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Original article

The Effect of Retinoic Acid on Development of Chicken Embryos

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ABSTRACT

Background and aims. *Retinoic acid (RA) is important morphogen for promoting normal vertebrate development, its work in critical gradient in most organs and tissues. Exogenous of RA can cause malformation in these organs and tissues. The current study aimed to find out the effect of application of different concentrations 6 ,10mg/ ml of retinoic acid dissolved in dimethyl sulphoxide (DMSO) on chicken development at different embryonic stages.* ***Methods****. Fertile domestic Gallus gallus eggs sullied from local poultry farm, eggs were cleaned and sterilized, then divided into two groups of experiments, one group for each concentration. Each experiment contains three groups, 10 eggs for each. These groups repeated four time for four different stages HH8, HH10, HH15 and HH18. Eggs were incubated in the incubation for require stage, then removed from incubation and injected with RA or (DMSO) in air sac or kept without injection as untreated control, then eggs were incubated for another 24 h. Eggs were opened after 24 and 48 h of incubation, survive embryos were collected and evaluated morphologically and histologically.* ***Results****. The study showed that RA cause general growth retardation. In addition, it causes, microcephaly, cranial bifida, cardiomegaly, forelimb induction, straight trunk. The degree of malformation depended on the developing stage and RA concentration, were malformation increases with high concentration and early stages. Significant effects observed in embryos treated with 10mg/ml at early stage. Moreover, effects of RA in HH8 and HH10 was sharper than that observed at embryos injected at HH15 and HH18 in two concentrations.* ***Conclusion****. This study demonstrates that exogenous RA treatment at doses above those necessary to ensure normal embryonic development results in severe abnormalities. This suggests that the embryonic response to rheumatoid arthritis is extremely sensitive, particularly during fetal neurogenesis.*

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INTRODUCTION

During the development of multi-cellular organisms, cell fate and behavior are regulated by several morphogens its works in precise gradient. Retinoic acid (RA) is an important morphogen that aids in the development of vertebrate embryos. It is made from provitamin A in mesodermal tissues that include members of the retinaldehyde dehydrogenase enzyme family [1,2].

RA and other retinoids, as well as vitamin A and its physiological metabolites, have a strong influence on pattern development, and may be one of the morphogens that regulate embryonic development [3-6].

While retinoids are required for proper embryo development, too much or too little, at the wrong stage or at the wrong time, can harm the developing embryo [7].
RA play a role in the anterior/posterior development of the central body axis and the limbs of vertebrates [8]. 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 axis. Also, RA receptors regulate many developmental control genes, including home box genes and growth factor genes [9].
In this study, we were use different concentrations of RA 6,10 mg/ml at different embryonic stages HH8, HH10, HH15and HH18 of chicken embryo growth and development, to observe effect of RA on morphological shape and influence it on some organs.

MATERIALS AND METHODS

Chemicals

Stock solutions of RA were prepared in dark room by dissolving it into DMSO for in ovo experiments. These solutions were protected from extended exposure to light when being prepared and used and then kept in aliquots at -20°C

Egg Injections

Fertilized white chicken (Gallus gallus) eggs were purchased from local breeder. Total eggs were 240, all the eggs were healthy and pathogens free. eggs cleaned with 70 % ethanol for sterile condition and labeled, eggs incubated on their side at 37.5C and 80% humidity for the required time, until appropriate developing stages. All embryos were staged according to Hamburger and Hamilton (HH) definitions [10], and embryos were harvested HH8, HH10, HH15, HH18.

Experimental design

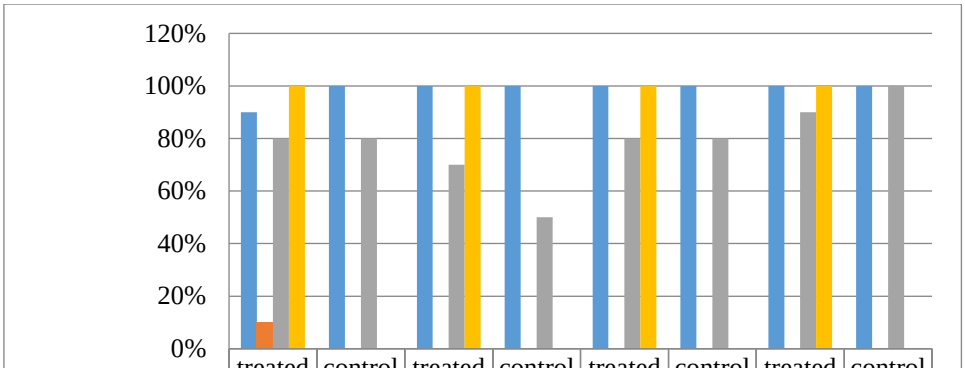
Eggs in each cons (6,10 mg/ml) divided into four groups, each group divided into four stages (HH8, HH10, HH15, HH18), each stage divided into three groups consist of 10 eggs (n=10).
Two groups were control groups, one of them without any treatments and the other control injected with DMSO. The third group treated with 6,10 mg/ml of RA

Specimens' preparation

Injection of embryos at (stage HH8) After, about 26-29 hours of incubation the eggs were left at room temperature and punctured at the blunt end of the egg and pulled about 1.5 to2 ml from albumin to allow the embryo float faraway about eggshell and injected RA(6,10mg/ml) 0.1 to 0.2 ml of solution was injected in the yolk sac with needle of outer diameter 0.60 MM (size23G x1.1). after the injection, holes made in the eggs were sealed with tape and the eggs were return to incubator to continued developed. The same methods as described above were used with another stages HH10 after 33 hours of incubation, HH15 after 50 hours of incubation; HH18 after 69hours of incubation
Embryos Collections and fixation: After incubation about three ,four and five days ,eggs were removed from incubator ,upper surface of shell was opened by micro scissor , embryos transferred with spatula to petri dish and phosphate buffer slain (PBS) was added to remove excess embryonic membranes using micro scissor , embryos were assayed under the dissected optical technology microscope to detected the morphology shape, embryos were photographed by digital camera (olympus) then embryos placed in formalin 10% for fixed. Then embryos were embedded into paraffin wax. Sections of 4-micron thickness were prepared and stained with hematoxylin–eosin [11] for light microscopic examination.

