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Histological Study of Lumbosacral Spinal Cord of Mice Embryos who's Mothers were Administered Retinoic Acid

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Abstract
The usefulness of retinoic acid (RA) in reproduction, embryonic and fetal development, growth and tissue maintenance has been established. Excess/low consumption of RA by pregnant rat also leads to congenital malformations affecting the musculoskeletal system and nervous system.
The aim of this study was to investigate the histological changes of the lumbosacral spinal cord in mice embryos whose mothers were administered RA on day 8 post coitum (dpc). Twenty pregnant albino mice were divided into two groups of 10 each. The experimental group received a single dose (70mg/kg) of RA dissolved in vegetable oil by gastrointestinal route, delivered by gavage on gestational day 8, while the control group received only vegetable oil by the same route on gestational day 8. The animals were all sacrificed on gestational day 14 and their embryos harvested and studied. Gross malformations were observed around the lumbosacral region. Microscopic observations revealed reduction in left and right anterior horn thickness (diameter) of the lumbosacral spinal cord region of experimental group (0.01885mm ± 0.00045mm and 0.01872mm ± 0.00054mm) against control (0.02015mm ± 0.00065mm and 0.02002mm ± 0.00054mm) $P<0.05$. Quantification of left and right lumbosacral anterior horn cell density revealed reduction in cell density of experimental group (354.4 ±4.77493 and 351.6 ± 6.542) $P<0.05$ against control (366.0 ± 6.245 and 366.4 ± 7.056). This study showed that one dose of 70mg/kg RA administered through the gastrointestinal route by gavage to pregnant mice on 8dpc caused neural tube defect such as spina bifida occluta, reduction in embryonic neural cell proliferation, reduction of lumbosacral anterior grey horn density and thickness.

Key Words: Administration, Embryonic, Gestational, Malformation, Spina bifida.

1. Introduction

Neurulation is described as the developmental process that results in the rolling up of a flat sheet of epithelial cells into an elongated tube (Colas *et al*., 2001). This process is a fundamental event of embryogenesis that culminates in the formation of the neural tube, which is the precursor of the brain and spinal cord (Copp, 2003). Neurulation has been studied extensively in amphibians, avian and mammalian embryos and results from such studies provide a more comprehensive picture of the intricate morphogenetic process (Colas *et al*., 2001). Neurulationis conventionally divided into primary and secondary phases.

Primary neurulation occur in four stages. These include formation of the neural plate, shaping of the neural plate, bending of the neural plate and fusion of the neural folds (Smith, 1997, Colas *et al*., 2001).

Secondary neurulation occur at more caudal levels, the neural tube is formed in the tail bud without neural folding. The tail bud (caudal eminence), comprises a stem cell population that represents the remnant of the retreating primitive streak. Mesenchymal cells in the dorsal part of the tail bud undergo condensation and epithelialization to form the secondary neural tube, the lumen of which is continuous with that of the primary neural tube. Secondary neurulation creates the lowest portion of the spinal cord, including most of the sacral and all of the coccygeal regions (Copp, 2003).

During the onset of the development of the neural tube, Neural plate gene expression is at first antagonized by endogenous repressors; the bone morphogenetic proteins (BMP-2 and/or BMP-4), which favor epidermal fate (Kerszberg *et al*., 1998). In the presence of BMP-4, which permeates the mesoderm and ectoderm of the gastrulating embryo; the ectoderm becomes epidermis, and mesoderm forms intermediate and lateral plate mesoderm (Sadler, 2008). The bone morphogenetic proteins are however, inhibited in turn by chordin, (Sasai *et al*., 1994, Piccolo *et al*., 1996) an extracellular matrix protein synthesized in the underlying dorsal mesoderm (Spemann's organizer), particularly in the notochord. The reduced inhibition leads to neural induction (Jessell *et al*., 1992) and to the formation of the neural plate. This basic mechanism is supplemented by dorsalizing, neuralizing, and antineuralizing contributions from other secreted factors such as activin (Gurdon, *et al*., 1992), noggin, (Ferreiro *et al*., 1994) fibroblast growth factor (FGF), or the hormone follistatin, all of which

originate in or near the notochord as well. However, these neural inducers induce only forebrain and midbrain types of tissues. Induction of caudal neural plate structures (hindbrain and spinal cord) depends upon two secreted proteins, WNT-3a and FGF (fibroblast growth factor) (Sadler, 2008). In addition, retinoic acid appears to play a role in organizing the cranial to caudal axis because it can cause respecification of cranial segments into more caudal ones by regulating expression of homeobox genes (Sadler, 2008). After induction, neural plate homeogene expression becomes largely cell autonomous. Under the concentration dependent control of yet another notochord-secreted polypeptide, Sonic hedgehog (SHH), and subject to BMP mediated contact interactions with the epidermal ectoderm, homeobox genes such as Msx and Nkx-2 then become expressed and play a role in finer-grained dorsoventral subdivision of the neural plate, as do the Pax genes, whose expression becomes topographically diversified at this time (Sadler, 2008).

