The Effect of Retinoic Acid on the Development of Neural Tube at Early Stages of Chicken Embryo

Kawakeb A. Saad[1*] and Eman A. Alsageer[1]

[1]*Department of Zoology, Faculty of Sciences, Umar Al Mukhtar University, Albyda, Libya.*

Authors' contributions: Please write this section

This work was carried out in collaboration between both authors. Author KAS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author EAA managed the literature searches. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: this study aimed to find out the effect of application of 1.5 mg\ml of retinoic acid on chicken development at different stages of neural tube development.
Methodology: thirty fertile domestic Gallus gallus eggs were divided into three groups of 10 eggs for each. These groups repeated four time for four different stages HH8, HH10, HH15 and HH18. Retinoic acid (RA) or dimethyl sulphoxide (DEMSO) was injected through the air sac, and the eggs were incubated for another 24 h. Eggs were opened after 24 or 48 h of incubation, and the embryos were evaluated morphologically and histologically.
Results: The results of this experiment indicated that, In control group and DEMSO group none exhibited neural tube defects neural tube (NTDs). However group treated with 1.5mg/ml of RA exhibit sever NTDs and malformed at the head, cranial and cardiac regions. Shape of the lumen and spatial arrangement of the cell populations of floor plate and roof plate of neural tube has been changed after RA treatment.
Conclusion: Association of excises RA with neural tube defects was demonstrated in the present study on neurulation process during embryogenesis.

_____

Corresponding author: E-mail: Kawakeb.omran@gmail.com;

1. INTRODUCTION

Retinoic acid (RA) is a morphogen derived from retinol (vitamin A) that plays important roles in cell growth, differentiation, and organogenesis [1]. It acts as ligand for nuclear RA receptors (RARs), converting them from transcriptional repressors to activators. The only source of retinoids in most animals is diet derived, as these compounds cannot be synthesized de novo [2].

High levels of endogenous retinoid have been detected in proximity to these developing axes in a variety of vertebrate fetuses. Teratogens studies suggest that both retinoid excess and deficiency are capable of disrupting the development of these axes. RA receptors regulate many developmental control genes, including homeobox genes and growth factor genes [2]. It works via Hox genes that ultimately control anterior / posterior pattern in early developmental stages [3]. Vitamin A has been the focus of extensive research as a fat-soluble nutrient. Due to the nutritional value of vitamin A, its role in vision, epithelial differentiation and spermatogenesis was identified, so vitamin A deficiency or excess in the diet contributes to destruction of these tissues and organs. RA is required in chordate species, which includes all the higher animals from fish to humans. Vitamin A (retinol) is an essential adult safety nutrient [4,5] and embryonic development [6]. RA is important for maintaining epithelial homeostasis in adults [7], and for spermatogenesis[8], immune function and brain function[9].

Neural tube formation and closure is a critical step of embryonic development, and neural tube defects (NTDs) are one of the most common birth defects, affecting on average 0.5–2 newborns per 1000 births [10]. Among the possible genetic and environmental causes of NTDs, there is clear evidence that some pharmacological treatments, including therapies with cumarin derivatives [11], Valproic acid [12],RA [13], and exposure to toxic substances such as solvents or alcohol [14], during pregnancy can result in NTDs or in altered neural development syndromes. A clear example is given by the case of retinoids exposure during early gestation.

Vitamin A, and its biologically active metabolite RA, is also thought to be involved in neurulation and subsequent neural tube patterning. This might be expected because the developing neural tube is the part of the embryo that contains the highest levels of endogenous RA [15]. A role for RA in rostrocaudal patterning of the nervous system has been well established in Maden's sudy (2002) [16] and in dorsoventral patterning Rantz, a., et al (2003) provided evidence that RA sets the dorsal, interneuron and ventral neuron boundaries within the rostral spinal cord [17]. Here we investigate in what way RA is involved in neurulation, shaping and growth of the neural tube by examining the morphology of the chicken neural tube that has developed in the excises of RA.