RESULTS

Embryos treated with 6mg/ml: Data for the overall survival, mortality, fertility and malformation at all experimental stages were showed in Fig 1 presented as percentage. At all
stages, fertility rate was between 50 to 100%, survival rate was between 90 to 100%, and death rate was 0 to 10%, and malformation rate was 0% in all control groups however, it was 100% in treated groups.



	treated	control	treated	control	treated	control	treated	control
	HH18		HH15		HH10		HH8	
survival	90%	100%	100%	100%	100%	100%	100%	100%
death	10%	0%	0%	0%	0%	0%	0%	0%
fertility	80%	80%	70%	50%	80%	80%	90%	100%
malformation	100%	0%	100%	0%	100%	0%	100%	0%

Figure 1. Histogram showing the Percentage of survival, death, fertility, malformation of embryos injected with 6 mg/ml (RA) at HH8, HH10, HH15, HH18

Morphometric Measurement

Morphometric measurement observations presented as surface area of whole embryos showed in figure 2, showed sever reduction in the surface area of whole mount embryo treated with RA 6mg/ml compared with control and DMSO treated. Embryos injected with RA at HH8 and HH 10, surface area were reduced by four times compared with control and Dmso treatment, RA = 3 and 4mm, DMSO = 6 and 10 mm, control = 12 and 18 mm respectively. Injection of RA at HH15and HH18, surface area was reduced three times compared with control and DMSO treatment, RA= 10 and 10 mm, DMSO = 28 and 30 mm, control = 28 and 30mm

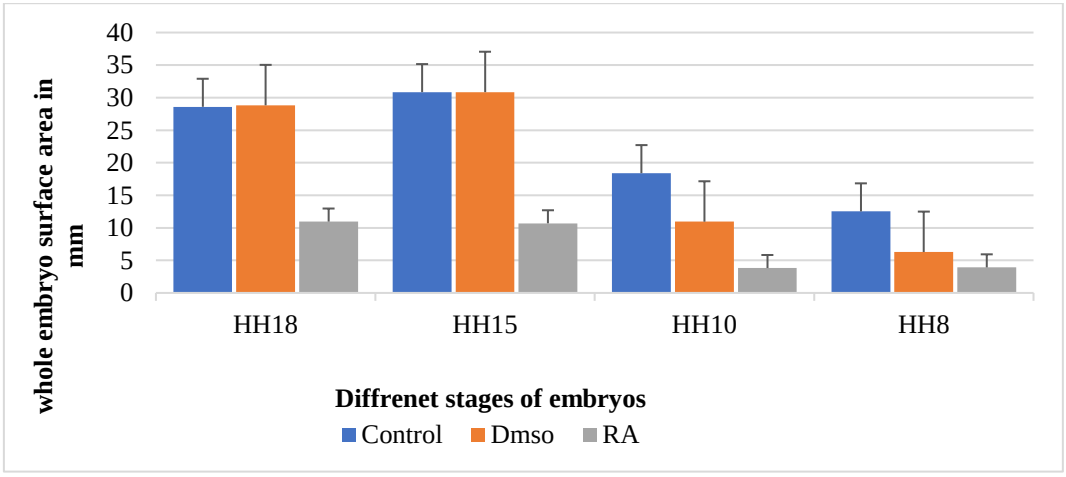


Figure 2. Histogram showing the whole surface area in mm of embryos measured by IMAGE J at different stages and with different treatments errors bars presented as SED.

Morphological Observation

Embryos at HH8

Control and dmso group: control embryos without treatment or injected with DMSO at HH8 were the first collected at HH17 (52-64 hr of incubation), all characteristic features in this stage are normal development observed fig 3 A, A1. the second embryos collection were at HH18, HH19 (52-64 hr. of incubation) were normal organs (fig3 B, B1). Embryos treated with 6 mg/ml RA at HH8: first were collected at HH17, embryos showed had retardation in growth compared with the control and DMSO embryos, delay in growth in all organs, the brain did not develop to it segmentation, heart was open and caudal region fail to form showed in (fig3, A2). Second collection was at HH18, HH19, the head was up did not toward to tail, brain fail to developed, cardiomegaly and tail toward to right unlike the normal situation and there was induction in forelimb bud unlike the rest of other organs (fig3, B2).

Embryos at HH10

Control and DMSO group: Control embryos without treatment or injected with DMSO at HH10 were the first collected at HH19 (68-72 hr incubation), Second collection (fig 3 D, D1) was at HH23 were normal development (fig 3 C, C1)
Embryos treated with 6mg/ml of RA at HH10: in tow collection had delay in growth in all organs exception forelimb bud was induction, anencephaly, cardiomegaly and tail were in a straight direction did not bend (fig3, C2, D2).

