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The Effect of Retinoic acid on blood vessel of chicken embryos

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ABSTRAC
Formation of the vasculature is an essential developmental process, delivering oxygen and nutrients to support cellular processes needed for tissue growth and maturation. Retinoic acid (RA) and its downstream signaling pathway is vital for normal pre- and post-natal development, playing key roles in the specification and formation of many 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 1.5, 6 ,10mg/ ml of retinoic acid dissolved in dimethyl sulphoxide (DMSO) on chicken blood vessels development at different embryonic stages.
Domestic fertile Gallus gallus After being cleaned and sterilized, eggs from a nearby poultry farm were split into two experimental groups, one for each concentration. Three groups of ten eggs each are used in each experiment. These groups repeated four time for four different stages HH8, HH10, HH15 and HH18After being incubated for the necessary of required time, the eggs were taken out and then either injected with RA or DMSO in an air sac or left untreated as a control. They were then incubated for an additional twenty-four hours. Eggs were opened after 24 and 48 h of incubation, surviving embryos were collected and blood vessels formation were evaluated morphologically. The results of this study showed that RA causes general growth retardation of blood vessels. The degree of malformation depended on the developing stage and RA concentration, where malformation increases with high concentration and early stages.

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INTRODUCTION

Vasculogenesis is the formation of primitive vascular networks mainly through differentiation of vascular progenitor cells (VPCs) into endothelial cells (ECs), while angiogenesis is the growth and sprouting of additional blood vessels from pre-existing blood vessels (Patan, 2000)
In either case, formation of blood vessel networks is completed by branching (remodeling) of blood vessels through interaction between ECs and surrounding mural cells (MCs; smooth muscle cells or pericytes), and tightly regulated by various factors (eg, vascular endothelial growth

factor [VEGF], fibroblast growth factor [FGF], angiostatin, endostatin, and angiopoietins [Ang-1 and Ang-2](Willet et al., 2000).
Retinoic acid (RA), a lipid soluble hormone derived from Vitamin A, has numerous well documented functions in embryonic development. RA's intracellular signaling is mediated through binding to nuclear hormone receptors retinoic acid receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ(Mark et al., 2009)
Retinoic acid (RA) and its downstream signaling pathway is vital for normal pre- and post-natal development, playing key roles in the specification and formation of many organs and tissues (Maden, 2000)
Although retinoids are necessary for healthy embryonic development, too much or too little, at the incorrect time, or at the incorrect stage can damage the growing fetus over time. both retinoid excess and deficiency are capable of disrupting the development.
In the current study, investigate the role of different concentration of RA in vascular development, discussing RA in early blood vessel formation.

MATERIALS AND METHODS

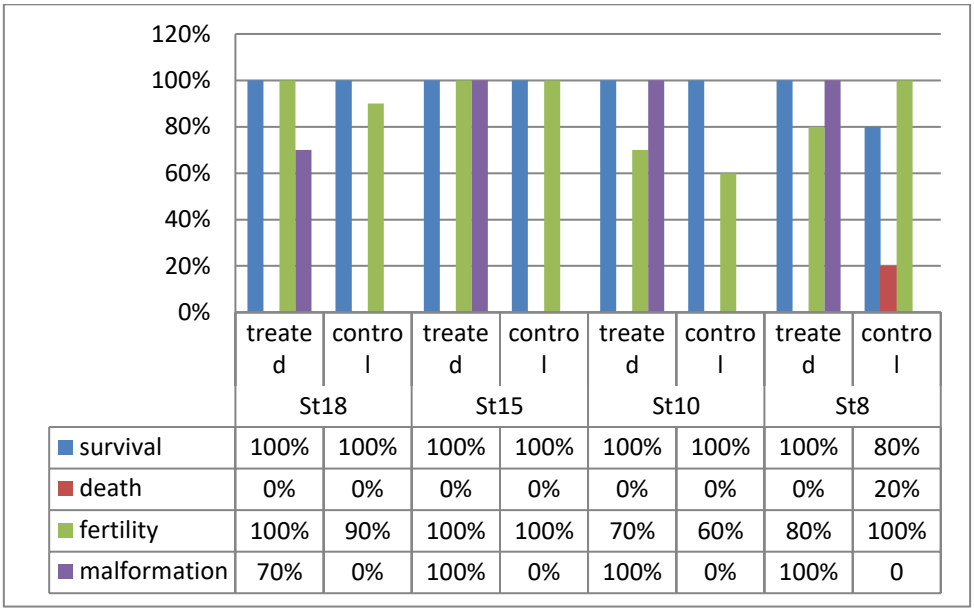
Chemicals:
In order to prepare stock solutions for in ovo investigations, RA was dissolved in DMSO in a dark room. After being made and used, these solutions were shielded from prolonged exposure to light and stored at -20°C in aliquots
Egg Injections:
Eggs from a local breeder of fertilized white chickens *(Gallus gallus*) were bought. The eggs were cleaned with 70% ethanol to ensure sterility, then labeled, and incubated on their side for the necessary desired time of development at 37.5C and 80% humidity until the proper phases of development were reached. All embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951),and embryos were harvested at HH8,HH10 ,HH15,HH18.