2. MATERIALS AND METHODS

Fertilized eggs of chicken domestic Gallus gallus purchased from local Breeding Farm. Total eggs were 120, all the eggs were healthy and pathogens free. Eggs weighed, that ranges from 54 - 60 g. eggs cleaned with 70 % ethanol for sterile condition and labeled. The eggs were incubated at 38ºC and 80% relative humidity for required time until the embryos reached stage 8, 10,15 and 18 of development according to Hamburger and Hamilton [18].

At these stage eggs were divided into three groups for each stage; each group consist of 10 eggs (n=10). Two groups were control groups, one of them without any treatments and the other control injected with DEMSO. The third group treated with 1.5 mg/ml of RA.

The injection procedure was done as following; eggs were removed from the incubator and wiped with 70% ethanol, followed by introduction of a 1.5-mm-diameter hole in the blunt end using a sharp forceps. Using a 20-gauge needle, injections were slowly introduced directly into the air sac of the egg, after which the injection aperture was sealed with seal tap, and eggs returned to the incubator for another 24 hrs.

Embryo collection: The eggs were opened at three, four and five days of incubation. They were cracked open and the outer shell was cut out to create a wide opening for visualization of the embryo. The viability of the embryos was measured by the heartbeat. The embryos were transferred to a petri dish. All the embryos were fixed with 10% formalin and examined under stereomicroscope to evaluate any developmental deformities. Then embryos were embedded into

paraffin. Sections of 4 micron thickness were prepared and stained with hematoxylin–eosin for light microscopic examination.

3. RESULTS AND DISCUSSION

Data for the overall survival, mortality, fertility and malformation of embryos at all experimental stages were showed in Fig. 1 presented as percentage. At all stages fertility rate was between 60 to 100%, survival rate were between 80 to 100%, and death rate was 0 to 20%, and malformation rate was 0% in all control groups however, it was 70 to 100% in treated groups. These results suggested that 1.5 mg/ml of RA has teratogenicity effect on chicken embryo.

3.1 Morphometric Measurement

Surface area of whole embryo were measured by using image j software was illustrated in Fig. 2 errors bars present the stander error deviation (SED). Figure showed that the surface area of whole embryo treated with 1.5 mg/ml at all experimental stages were significantly decreased compared with the control and DEMSO groups.

3.2 Morphological Observation

Control embryos without treatment or injected with DEMSO at stage HH 8 were collected at stage HH15 (50-55 hr of incubation), all characteristic features of development in this stage are observed. Lateral body-folds extend to anterior end of wing-level (somites 15-17), Optic cup is completely formed visceral arch 3 and cleft 3 are distinct, Fig. 3 A, A1.

3.2.1 Embryos treated with 1.5 mg/ml RA at HH8

Fig. 3 A2 showed all embryos in this group showed retardation in growth and defect in brain, it was small in addition forebrain, midbrain did not develop comparison with control. Also embryos showed, delay in heart development and trunk was abnormal. Moreover, somites numbers was less, compared with control as showed in Fig 3 A2 black arrow.

3.3 Embryos at HH10

Control embryos without treatment or injected with DEMSO at stage HH 10 were collected at stage HH18 (65-69 hr of incubation), all characteristic features of development in this stage are observed. Somites: 30-36; extend beyond level of leg-bud, at the cervical flexure, the axis of the medulla forms approximately a right angle to the axis of the posterior to trunk, as showed in Fig 3 B and B1.