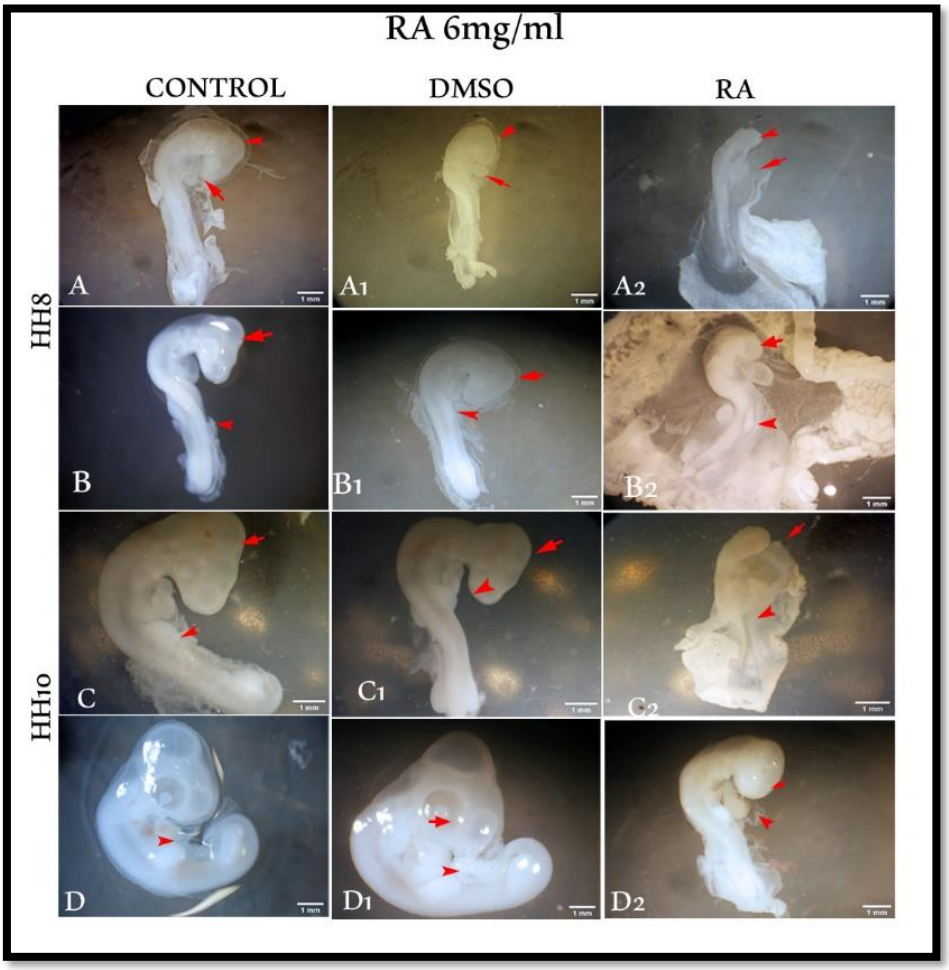


Figure 3. Effect of injection 6 mg/ml of RA on developing chicken embryo at HH8 and HH10

Lateral view of developing chicken embryo showed(A), (B), (C). (D) control embryos were normal development, red arrow heads indicate to brain and forelimb, red arrows indicate to heart, (A1), (B1), (C1), (D1) Embryo injected with DMSO was normal growth, red arrowheads indicate to forelimb and brain, red arrows indicate to heart and eye. (A2), (B2) (C2), (D2) Embryo injected with RA 6 mg/ml delayed in growth in brain(microcephaly)indicated by red arrows, induction in forelimb indicated by red arrowheads, caudal region not formed, HH 8, HH10 (The collection in second and third day of injected).

Embryos at HH15

Control and DMSO group: Control embryos without treatment or injected with DMSO at HH15 were collected at HH23(96 of incubation) were normal development (fig4 A, A1).
Embryos treated with 6mg/ml of RA at HH15: all embryos where retardation in growth whereby did not arrive to HH23, defect in brain whereby did not bend, eye un-pigmentation, cardiomegaly (Fig 4 A2).

Embryos at HH18

Control and DMSO group: Control embryos without treatment or injected with DMSO at HH18 (Fig4 B, B1) were collected at HH25 (4.5 to 5 day of incubation) all characteristic features of normal development in this stage were observed.

Embryos treated with 6mg/ml of RA at HH18: all embryos had retardation in developed, microcephaly, eye unpigmentation, cardiomegaly, forelimb, hindlimb did not appear and trunk region was s shape unlike normal case (Fig4 B2).

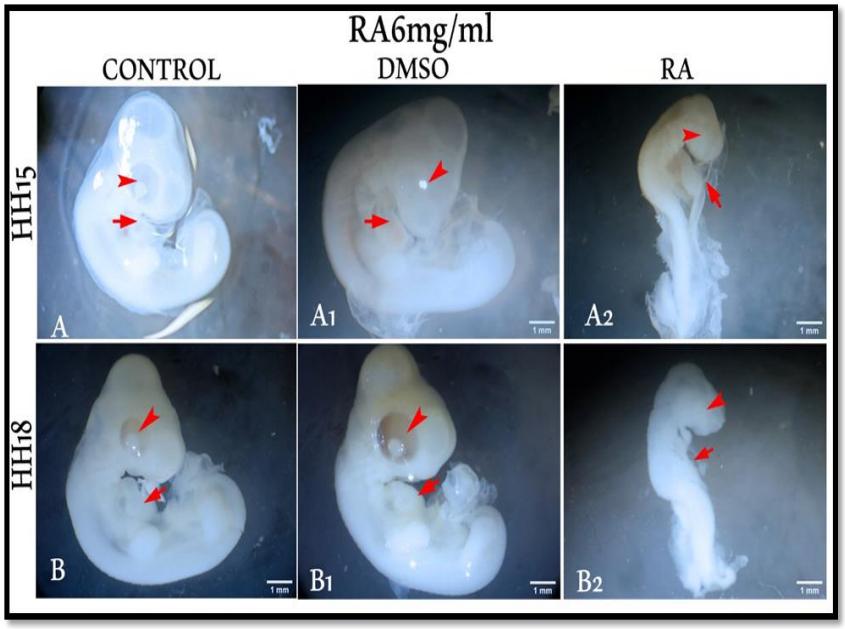


Figure 4: Effect of injection 6 mg/ml of RA on developing chicken embryo at HH15 and HH18

Lateral view of developing chicken embryo showed(A), (B)control embryos were normal development, red arrowheads indicated to eye, (A1), (B1) embryos injected with DMSO was normal growth, red arrows indicate to forelimb, red arrowheads indicate to eyes, (A2), (B2). Embryos injected with RA 6 mg/ml unpigmentation indicated by red head arrow, red arrows indicate to cardiomegaly, abnormal caudal region HH15, HH18 (The collection in next day of injected).