Development of the central nervous system (CNS) involves the specification of distinct classes of neurons at defined locations within the neural tube (Lumsden & Krumlauf, 1996, Tanabe & Jessell, 1996). Recent studies have identified several mechanisms mediated by extracellular signals that instruct the differentiation of cell types along the dorsoventral (DV) and anteroposterior (AP) axes of the neural tube (reviewed in Jessell, 2000; Lee & Pfaff, 2001). In the DV axis, current theory suggests that the generation of neuronal populations involves opposing gradients of morphogens. Sonic hedgehog protein (SHH), generated ventrally in the notochord and floor plate, acts in a concentration dependent manner to induce several classes of ventral interneuron progenitors (V0–V3) as well as helps specify the identity of motor neuron (MN) progenitors (Ericson *et al*., 1995, 1997a). It does this by regulating the pattern of expression of a set of homeodomain (HD) and basic helix– loop–helix (bHLH) transcription factors that are categorized into two major groups based on their modulation by SHH: class I proteins that are repressed by SHH and class II proteins that are activated by SHH (Briscoe *et al*., 1999, 2000; Jessell, 2000). The combinatorial expression of these two classes of HD proteins serves to establish individual neural progenitor domains, the boundaries of which become sharpened through selective cross-regulatory interactions (Pierani *et al*., 2001). In the dorsal spinal cord, the expression and inductive activities of bone morphogenetic proteins (BMPs) in the over-lying ectoderm, and roof plate provides an opposing gradient to SHH and plays a major part in the mechanisms defining the dorsal neuronal progenitor cell populations (reviewed in Helms & Johnson, 2003).

In addition to these signalling molecules, retinoic acid (RA) has been implicated in two aspects of spinal cord DV patterning. Firstly, in the induction of a subset of ventral interneurons (Pierani *et al*., 1999) and secondly, in the specification of MN subtypes (Sockanathan & Jessell, 1998). Evidence for the role of RA in the induction of interneurons comes from experiments in which naive neural plate tissue cultured in the presence of retinol (the metabolic precursor of RA) results in the appearance of subsets of interneurons, characterized by the expression of the homeobox genes (Pierani *et al*., 1999). A later role for retinoid signalling in MN patterning was initially indicated by the expression of the RA-synthesizing enzyme Raldh2 in the MNs at limb levels (Niederreither *et al*., 1997).

2. Materials and Methods

10 male and 20 female albino mice weighing 20 – 30g were obtained from breeders in Veterinary Anatomy Department Animal house, University of Ibadan. The animals were acclimatized for two weeks and were assigned to the experimental (group 2) and control (group1) groups by applying random sampling technique. Each group consisted of 10 females and 5 males. Animals in each group were further divided into five subgroups of 2 females and a male and kept in separate cages for mating. The animals were fed standard mice diet and given water ad libitum.

Retinoic acid (all trans-RETINOIC ACID) was purchased from Sigma-Aldrich. 50mg of retinoic acid (powder) Sigma prod. No. R 2625 was supplied by Zayo-Sigma-Aldrich. Vegetable oil was also purchased.

2.1. Determination of Vaginal Plug

After overnight exposure to male, the female mice were examined to determine the presence of vaginal plug. Mice with definitive vaginal plug (a white coloured solid like a grain of boiled rice) were recorded as mated, and the morning of vaginal plug designated day “0”

2.2. Administration of Retinoic Acid

The pregnant experimental animals where gavage fed 70mg/kg body weight of retinoic acid suspended in vegetable oil, which is equivalent to 0.07ml of solution per 25g body weight of mice on the eighth day post coitum (8dpc). The control groups were gavage fed the same quantity of only vegetable oil on 8dpc.Animals in both experimental and control groups were sacrificed on day 14 post coitum under chloroform anesthesia and their litters were carefully removed by cesarean section and fixed in Bouin’s fluid.Each embryo was dissected out of the bead-like uterus, transferred to a separate bottle containing Bouin’s fluid and labeled. Each embryo was blotted dry and their weights taken using the Metler Analytical Balance.