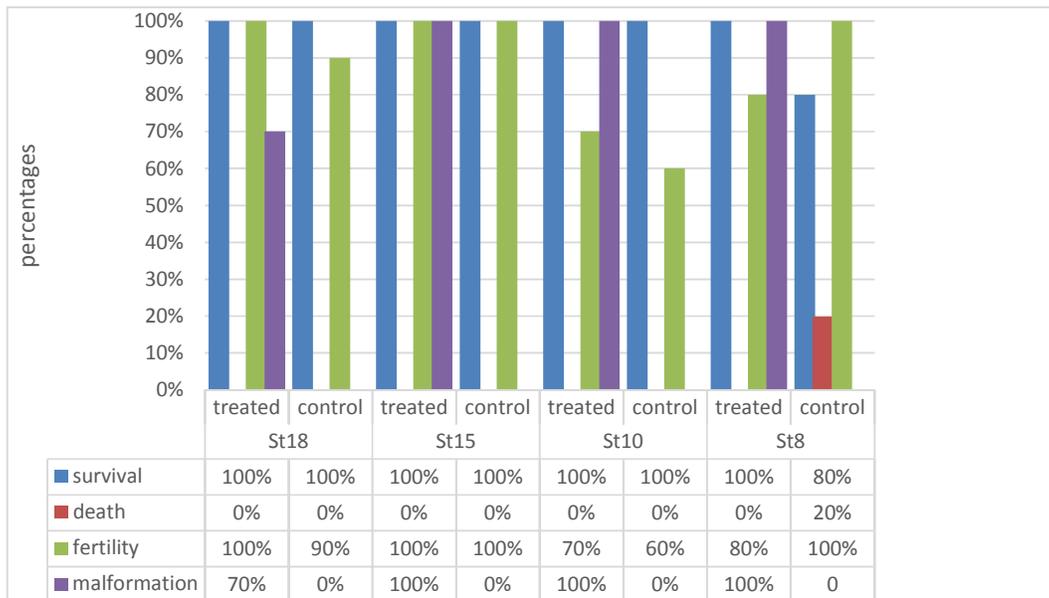|	treated St18	control St18	treated St15	control St15	treated St10	control St10	treated St8	control St8
survival	100%	100%	100%	100%	100%	100%	100%	80%
death	0%	0%	0%	0%	0%	0%	0%	20%
fertility	100%	90%	100%	100%	70%	60%	80%	100%
malformation	70%	0%	100%	0%	100%	0%	100%	0

Fig. 1. Histogram showing the Percentage of survival, death, fertility, and malformation of embryos injected with 1.5 mg/ml (RA) at HH8, HH10, HH15, and HH18

Embryos treated with 1.5 mg/ml RA at HH10:Fig 3 B2 showed all embryos in this group showed retardation in general growth, heart turning upward optic vesicles losing and abnormal trunk Fig 3 B2.

3.4 Embryos at HH15

Control embryos without treatment or injected with DEMSO at stage HH 15 were collected at stage HH21 (3.5 days of incubation), both wing- and leg-buds are slightly asymmetrical. Somites were 43-44, eye-pigmentation: Faint, as showed in Fig 4 A and A1.

Embryos treated with 1.5 mg/ml RA at HH10: as showed in Fig. 4 A2, very small head, open neural tube, eyes lose and induction of fore limb bud was observed.

Embryos treated with 1.5 mg/ml at HH18 showed the following:

3.4.1 Control group

The collected embryos were at stage HH23 all embryos were survive, survival rate was %100 n=10 normal growth in all organs.

The head segmentation were complete, eye pigmentation observed, heart was normal, tail toward to head and the limb buds are as long as they are wide and there is a slit in the fourth cleft.

3.4.2 Demso group

Similar observation seen in embryos at the control group, collected at the same stage23.

3.4.3 Treated groups

collection was at stage HH23 retardation in growth was observed in all embryos (n=10) the head was upward (Fig 4 red arrow), eye pigmentation lost ,brain not developed, bronchial arch was up normal, the heart was enlarged Fig 4 red arrow head, and tail did not bend toward to head and it was very thin.