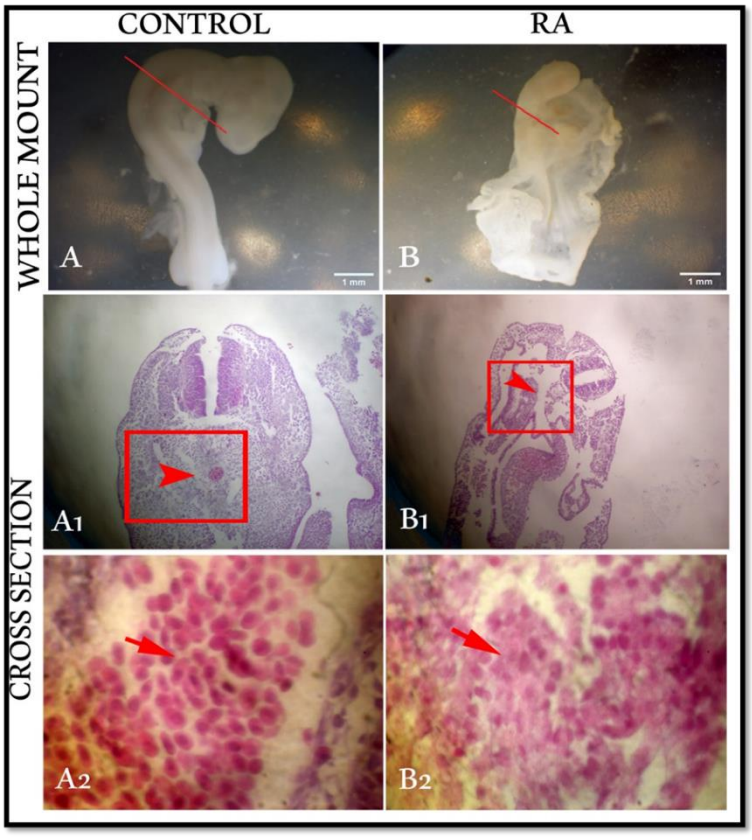
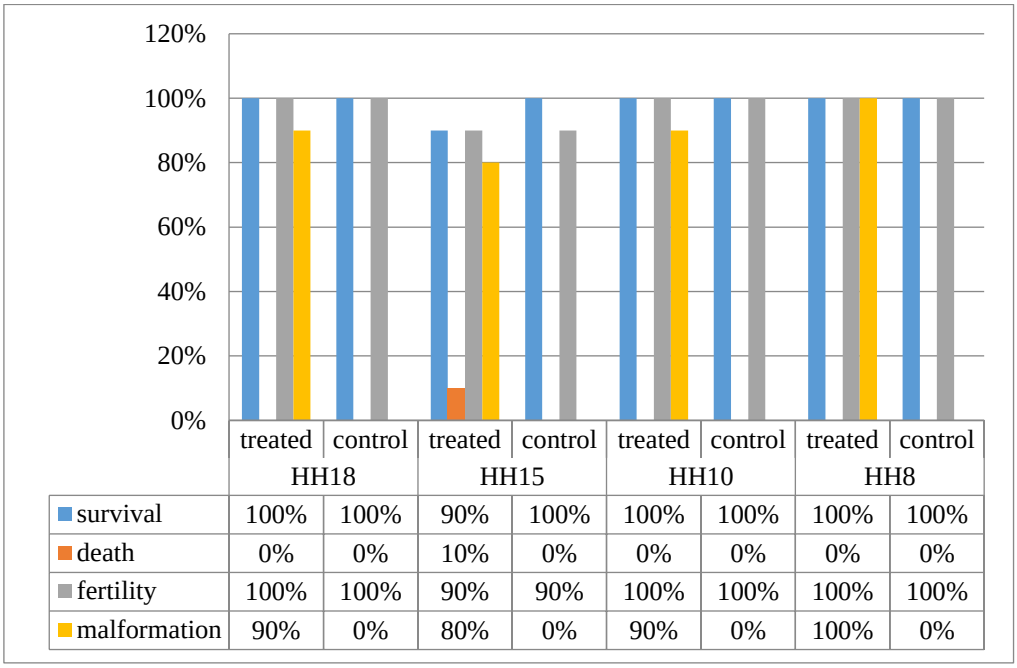


Figure 5. RA induced defects in Heart at HH8.

A: The control embryo, B: RA-treated embryo 6 mg/ml A1–B1 and A2-B2, H&E staining of transverse sections of whole embryos at the level indicated by the red arrows. A, A1, A2 control and B-B1, B2 RA-treated embryos at the cardiac level, which the structure of the Heart was damaged.