Experimental design:
In this study, two groups of eggs were created, each of which was divided into four distinct stages: HH8, HH10, HH15, and HH18. For every stage, three groups were designated: two control groups, one that did not receive any treatment, and a control group that was given a DMSO injection. The third group was treated with retinoic acid (RA) at varying concentrations of 1.5, 6, and 10 mg/ml.

Control embryos
The first group had no treatment but, the second group injected with 0.1 to 0.2 ml of DMSO solution
Treatment embryos
HH8: Following roughly 26 to 29 hours of incubation, the eggs were allowed to set at room temperature before being perforated at the blunt end, 1.5 to 2 milliliters of albumin were extracted to enable the embryo to float out of the eggshell, and RA (1.5,6 to 10 mg/ml) was injected. The yolk sac was punctured with a 0.60 mm outer diameter needle (size 23G x1.1) containing 0.1 to 0.2 ml of fluid. The holes created by the injection were taped shut, and the eggs were put back in the incubator to continue developing, the same method were used for the other stages
Embryos Collections and examination:
After three, four, and five days of incubation, the eggs were taken out of the incubator, the top of the shell was cut open with a scissors, the embryos were examined under a dissected optical technology microscope to determine the shape and morphology of the blood vessels, and then were photographed using an Olympus digital camera.

RESULTS:

Embryos treated with 1.5 mg/ml: Data for the overall survival, mortality, fertility and malformation of embryos at all experimental stages were shown in Fig. 1 presented as percentage. At all stages fertility rate was between 60 to 100%, survival rate was 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.



	St18 treated	St18 control	St15 treated	St15 control	St10 treated	St10 control	St8 treated	St8 control
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

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

Morphological Observation

Embryos at HH8, HH10, HH15, HH18:

Control and DMSO group: control embryos without treatment or injected with DMSO at HH8 were collected at HH15 (50-55 hr of incubation) and at HH10 were collected at stage HH18 (65-69 hr of incubation),and at HH15 were *3.1.1* collected at stage HH21 (3.5 days of incubation),and at HH18 embryos were collected at stage HH23 the blood vessels were normal growth and development, blood vessels were thic wall and easy eye naked with high rate beat in all stages.

Embryos treated with 1.5 mg/ml RA: Embryos were obtained at HH15 (50–55 hours of incubation), HH10 (65–69 hours of incubation), HH15 (3.5 days of incubation), and HH18 (stage HH23). When compared to the control and DMSO, these embryos showed reduced beating, thin blood vessel walls, and blood vessel retardation. There were blood islands everywhere (fig. 2).

