicate that a higher dose (16mg/kg dose in the present study) of isotretinoin at early gestational treatment will have its neurotoxic effect at postnatal day 21 (Fig.4a, 4b &4c).

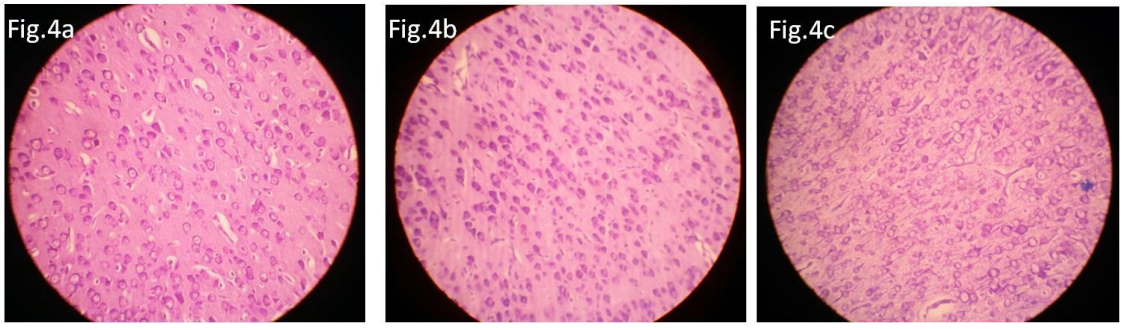

Figure-4: Histomicrograph of rat prefrontal cortex at day-21, stained with cresyl violet under 40X. Fig.4a-Control, Fig.4b-8mg kg/dose (day 1-5 treatment), Fig.4c-16mg/kg dose (day 1-5 treatment)

Comparison between 8 and 16mg/kg dose at early gestation treatment showed a significant decline ($p<0.001$) in neuronal population at 16mg/kg dose. Similar comparison at late gestation treatmentshowed a significant ($p<0.001$) decline in neuronal number at 8mg/kg dose group.Similar results were observed in day 7 analysis. Hence from these results, it cannot be concluded that, isotretinoin-induced neurotoxic effects was dose dependent.

DISCUSSION

10% mortality at birth and 5% during preweaning period is a very high rate and raises serious questions about the usage of isotretinoin during pregnancy. A highly significant weight loss during preweaning period and also a reduced birth weight also raises serious questions about the usage of isotretinoin during pregnancy. A study by Holson et al [11] showed a significant weight loss in pups after prenatal exposure of all-trans retinoic acid, but the loss of weight was depended on the days of gestational treatment. But in the present study the loss of weight was not depended on the gestational days.

The results of the present study clearly demonstrate that isotretinoin has a teratogenic effect by declining the neuronal population of the prefrontal cortex during postnatal day 7 and 21 in rats. Only early gestational treatment affected frontal cortex neurons (at both the doses and also at postnatal day 7 and 21).

The cognitive deficits associated with prenatal exposure of isotretinoin in children can be correlated with loss of neurons in the prefrontal cortex in this animal model study. Several lines of data support this hypothesis, for example cognitive disruption, such as that seen in humans including schizophrenic patients is known to involve alteration in structure and function of prefrontal cortex [12]. Most of the cortical neurons originate in the ventricular and subventricular zones. From these proliferating regions, the cells migrate to their targets where they differentiate into neurons [13]. These cell migrations occur during gestational day 11 to 17 in rats [14]. In the present study isotretinoin during early and mid-gestational period itself has affected neuronal population. Hence it appears that isotretinoin has affected neuronal stem cells (neuroepithelial cells) rather than its migration. Neurogenesis primarily occurs during embryogenesis and early development and persists in restricted regions like hippocampus in mature CNS. Embryonic neurogenesis is closely regulated by retinoic acid at various time points and regions; for example retinoic acid signaling controls the number, timing and subtype produced during motor neuronal differentiation in spinal cord [15]. Retinoic acid is known to promote neuronal differentiation in *invitro* studies, but also known to be a teratogen in embryonic brain. A study by Deborah Smith [16] demonstrates that retinoic acid influence the early telencephalon before the beginning of neurogenesis as well as differentiation and migration of neurons into the cerebral cortex. Another study by Irving et al [17] shows that prenatal isotretinoin resulted in failure of neural tube closure in the midbrain and the hind brain regions of the embryo. Hence the results of the present study demonstrate that prenatal isotretinoin has a direct effect on neuroepithelial cells.

The function of retinoids (growth, vision, reproduction immune function etc.) 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 frontal cortex, an area of the brain associated with higher intellectual functions. Hence the loss of neurons in prefrontal cortex can be attributed to the teratogenic effect of retinoic acid.