Embryos treated with 10mg/ml

Observations for the overall survival, mortality, fertility and malformation at all experimental stages were showed in (Fig6) presented as percentage. At all stages, fertility rate was between 90 to 100%, survival rate was between 90 to 100%, and death rate was 0 to 10%, and malformation rate was 0% in all control groups however, it was 80 to 100% in treated groups.



	treated	control	treated	control	treated	control	treated	control
	HH18		HH15		HH10		HH8	
survival	100%	100%	90%	100%	100%	100%	100%	100%
death	0%	0%	10%	0%	0%	0%	0%	0%
fertility	100%	100%	90%	90%	100%	100%	100%	100%
malformation	90%	0%	80%	0%	90%	0%	100%	0%

Figure 6: Histogram showing the Percentage of survival, death, fertility, malformation of embryos injected with 10 mg/ml (RA) at HH8, HH10, HH15, HH18

Morphometric Measurement

Embryos at HH8 Morphometric measurement for surface area of whole embryos showed in (figure 7), showed sever reduction in the surface area of whole mount embryo treated with 10 mg/ml of RA compared with control and DMSO treated. Embryos injected with RA at HH8 and HH 10 surface area reduced by six times compared with control and DMSO treatment, RA = 6 and 6mm, DMSO = 35 and 49 mm, control = 37 and 50 mm respectively. Injection of RA at HH15and HH18 surface area reduced by ten times compared with control and DMSO treatment, RA =6 and 10mm, DMSO 43 and 58 mm, control 47 and 52 mm respectively.

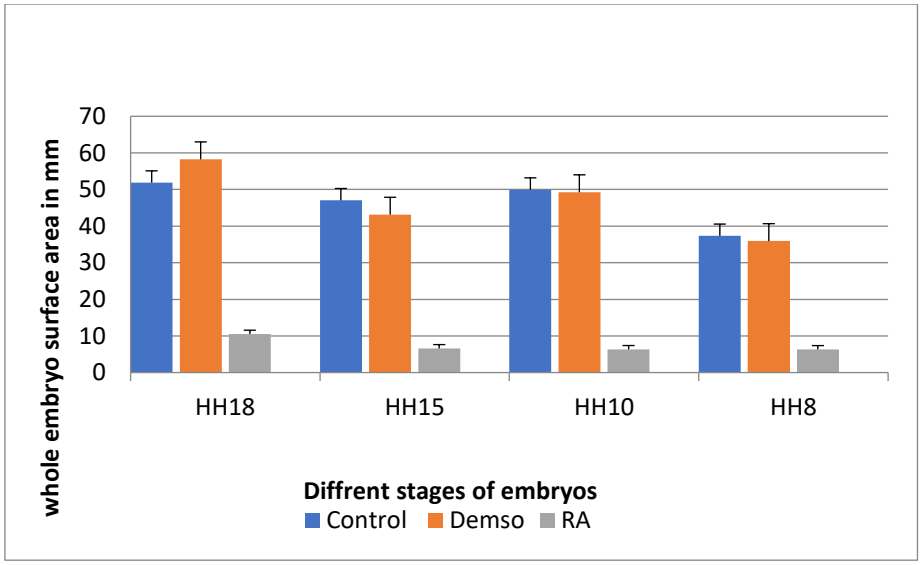


Figure 7: Histogram showing the whole surface area in mm of embryos measured by IMAGE J at different stages and with different treatments errors bars presented as SED.

Embryos at HH8

Control and DMSO group: control embryos without treatment or injected with DMSO at HH8 were first collected at stage HH19, (68-72 hr of incubation), all embryos was normally. The somites extend to the tail, the eyes were unpigmented and the tail bud curves towards the head as showed in (fig8 A and A1). Second set of collection was at stage HH21-22 (70-72 hr of incubation), embryos showed normal development, eye pigment and somites that have fully extended into the tail (fig8 B, B1).

Embryos treated with 10mg/ml of RA at HH8: the first collected (fig8 A2), all embryos were survived, with retardation in growth and weak heartbeat, blood vessels were weak, the head did not form fully and the trunk was straight. The second set of embryos collected, showed survival embryos with obvious growth delay, blood vessels decayed, the malformation similar to the first collected embryos but here the forelimb bud induction (fig8 B2).

Embryos at HH10

Control and DMSO group: the first embryos collected were at HH20 (70 -72 hr of incubation) indicated in fig8 C, C1, all embryos survival were %100 with normal growth. Second collected were at stage HH23 (96 hr of incubation). All characteristic features of embryos were normal, (fig8 D, D1).

Embryos treated with 10mg/ml of RA at HH10: the first collected indicated in (fig8 C2), it was at HH20, the embryos with delay growth, bleeding and defect in the brain whereby fail to develop, also the cardiomegaly, tail did not toward to the head, eye unpigment. Second collected were at HH23 (96 of incubation) the characteristic features in all embryos were retardation in growth in all organs, anencephaly, tail was striate and observed induction in the forelimb bud unlike the other organs whereby it does not appear at this stage (fig8 D2).