2.3. Tissue Processing

Tissue processing was done at the Histology Laboratory of the Anatomy Department, college of Basic Medical Sciences, University of Ibadan. Transverse section through the lumbosacral region of each embryo was made using a sharp blade and the caudal end of the embryos were passed through the processes of fixation, dehydration, clearing, infiltration, embedding, sectioning and staining.

Bouin's fluid was the fixative of choice because of the nature of the tissue to be processed; The caudal ends of the embryos were fixed in Bouin's fluid for 48hours this was followed by dehydration, The embryonic tissues were transferred to 50%, 70%, 90% alcohol and two changes of absolute alcohol for one hour each on each concentration of alcohol. Clearing was done using cedar wood oil allowing it to stand for 48 hours. This was followed by passing the tissues through two changes of xylene for 15 minutes each. Following this were infiltration and embedding in molten paraffin wax. The tissues were sectioned and stained with Haematoxylin and Eosin (H & E).

2.4. Measurement of Microscopic Parameters

The microscopic parameters measured and the methods used include:

i. Thickness measurement of the anterior grey horn of lumbosacral spinal cord, using a microscope with a graticule attached to the eyepiece
ii. Counting of neurons in anterior grey horn, using a microscope with a graticule attached to the eyepiece, a pointer and a hand counter. The method of counting employed, was the Profile count method as described by Coggeshall *et al*., (1996).

The photomicrographs were taken with Leitz Photomicroscope manufactured by Ernst Leitz Wetzlar GmBH.

2.5. Statistical Analysis

The data were analyzed using Student's T-test. Confidence interval was calculated at 95% level. The level of significance was fixed at less than 5%.

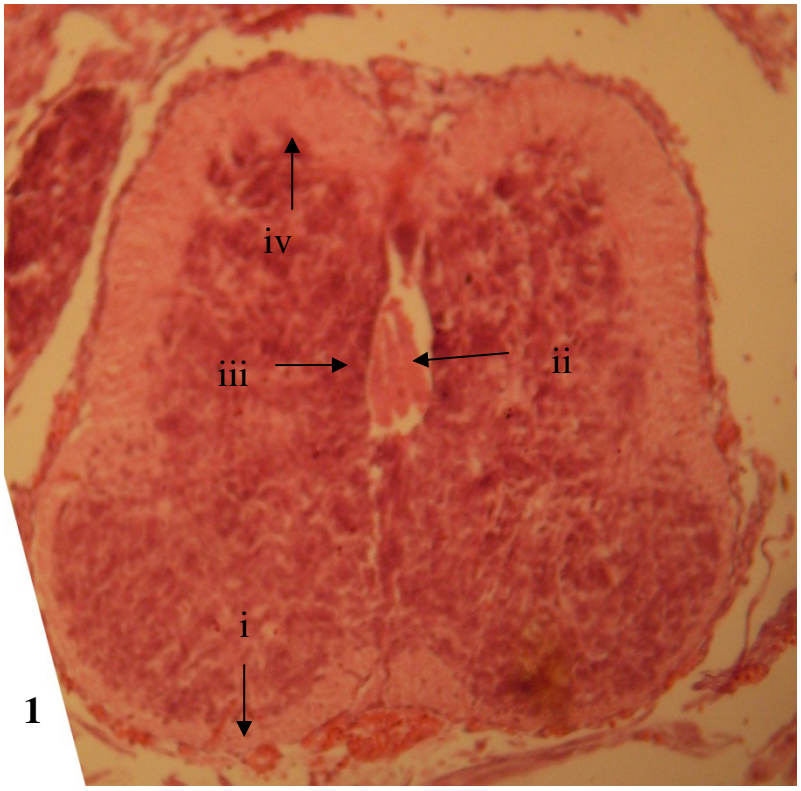
3. Results and Discussion

In this study, eighty nine (89) mice embryos of 14 days gestational age were studied.
Treatment was carried out on pregnant female mice on the eighth day post coitum (8dpc).
From the control group, 6 (six) litters containing 56 normal, non malformed embryos were collected.
From experimental group, 5 litters containing 33 embryos were collected.
No gross malformation was observed on any of the control embryos while Most of the experimental embryos had gross malformations.