Current results showed that RA even in the low dose has teratogenicity. RA affects
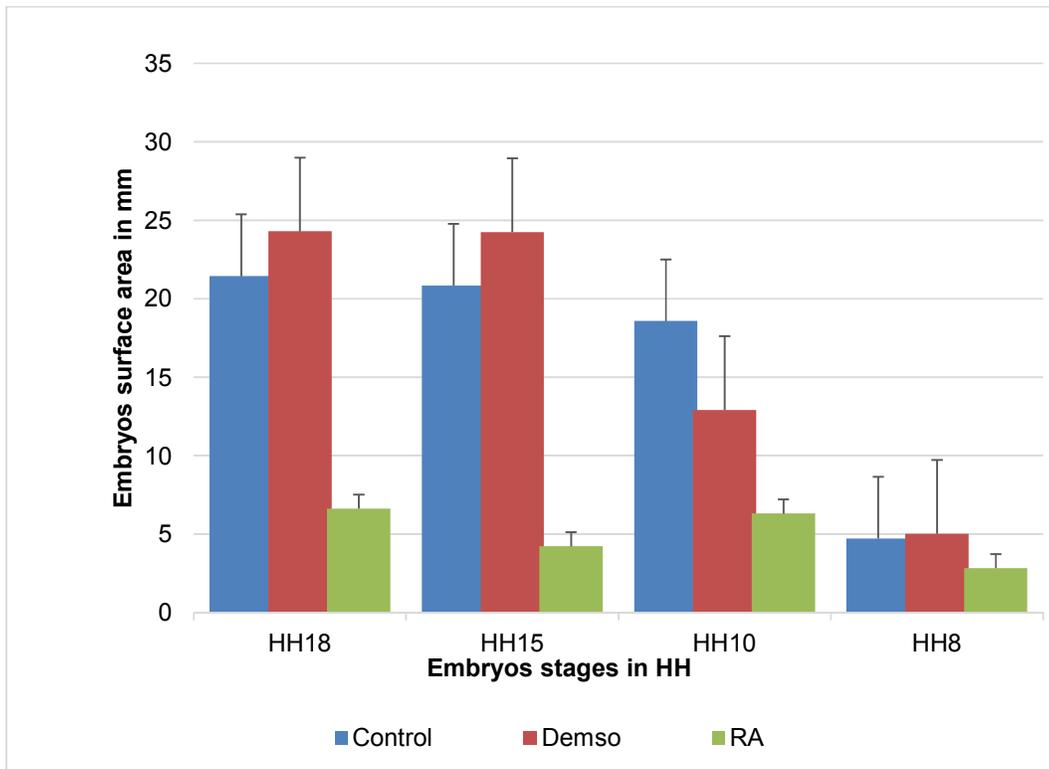

Fig. 2. Histogram showing the whole surface area in mm of embryos measured by IMAGE J at different stages and with different treatments errors bars presented as SED
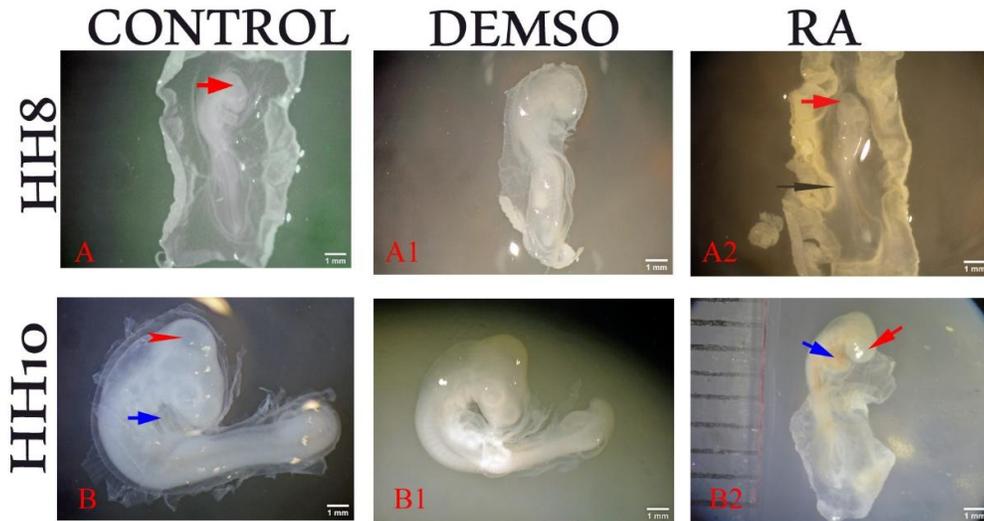
Fig. 3. Lateral view of chick embryos A and B control embryo injected at HH8, and 10, A1 and B1 embryos injected with DEMSO. A2 and B2 lateral view of embryo injected with RA1.5 mg/ml showed abnormal head developing
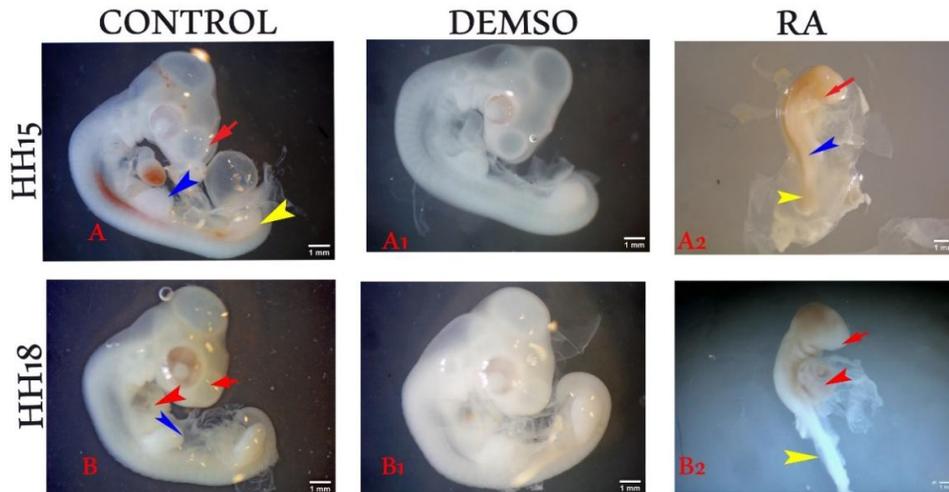