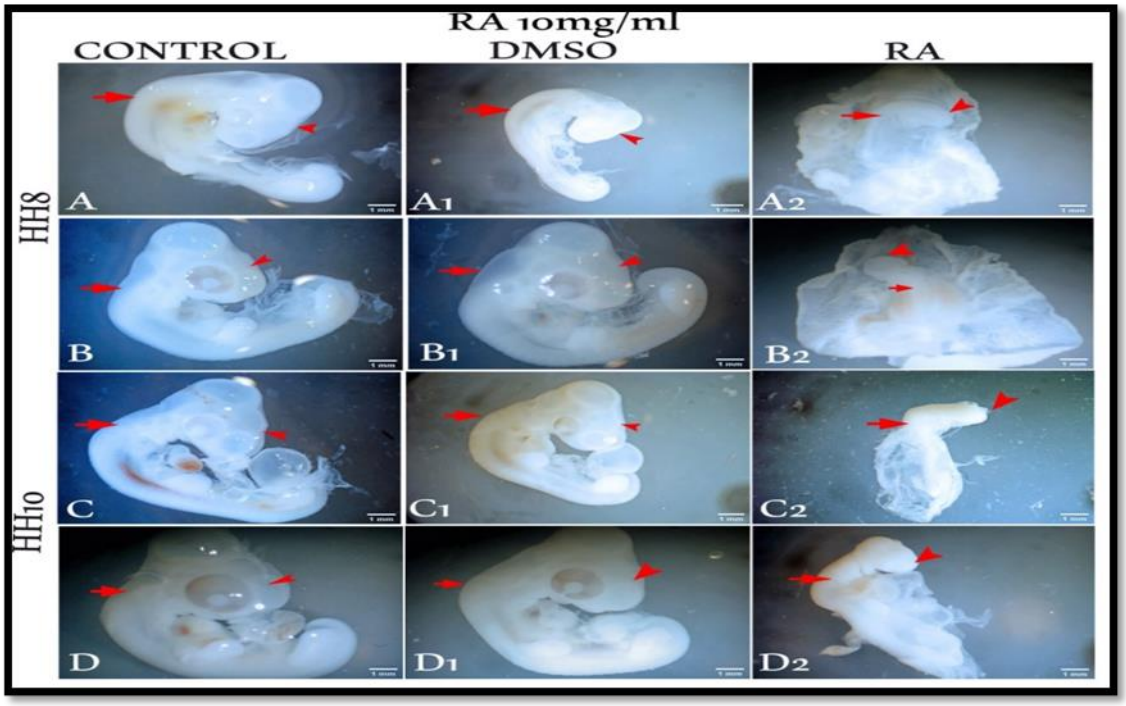


Figure 8: Effect of injection 10 mg/ml of RA on developing chicken embryo at HH8 and HH10

Lateral view of developing chicken embryo showed(A), (B),(C),(D)control embryos was normal development, (A1), (B1), (C1), (D1) embryos injected with DMSO was normal development, (A2), (B2) (C2), (D2). Embryo injected with RA 10mg/ml, delayed in growth red head arrow indicate to microcephaly, induction in forelimb bud bleeding in heart, abnormal bronchial arch indicated by red arrows and caudal region failed to form, HH8, HH10 (The collection in second and third day of injected).

Embryos at HH15

Control and DMSO group: The first collected in Control and DMSO groups showed in (fig9 A, A1), it was at HH19 all characteristic features are normal development observed. The second collected were at stage HH23, observed normal embryos (fig9 B, B1).

Embryos treated with 10mg/ml of RA at HH15: the first collected indicated in (fig9 A2), it was at HH19, survival embryos was %70, defects in blood vessel was weak, cardiomegaly and bleeding, the brain did not complete formed (microcephaly) and induction forelimb bud unlike other organs did not developed such as head, heart and trunk. Second collected were at stage HH23, observed embryos with the same defects in first collected, microcephaly, unpigmented eyes, cardiomegaly and induction in forelimb bud (fig9 B2).

Embryos at HH18

Control and DMSO group: Control and injected embryos with DMSO at HH18, the first collected indicated in (fig9 C, C1), it was at HH23, embryos were normal development, the same characteristics in this stage. Second collection were at HH26, HH27 (5-5.5 days of incubation) all embryos were normal growth and development. (fig9 D, D1).

Embryos treated with 10mg/ml of RA at HH18: the first collected was at HH23, the second collected was at HH26-27, observed retardation in growth,forebrain was open, eye unpigment, bleeding in heart, delay in blood vessel, but observed induction forelimb bud whereby as well as the hindlimb in size, (fig9C2 and D2).

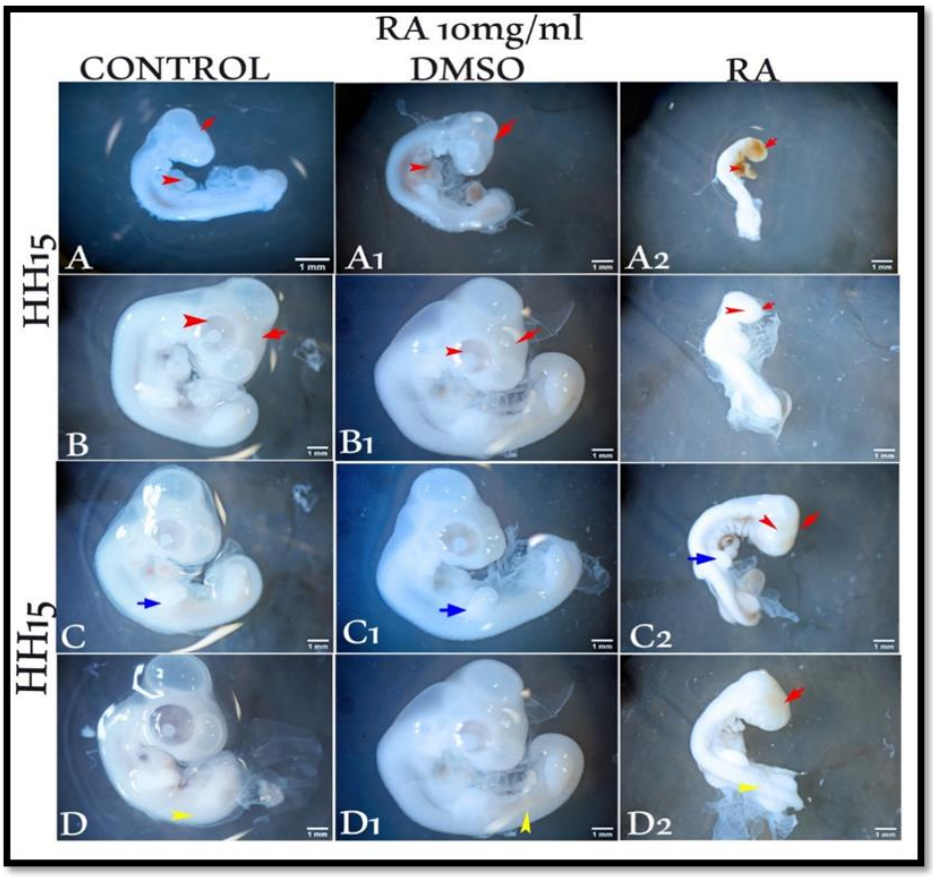


Figure 9: Effect of injection 10 mg/ml of RA on developing chicken embryo at HH15 and HH18