3.1. Microscopic Observations

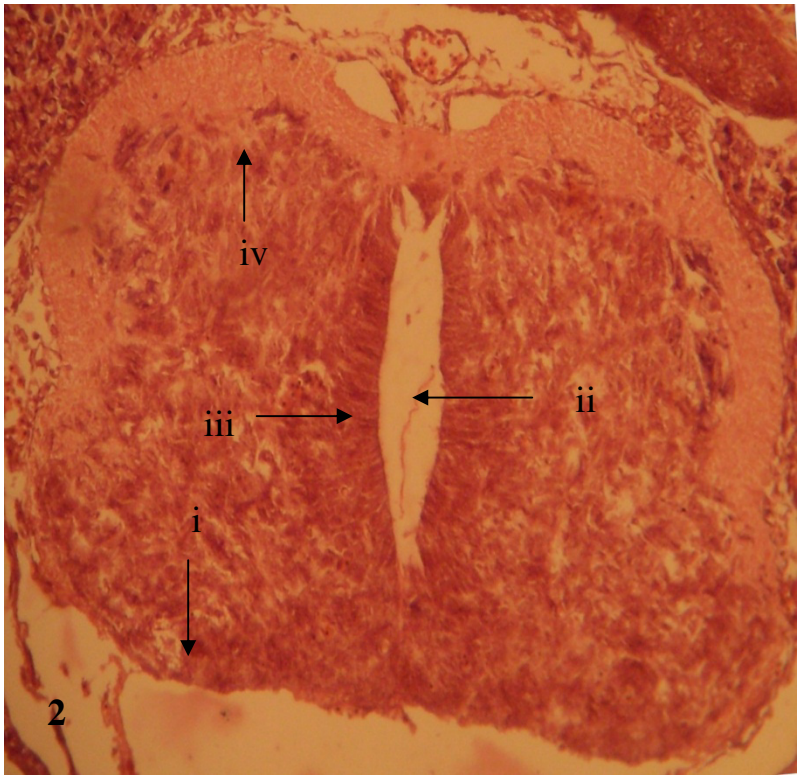
Following histological preparations with H & E staining methods, microscopic observations showed that the thickness of the right and left anterior grey horn of the lumbosacral region of the spinal cord of retinoic acid treated group (0.01885mm ± 0.000459mm and 0.01872mm ± 0.000543mm), (see figure 1 and 2) respectively were reduced and statistically significant at $P<0.05$ when compared with the right and left anterior grey horn of control group (0.02015mm ± 0.000650mm and 0.02002mm ± 0.000544mm).

Quantification of the cells in the right and left anterior grey horn of the lumbosacral region of the spinal cord of RA treated group (354.4 ± 4.775 and 351.6 ± 6.542) respectively, showed reduction in cell count and statistically significant at $P<0.05$ when compared with the right and left anterior grey horn of control group (366.0 ± 6.245 and 366.4 ± 7.056).



i) Anterior horn
ii) Central canal
iii) Ependymal layer
iv) Posterior horn

Figure 1: Photomicrograph of lumbosacral spinal cord of the experimental embryo



i) Anterior horn
ii) Central canal
iii) Ependymal layer
iv) Posterior horn

Figure 2: Photomicrograph of lumbosacral spinal cord of control embryo

Table 1: Right and left anterior grey horn thickness in millimeters

S/n	Right		Left	
	Experimental	Control	Experimental	Control
1	0.01885	0.02080	0.01885	0.02080
2	0.01885	0.02015	0.01820	0.02015
3	0.01820	0.02080	0.01820	0.02015
4	0.01885	0.01950	0.01885	0.01950
5	0.01950	0.01950	0.01950	0.01950
Mean	0.01885	0.02015	0.01872	0.02002
SD	0.000459	0.000650	0.000543	0.000543
P value	.006		.005	

Table 2: Right and left anterior grey horn cell count

S/n	Right		Left	
	Experimental	Control	Experimental	Control
1	359.00	363.00	357.00	371.00
2	347.00	367.00	341.00	359.00
3	358.00	370.00	351.00	365.00
4	355.00	357.00	352.00	361.00
5	353.00	373.00	357.00	376.00
Mean	354.40	366.00	351.60	366.40
SD	4.77	6.24	6.54	7.05
P value	.011		.009	