Fig. 4. Lateral view of chick embryos A and B control embryo injected at HH15, and 18, A1 and B1 embryos injected with DEMSO. A2 and B2 lateral view of embryo injected with RA1.5 mg/ml showed failure of neural tube closure in the cranial region

morphological organization & cellular behavior of developing neural tube. Morphologically the general shape, shape of the lumen was very wide in treated group Fig 5 B1 red arrow. Spatial arrangement of the cell populations of floor plate and roof plate of neural tube appears to be abnormal in treated group of chicken embryo as showed in Fig 5 B2 red arrowhead. A variety of cellular effects have been Suggested as being responsible for the appearance of spina bifida following RA administration. These include vascular damage, malformation of the notochord, distortion of the neural folds, cell death in the neural tube, delayed posterior neuropore closure [19,20] Scientists have shown that prenatal RA exposure induces several structural abnormalities, including reduced cerebellar size and impaired foliation profile [21]. .
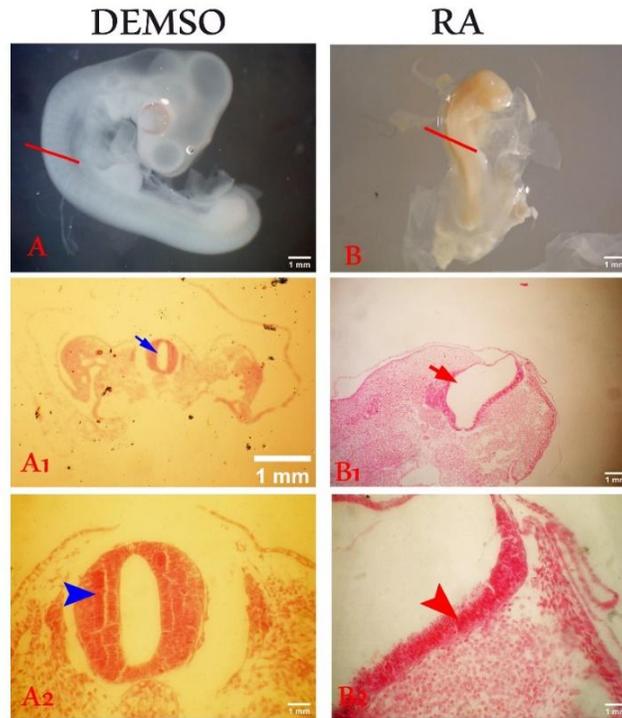
Fig. 5. RA induced defects in neural tube at HH15. A: The control embryo, which was treated DEMSO solution. B: RA-treated embryos 1.5 mg/ml A1–A2 and B1,B2, H&E staining of transverse sections of whole embryos at the level indicated by the red line. Scale bars 1mm .A1,A2 control and B1-B2 RA-treated embryos at the cranial level, which the structure of the neural tube was damaged