Lateral view of developing chicken embryo showed (A), (B), (C), (D) control embryos was normal development in all organs red arrows indicate to forebrain, red arrow heads indicate to eye, blue arrows indicate to forelimb ,yellow arrowhead indicated to trunk, (A1), (B1), (C1), (D1) embryos injected with DMSO, was normal growth red arrows indicated to fibrined arrowhead indicated to heart and eyes, blue arrows indicate to forelimb, yellow arrowhead indicated to trunk ,(A2),(B2),(C2)(D2)embryo injected with RA 6 mg/ml retardation in growth ,red arrows indicate to microcephaly and eye lost, blue head arrow indicate to cardiomegaly, blue arrows indicate to induction in forelimb bud, yellow arrows indicate to abnormal trunk, HH15, HH18 (The collection in second and third day of injected).

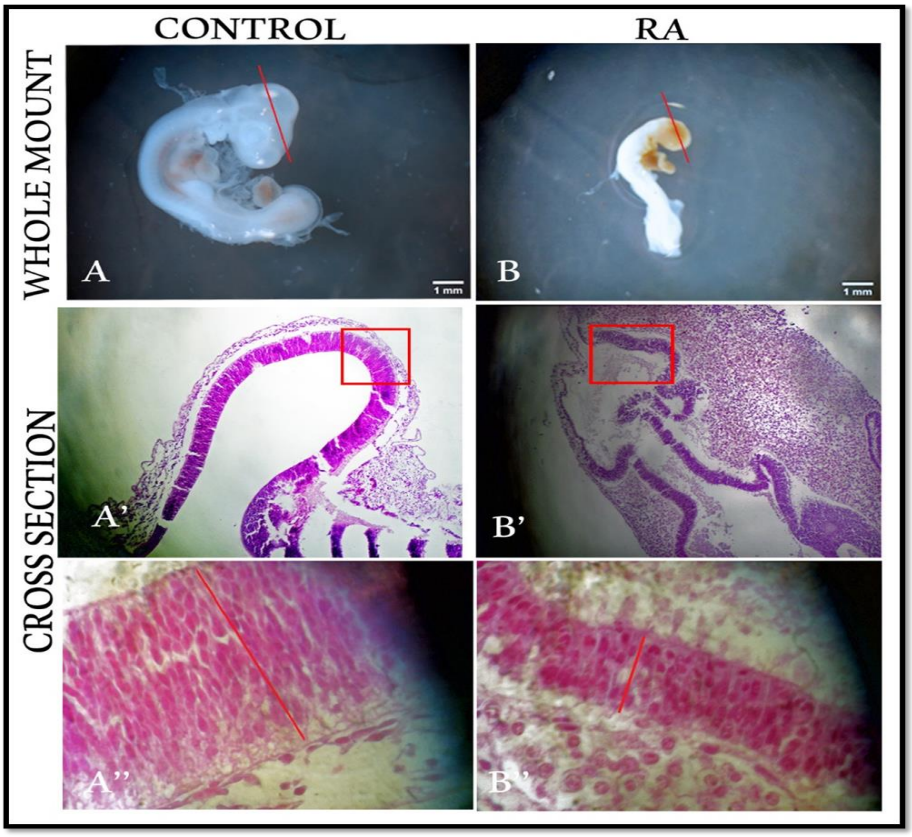


Figure 10: The effects of RA on Brain chick embryo at HH15.

Control embryo, B- RA treated embryo 10mg/ml, A', A'' and B', B'', H&E staining of transverse sections of whole embryos at the level indicated by the red line. A', A'' control and B', B'' RA-treated embryos at the cranial level, which the structure of the brain was reduced.

DISCUSSION

Retinoic acid is important morphogen in body axis formation by control many of genes in early development [12], Its work in certain gradient, so excess amount cause malformations in tissues and organs. Effects of RA on the central nervous system were showed in this study, where RA cause forebrain, midbrain and hindbrain reduction in size, microcephaly indicated in (figure3 A2, B2). These effects increase with concentration that's agreed with some studies, found that there is relation between concentration and brain malformation [13]. Gale and others suggested that, malformation in the head caused by influences of RA on specification of r rhombomere 4 in some but not all of its of characteristics at the time of r4 boundary formation, also they said contralateral vestibuloacoustic neurons, the pathways of a subpopulation of crest and the pathways of facial ganglia extensions are influenced by RA while other elements of the r4 phenotype, mesenchymal crest migration, cartilaginous elements and motor neuron extensions, were not influenced [14].

The current study found excess amount of RA cause malformation in some organs, brain, heart and forelimb bud. RA is essential for cardiac progenitor specification and organogenesis, RA signaling and its establishment of networks that drive both early and later steps of normal vertebrate heart development. Although RA signaling is necessary for normal vertebrate heart development, it is needed at critical levels in embryonic period, RA are maintained within an appropriate range as both decreases and increases in RA signaling can result in congenital heart malformations[15].

High doses of RA can inhibit the specification of both ventricular and atrial cardiomyocyte[16] this study showed that, effect exogenous RA on heart development , caused cardiomegaly ,whereby red blood cells scattered not arrangement and bleeding ,RA promote atrial identity at the expense of ventricular identity, so excess amount of RA may inhibit ventricular specification [17], and influence endocardia cushion volumes by inhibit proliferation of myocardial cell ,this effect on ventricular wall lead to abnormal out blood flow that is may be explain heart weak beat and blood vessels as discussed by Bouman and others [18].

full cardiac bifida showed in (fig8 B2) embryos treated with 6mg/ml indicated RA affect precordial cell, that may lead to cell death, this effect similar to work done by Osmond and others, were remove a piece of precordial that cause to heal in crescent and distrust heart beat where fast in cases and weak in another [19]. Degrees of cardia bifidia depended

in concentration were illustrated in this study ,where embryos treated with 10mg/ml full cardia bifidia, while embryos treated with 6 mg/ml showed bifurcation heart, that agree with Osmond and others study , they found that high concentration inhibit the migration of the entire right heart-forming area and, providing the two heart- forming regions have not already joined in the mid-line, this will result in two completely separate hearts, on other hand lower concentration, may not affect the precordial cells at the cranial end of the heart-forming area, allowing them to migrate and join up with the left-hand branch. They suggested that RA cause disrupting in the fibronectin gradient during heart formation, suggest that RA interfere with the interaction between the precordial cells and fibronectin in the extracellular matrix [20].