In adult rat brain therapeutic dose of Isotretinoin (Roaccutan) has influenced 'embryonic-like' regions where there is neurogenesis throughout the life.It was found to inhibit the growth of neurons in hippocampus that is involved in memory and emotion.The report by Crandall et al [18] concluded that 13-cis-RA in mice significantly reduced cell proliferation in the hippocampus and the subventricular zone, suppresses hippocampal neurogenesis, and severely disrupts capacity to learn a spatial radial maze task. The results demonstrate that the regions of the adult brain where cell proliferation is ongoing are highly sensitive to disruption by a clinical dose of isotretinoin.Our other completed study regarding the teratogenic effect on hippocampal neurons (unpublished) clearly demonstrated a hippocampal neuronal loss during early as well as mid-gestational treatment. In the present study the neurotoxic effecton prefrontal cortex was observed in early gestational treatment. Hippocampus is an area where there will be active neurogenesis throughout the prenatal development and also it extends to postnatal development. However the cortical areas like frontal cortex, the active neurogenesis occur during early prenatal development (during embryogenesis), hence in the present study the neurotoxic effect was restricted to early gestational treatment schedules only.

The works of Webster et al. [19] and Ritchie and Webster [20] revealed that this drug in vitro or in vivo administered 6 h before cell migration from the neural crest was sufficient to induce severe defects in the second branchial arch in the great majority of the exposed embryos causing mild malformation both in the primary and secondary palate. The fibroblast growth factor and retinoic acid receptors of neural crest cells play a major role in the development of skeletal portions of the cranio-facial region and altered level of retinoic acid receptor by isotretinoin was attributed the cause for cleft palate. Though the present study does not focus on migration of neural crest cells, but demonstrates that isotretinoin affect the neuroepithelial cells, as confirmed by neuronal loss in the frontal cortex.

In this animal model study, isotretinoin has proved to be teratogenic at early & mid-gestational consumption by affecting the neurons of prefrontal cortex. As prefrontal cortex play a major role in cognitive functions in humans, consumption of isotretinoin during pregnancy must be avoided.

CONCLUSION

Dermatologists should take every action to ensure that all women being considered for treatment understand the risks and consequences of pregnancy. All women of childbearing

potential must be fully counseled about this effect of the drug and must also receive the patient information brochure provided by the manufacturer of the brand that is being prescribed.

REFERENCES

[1] Berarda A, Azoulay L, Koren G, Blais G, Blais L, Perreault S & OraichiD. Br J Clin Pharmacol 2007; 63: 196-205.
[2] Sladden MJ, Harman KE. Arch Dermatol 2007; 143: 1187-88.
[3] Hendrickx AG, Silverman S, Pellegrini M, Steffek AJ. Teratology 1980; 22: 13-22.
[4] Eleny BR, Karina GS, Maria AJ. Braz Dent J 2001; 12(2): 115-119.
[5] O'Donnell J. Am J Ther 2003; 10(2): 148-59.
[6] Bermer JD, Negar Fani, Ali Ashraf et al. Am J Psychiatry 2005; 162: 983-991.
[7] Kally Reilly, Jason Shumake, Sarha J Baiely et al. Neuropsychopharmacology 2006; 31: 1919-1927.
[8] O'Reilly KC, Shumake J, Gonzalez-Lima F, Lane MA, Bailey SJ. Neuropsychopharmacology 2006; 31(9): 1919-27.
[9] Moskop JC, Smith ML, DeVille K. J Clin Ethics 1997; 8: 264-78.
[10] Madhyastha S, Bairy KL, Nalini K, Somayaji SN. Canadian Journal of Physiology and Pharmacology 2002; 80 (1): 1076-84.
[11] Holson RR, Gazzara RA, Ferguson SA, Ali SF, Laborde JB, Adams Jane. Neurotoxicology & Teratolgy 1997; 19(5): 335-346.
[12] Elsworth JD, Morrow BA, Roth RH. Synapse 2001; 42:80-83.
[13] Rakic P. Experientia 1990; 46: 882-891.
[14] Takahashi T, Nowakowski RS, Caviness VS. J Neurosci 1996; 16: 5762-5776.
[15] Sockanathan S, Jessell TM. Cell 1998; 94:503-514.
[16] Deborah Smith, Elisabeth Wagner, Omanand Koul, Peter McCaffery. Cerebral cortex 2001; 11(10): 894-905.
[17] Irving DW, CC Willhite and DT Burk. Teratology 1986; 34:141–153.
[18] Crandall J, Sakai Y, Zhang J, Koul O, Mineur Y, Crusio WE, McCaffery P. 13-cis-retinoic acid suppresses hippocampal cell division and hippocampal-dependent learning in mice. Proceedings of the National Academy of Sciences USA 2004; 101:5111-5116.
[19] Webster WS, Johnston MC, Lammer EJ & Sulik KK. J Craniofac Genet Dev Bioi 1986; 6: 211-222.
[20] Ritchie H and Webster WS. Teratology 1991; 43: 71-81.