The central nervous system (CNS) is a major site of retinoid action, as both vitamin A deficiency and excess cause abnormal neural patterning and development (Durston *et al*., 1989, Altaba & Jessell, 1991, Maden *et al*., 1997, Niederreither *et al*., 2000). Retinoid excess affects the normal proliferation and differentiation of the neural epithelium leading to brain and spinal cord deformities (Lammer & Armstrong, 1992). Retinoids have been implicated as pivotal regulators of the normal determination and differentiation of neurons. Experimental manipulation of retinoid synthesis modifies gene expression patterns and the differentiation of specific neuron classes in the developing spinal cord (Forehand et al, 1998, Sockanathan & Jessell, 1998, Pierani *et al*., 1999). Embryopathy due to RA is being intensely investigated in view of the teratogenic potential of retinols and of the crucial role played by their receptors in embryo development (Paulo Roberto, 2007).

The results from this study focus on the effect of RA on structures derived from the neural crest and from the neural tube with emphasis on the anterior grey horn of the lumbosacral spinal cord region of mice when administered on gestational day 8 and the embryos allowed to survive in utero to the 14 day of gestation. However, apart from spina bifida which is a neural tube defect induced by retinoic acid in this study, other types of anomalies were observed such as, total resorption of tail, partial resorption of tail, tail retroflexion. Tomohiko *et al.,* (1997) in his studies on mice, recorded that the malformations in the tail and limbs induced by maternal administration of RA is developmental stage specific. Early in development (8.5dpc), RA treatment induced truncation of the tail in a dose-dependent manner, but no such malformation was observed with treatment at a later stage (10.5 dpc). Niederreither *et al*., (2002), using curly tail mice, demonstrated that alterations in RA availability have marked effects on the incidence of spina bifida, and defects of caudal morphogenesis and neural tube formation.

Results from microscopic observations made in this study, indicates a reduction in the neuronal cell population of the right and left anterior grey horn of sections taken from the lumbosacral region of the spinal cord of the retinoic acid treated mice embryo. Retinoids have been linked to the induction of apoptosis in several in vivo and in vitro models of cell death, but very little is known of the molecular mechanisms involved in the retinoid – mediated induction of this process (Nagy *et al*., 1995). In their study on HL-60 cells, Nagy et aldemonstrated that while RAR – specific compounds can trigger cellular differentiation, they are unable to induce apoptosis, apoptosis was observed only in cultures treated with agents capable of activating both RARs and RXRs. In these cultures, the appearance of differentiation was followed by the apoptosis of the differentiated cells. In the process, retinoic acid may primarily activate the RAR component of retinoid receptor heterodimers, activating the expression of genes linked to cellular differentiation. As differentiation proceeds, however, progressive accumulation of RA metabolites that activate the RXR component of the heterodimer (9-cis RA) and accumulation of the RXR receptors themselves could lead to activation of genes linked to apoptosis. The net effect would be a progressive increase in the frequency with which differentiating cells enter into apoptotic program (Nagy *et al*., 1995).

The reduction in the thickness (diameter) of the lumbosacral right and left anterior grey horn in the RA treated embryos as observed in this study also could be an effect of the reduction in the number of the neurons due to apoptosis in this area of the spinal cord studied. Results by Okuda *et al*., (1997), in his study on "Retinoic Acid Induces Malformations Related to Cell Death in the Developing Embryo" revealed that exogenous RA inhibits normal cell growth in embryos and induces excessive cell death in tail and limb buds. Sockanathan *et al*., (1998), demonstrated retinoic acid-dependent regulation of ventral progenitor proliferation and ventral neuron differentiation. Retinoic acid is also required for the differentiation of specific classes of ventral interneurons (Pierani *et al*., 1999).

4. CONCLUSION

One dose of 70mg/kg RA administered through the gastrointestinal route by gavage to pregnant mice on 8dpc caused neural tube defect such as spina bifida occulta. Reduction in lumbosacral spinal cord right and left anterior grey horn thickness and cell population as made evident by this study, indicates that retinoic acid in excess affects the normal proliferation and differentiation of the neural epithelium and since ventral neuron differentiation and proliferation is retinoic acid dependent as reported by Sockanathan *et al.,* (1998), excess retinoic acid might have inhibited normal cell growth and induced apoptosis in the ventral grey horn of the lumbosacral spinal cord of the mice embryos in this study.

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