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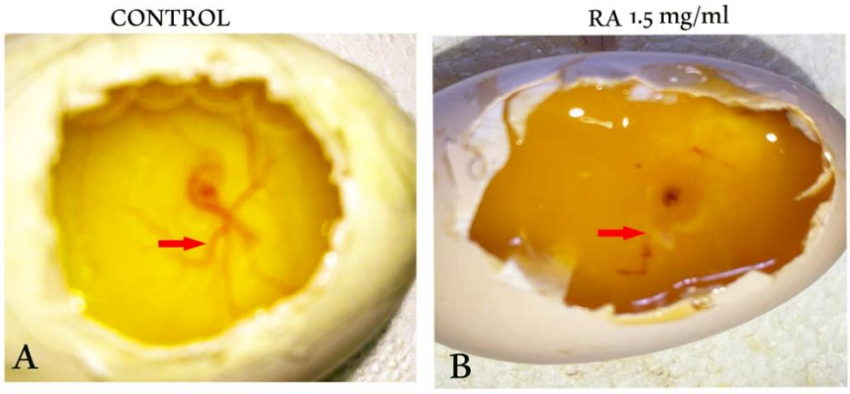
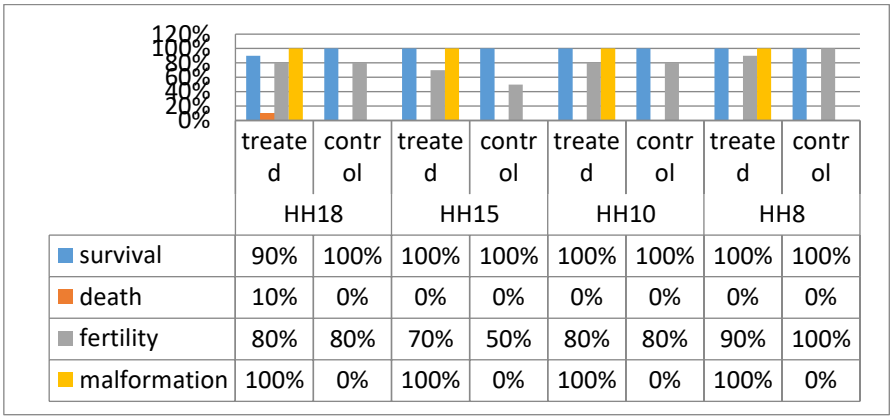


Figure 2: Effect of injection 1.5 mg/ml of RA on chicken embryo blood vessel

A: control embryo with normal growth, blood vessels were intact and blood flows normally with a normal heartbeat indicated by red arrow.
B: treated embryo with 1,5mg/ml, red arrow indicate to blood vessels retardation and embryo growth delay, no visible heartbeat

Embryos treated with 6mg/ml: Data for the overall survival, mortality, fertility and malformation at all experimental stages were showed in Fig3 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.



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

Morphological Observation

Embryos at HH8,HH10,HH15,HH18

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 include blood vessels were normal development growth .all embryos at all stages were collected after 24-48 hour and were normal growth with eye naked blood vessels.

Embryos treated with 6 mg/ml RA at HH8, HH10, HH15, HH18:

Embryos at HH8 showed had retardation in blood vessels growth compared with the control and DMSO embryos, also there were islands of blood scattererd around

all embryos at other stages in first and second collection observed delay and retardation in blood vessels growth and beating rate was weak and blood islands were scattered.