4. CONCLUSION

Current study shows that treatment with exogenous RA, dose greater than the level needed to maintain the normal embryonic development and that leads to severe malformation. It suggested that the response of embryos to ra is very sensitive especially during neurulation process during embryogenesis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kam RKT, et al. Retinoic acid synthesis and functions in early embryonic development. Cell & bioscience. 2012;2(1):11.
2. Means AL, Gudas LJ. The roles of retinoids in vertebrate development. Annual review of biochemistry. 1995;64(1):201-233.
3. Holland LZ. A chordate with a difference. Nature. 2007;447(7141):153-154.
4. Wolbach SB, Howe P.R. Tissue changes following deprivation of fat-soluble A vitamin. The Journal of experimental medicine. 1925;42(6):753-777.
5. Frazier CN, Ch'uan-K'uei H. Cutaneous lesions associated with a deficiency in vitamin A in man. Archives of Internal Medicine. 1931;48(3):507-514.
6. Wilson JG, Roth CB, Warkany J. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. American Journal of Anatomy. 1953;92(2): 189-217.
7. Delescluse C, et al. Selective high affinity retinoic acid receptor alpha or beta-gamma ligands. Molecular pharmacology. 1991; 40(4):556-562.

8. Raverdeau M, et al. Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. Proceedings of the National Academy of Sciences. 2012;109(41): 16582-16587.

9. Raverdeau M, Mills KH. Modulation of T cell and innate immune responses by retinoic acid. The Journal of Immunology. 2014;192(7):2953-2958.

10. Greene MR, Oliva A. The briefest of glances: The time course of natural scene understanding. Psychological Science. 2009;20(4):464-472.

11. Raghav S, Reutens D. Neurological sequelae of intrauterine warfarin exposure. Journal of Clinical Neuroscience. 2007; 14(2):99-103.

12. Jergil M, et al. Valproic acid–induced deregulation in vitro of genes associated in vivo with neural tube defects. Toxicological Sciences. 2009;108(1):132-148.

13. Coberly S, Lammer E, Alashari M. Retinoic acid embryopathy: Case report and review of literature. Pediatric Pathology & Laboratory Medicine. 1996;16(5):823-836.

14. Bowen SE, Hannigan JH. Developmental toxicity of prenatal exposure to toluene. The AAPS journal. 2006;8(2):E419-E424.

15. Maden M, et al. The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms. Development. 1998;125(21): 4133-4144.

16. Maden M. Retinoid signalling in the development of the central nervous system. Nature Reviews Neuroscience. 2002;3(11):843-853.

17. Frantz A, et al. Reliable microsatellite genotyping of the Eurasian badger (Meles meles) using faecal DNA. Molecular Ecology. 2003;12(6):1649-1661.

18. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. Journal of morphology. 1951;88(1):49-92.

19. Tibbles L, Wiley M. A comparative study of the effects or retinoic acid given during the critical period for inducing spina bifida in mice and hamsters. Teratology. 1988; 37(2):113-125.

20. Kapron-Brás CM, Trasler DG. Histological comparison of he effects of the splotch gene and retinoic acid on the closure of the mouse neural tube. Teratology. 1988; 37(4):389-399.

21. Coluccia A, Belfiore D, Bizzoca A, Borracci P, Trerotoli P, Gennarini G, et al. Gestational all-trans retinoic acid treatment in the rat: neurofunctional changes and cerebellarphenotype .Neurotoxicol Teratol. 2008;30:395-403.