RA acts as a graded morphogen by specifying the anteroposterior limb pattern through induction of downstream genes that are directly involved in generating the pattern of forelimb by conferring positional information to limb bud cells, local application of RA to the anterior margin of chick limb buds' results in pattern duplications by induce shh morphogen and Hoxd-11, a gene induced by the polarizing signal [21].

In our study (figure3 A2, B2) showed embryos injected with RA before limb bud initiation result in induction in forelimb bud, unlike control embryos where there is no forelimb bud formation

Suggest that RA induce the genes in lateral plate mesoderm (LPM) include patterning genes (Meis.shh). Pickering in his study demonstrate RA induction of the gene encoding Shh that

specifies anteroposterior positional values and promotes growth of the developing limb bud [22]. initiation forelimb bud before hindlimb bud attributed to Tbx5 is required upstream of FGF10 [23] and RA is required upstream of Tbx5[24] and Tbx5 is essential for forelimb initiation [18,19], via stimulation of epithelial-to-mesenchymal transition (20) and activation of Fgf10 [21].

RA functions upstream of these important regulators of forelimb bud initiation, but hindlimb buds do not express Tbx5, but instead use Tbx4 and Pitx1 to initiate expression of Fgf10 to stimulate outgrowth [22,23,34], so excess RA can lead to induction of Shh, Hoxb8, Hand2, and Meis1/2.

This study suggested that RA work in early embryonic development in certain gradient and interfere with the other important morphogens such as FGF, shh, Bmp, wnt and other which regulate the normal pattern formation and organogenesis. This study was done in the morphological and histological level, which make it consistent and fairly strong, but were not strong enough, indeed study on the molecular level were needed to confirm our results in order to find the interaction between these morphogens and RA during embryogenesis.

CONCLUSION

This study shows that treatment with exogenous RA at doses exceeding the levels required to maintain normal embryonic development causes severe malformations. This indicates that the fetal response to rheumatoid arthritis is highly sensitive, especially during neurogenesis during fetal development.

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تأثير حمض الريتينويك على تطور أجنة الدجاج

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المستخلص

الخلفية والأهداف. يعتبر حمض الريتينويك (RA) مورفوجينًا مهمًا لتعزيز التطور الطبيعي للفقاريات، ويعمل في التدرج الحرج في معظم الأعضاء والأنسجة. يمكن أن يسبب التهاب المفاصل الروماتويدي الخارجي تشوهًا في هذه الأعضاء والأنسجة. هدفت الدراسة الحالية إلى معرفة تأثير إضافة تراكيز مختلفة 6,10 ملغم/مل من حمض الريتينويك المذاب في ثنائي ميثيل سلفوكسيد (DMSO) على نمو الدجاج في المراحل الجنينية المختلفة. **طُرق الدراسة**. تم تطهير بيض جالوس جالوس المنزلي المخصب من مزرعة الدواجن المحلية، وتم تنظيف البيض وتعقيمه، ثم تقسيمه إلى مجموعتين من التجارب، مجموعة واحدة لكل تركيز. تحتوي كل تجربة على ثلاث مجموعات، 10 بيضات لكل منها. تكررت هذه المجموعات أربع مرات لأربع مراحل مختلفةHH8 ، HH10، HH15و .HH18تم تحضين البيض في الحضانة للمرحلة المطلوبة، ثم إزالتها من الحضانة وحقنها بـ RA أو (DMSO) في كيس هوائي أو الاحتفاظ بها بدون حقن كتحكم غير معالج، ثم تم تحضين البيض لمدة 24 ساعة أخرى. تم فتح البيض بعد 24 و 48 ساعة من الحضانة، وتم جمع الأجنة الحية وتقييمها شكليا ونسيجيا. **النتائج**. وأظهرت الدراسة أن التهاب المفاصل الروماتويدي يسبب تأخر النمو العام. بالإضافة إلى ذلك، فإنه يسبب صغر الرأس، وتشقق الجمجمة، وتضخم القلب، وتحريض الطرف الأمامي، والجذع المستقيم. تعتمد درجة التشوه على مرحلة التطور وتركيز التهاب المفاصل الروماتويدي، ويزداد التشوه مع التركيز العالي والمراحل المبكرة. لوحظت تأثيرات ملحوظة في الأجنة المعالجة بـ 10 ملغم/مل في مرحلة مبكرة. علاوة على ذلك، كانت تأثيرات RA في HH8 و HH10أكثر حدة من تلك التي لوحظت في الأجنة المحقونة في HH15 و HH18في تركيزين. **الخاتمة**. توضح هذه الدراسة أن علاج التهاب المفاصل الروماتويدي الخارجي بجرعات أعلى من تلك اللازمة لضمان التطور الجنيني الطبيعي يؤدي إلى تشوهات شديدة. يشير هذا إلى أن الاستجابة الجنينية لالتهاب المفاصل الروماتويدي حساسة للغاية، خاصة أثناء تكوين الخلايا العصبية الجنينية.

الكلمات الدالة. جنين الفرخ، حمض الريتينويك، تأثير الدماغ والأطراف والذيل.