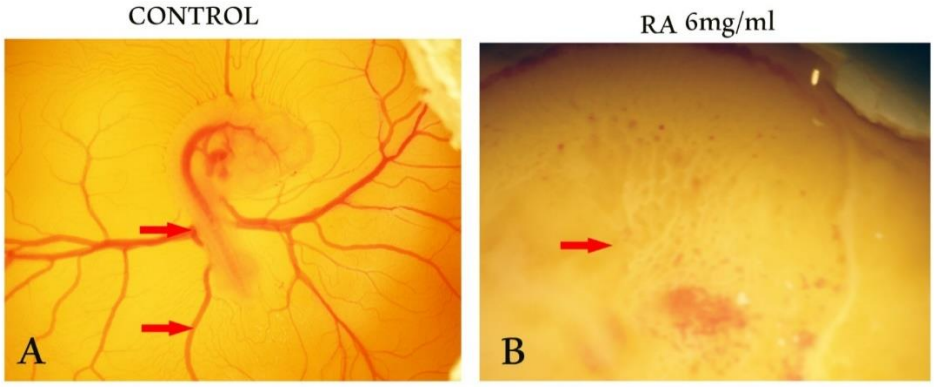


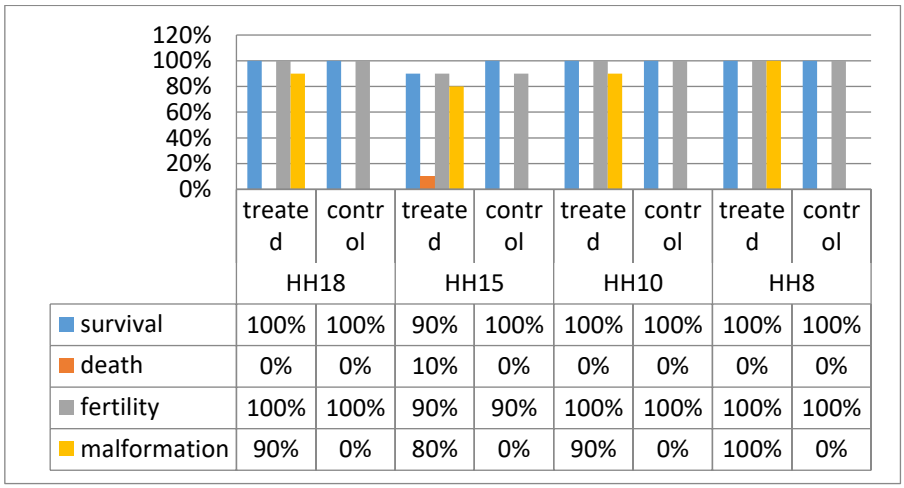
Figure 4: Effect of injection 6 mg/ml of RA on chicken embryo blood vessels

A: control embryo with normal growth in heart and blood vessels, which were eye naked and high-rate beat

B treated embryo with 6mg/ml was retardation in all organs include heart and blood vessels were weak and not clear to naked eye also there were scattered islands of blood around.

Embryos treated with 10mg/ml

Observations for the overall survival, mortality, fertility and malformation at all experimental stages were showed in (Fig5) 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 5: Histogram showing the Percentage of survival, death, fertility, malformation of embryos injected with 10 mg/ml (RA) at HH8, HH10, HH15, HH18

Morphological Observation

Embryos at HH8, HH10, HH15, HH18

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 were normally blood vessels

growth. Blood flow within the blood vessels was visible to the naked eye. as showed in (fig6 A, B). Second set of collection was at stage HH21-22 (70-72 hr of incubation), embryos showed normal development of blood vessels as showed in (fig6 C).
Other stages HH10, HH15, HH18 in both collections also were normal blood vessels growth.

Embryos treated with 10 mg/ml RA at HH8, HH10, HH15, HH18:
The first collected HH8 (fig6 A, A), all embryos were survived, with retardation in growth and weak heartbeat, blood vessels were weak and blood islands were scatterred. The second set of embryos collected, showed survival embryos with obvious growth delay, blood vessels decayed, the malformation similar to the first collected embryos as showed in (fig6 A)
The first collected HH10 indicated in (fig6 B), it was at HH20, the embryos with delay growth, bleeding and defect in most blood vessels
Second collected were at HH23 (96 of incubation) the characteristic features in all embryos were retardation in growth in all blood vessels showed in (fig6 B).

At HH15: the first collected indicated in (fig6 C), it was at HH19, survival embryos were %70, deffects in blood vessel was weak, and bleeding. Second collected were at stage HH23, observed embryos with delayed and weak blood vessels growth.
At HH18 the first collected was at HH23, the second collected was at HH26-27, observed retardation in growth, bleeding in heart, delay in blood vessel and scattered blood islands around.

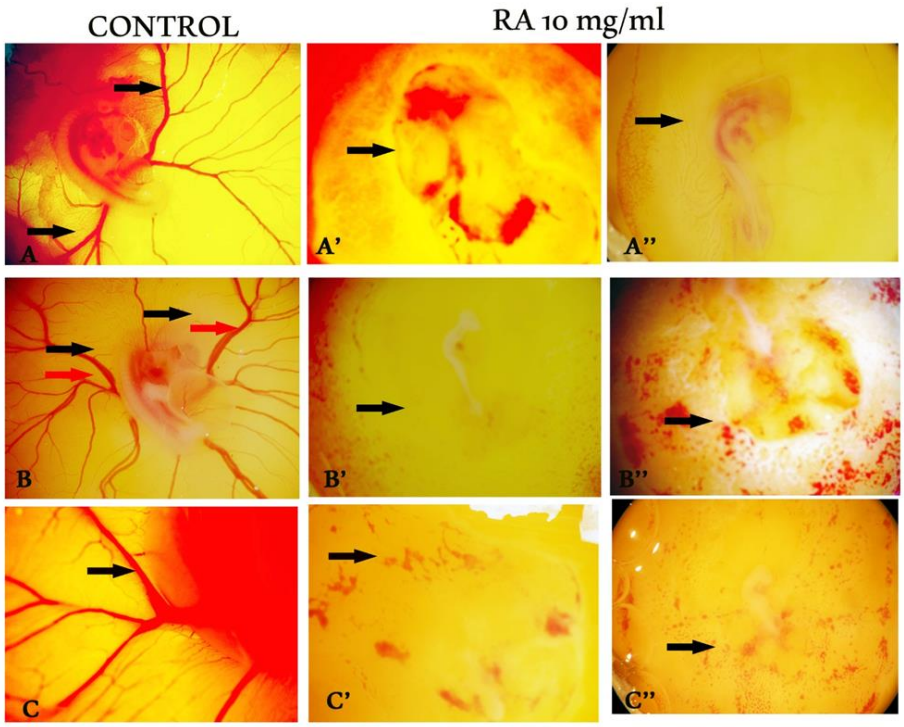


Figure 6: Effect of injection 10 mg/ml of RA on d chicken embryo blood vessels

A,B,C: control embryos at different stages HH8,HH10,HH18 , all embryos were normal growth , red and black arrow indicate to eye naked blood vessels with thick wall and high heartbeat also normal blood flow.

A,B,C: first collected of treated embryos with10mg/ml at HH8,HH10,HH15 all embryos showed growth retardation and delay in blood vessels also there were blood island scattered around indicate with black arrow.
A,B,C: second collected of treated embryos with10mg/ml at HH8,HH10,HH15, retardation growth was visible , black arrow indicate to blood island scattered around.

DISCUSSION:

In the present study investigated the effects of different concentrations of RA on blood vessel growth at different stages, it illustrated the effect of RA on blood vessel was sharp in high concentration that is correlated with our Previous Studies. Embryos treated with high concentration10mg/ml were had intense defects than embryos treated with RA at concentrations 1,5 ,6 mg/ml as showed in (fig 6,), which delay in blood vessel growth with scattered blood islands, also entire retardation in all organs development where the embryos failed to reach the required stage of development.
RA cause delay in blood vessel growth suggested RA contributes to a delay in the growth of blood vessels due to its function of suppressing the progression of the endothelial cell cycle, which is essential for the remodeling of vascular plexuses and the later phases of vessel assembly (Lai et al., 2003)

RA was needed during the entire period of vascular development to suppress endothelial cell growth and restore vascular remodeling, it appears that continuous regulation of the production or degradation (Bohnsack et al., 2004) RA is necessary for appropriate vessel formation and function, Our in vivo data collectively suggest that excess amount of RA signaling suppresses endothelial cell replication via the up regulation of Cdk inhibitors of the Cip/Waf family. Consistent with our findings, previous studies demonstrated that the expression of p21 and p27 is transcriptionally (Huss et al., 2004, Park et al., 2011) and post-transcriptionally (Huang et al., 2000) regulated by RA. the mechanism of RA control of proliferation has been previously demonstrated for lymphocytes (Naderi and Blomhoff,1999) and myeloid cells (Huang et al., 2000), as well as tumor cells (Chen et al., 2014, Ni et al., 2019, Costantini et al., 2020)

CONCLUSION:

Our research into the influence of different concentrations of retinoic acid on the blood vessels of chicken embryos has identified several critical malformations and effects on vascular development. It is evident that varying levels of retinoic acid can facilitate angiogenesis, enhancing the supply of nutrients and oxygen to the developing embryo. Conversely, excessive retinoic acid concentrations can lead to detrimental complications.

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