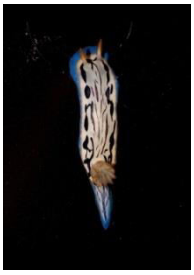

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Copper Sulphate Induced Embryological Anomalies in Avian Species *Gallus gallus*

Tendulkar S.S. and Kamble N.A.*

Department of Zoology, Shivaji University, Kolhapur- 416 004, India

**Corresponding Author*

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Abstract: In the field of developmental biology embryonic growth is accelerated by specific doses of biological and chemical factors. In the developing countries like India, contamination of metallic components like copper sulphate through food chain became serious issue to disturb developmental features of animals. Among vertebrates, chick embryo of *Gallus gallus* has dynamic features during development. Copper sulphate has been found to contaminate and bio-concentrate in the embryological tissues of experimental model *Gallus gallus.* A time- dependent exposure of copper sulphate up to five days of incubation showed prominent alterations in vascularisation and angiogenesis of developing embryo. The present investigation focus about egg weight and altered angiogenesis of avian embryo *Gallus gallus* after exposure to copper sulphate.

Keywords: Copper sulphate, Blood, Angiogenesis, Embryonic development, *Gallus gallus*

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Introduction

Augustin (2003) documented that angiogenesis in animals is fundamental process by which new blood vessels are formed as extensions from the existing vasculature. Ribatti *et al.* (2007) reported the history of the angiogenic pattern in vertebrates regarding the developmental physiology. Folkman *et al.* (2001) provided concept about angiogenesis research in which they enlighten the mechanism and guidelines for translation to clinical application of blood vessels formation. Murugesan *et al.* (2007) reported that in developing embryo carbon has inhibitory role in the vascular endothelial growth factor and fibroblast growth. Bhagwat *et al.* (2001) documented that angiogenic signals were essential for capillary tube formation during the vascularization. Andrés

et al. (2009) stated that FGF2-induction has pro-inflammatory mechanism in the angiogenesis of animals. Staton *et al.* (2004) reported number of methods for assessment of angiogenesis *in vitro* and *in vivo* during the development of animals. Brand *et al*. (2007) stated that angiotensinogen has inhibitory role to minimize the mechanism of angiogenesis in the chick chorio-allantoic membrane. Nyberg *et al.* (2005) noticed copper toxicity syndrome while studding number of endogenous inhibitors during angiogenesis of animal. Thapa *et al.* (2008) observed that clotrimazole ameliorates intestinal inflammation and disturbed angiogenesis by inhibiting interleukin-8 expression through a nuclear factor-kappaB-dependent manner.

Valdes *et al*. (2002) reported chick chorio-allantoic membrane as one of the accepted novel *in vivo* model for testing of biomaterials interaction in the developmental biology. Cao (2004) reported anti-angiogenic therapy for the neoplastic cell growth. Patan (2000) described mechanisms of vascular network formation in vasculogenesis and angiogenesis during the embryonic growth and remodeling. Lawrence *et al.* (2007) reported copper toxicity syndrome during development. Amelioration of ethanol induced growth retardation through all-trans-retinoic acid and alpha tocopherol in shell-less culture of the chick embryo was documented by (Satiroglu-Tufan and Tufan 2004). DeFouw and DeFouw (2000) noticed that vascular endothelial growth factor failed to acutely modulate endothelial permeability during early angiogenesis in the chick chorioallantoic membrane. Jinsong *et al.* (2006) determined amount of trace copper, lead, cadmium and iron in environmental and biological samples. Sinkovic *et al.* (2008) reported severe acute copper sulphate poisoning.

$CuSO_4$ is a toxic insecticide which is practically non-toxic to bees and moderately toxic to bird's. Studies on several aquatic species have found $CuSO_4$ to be highly toxic to fish and aquatic life. The clinical manifestation of $CuSO_4$ poisoning include erosive gastropathy, hepatitis, acute kidney injury and rhabdomyolysis, arrhythmias and seizures which are also reported probably secondary to other organ system involvement (Gamakaranage *et al*., 2011).

Copper sulphate is an inorganic compound and used as fungicide and bactericides. $CuSO_4$ is also used as a insecticides to control the various insects and pests in agriculture sector. $CuSO_4$ is listed on the National list of allowed synthetic substances for use in organic livestock production (Hoffman, 2012). Toxicity of copper sulphate depends on its significant entity, bioaccumulation and bio-reactivity with doses of ingestion (Gamakaranage, 2018).

Keeping above points into consideration, the present investigation focus about egg weight and altered angiogenesis of avian embryo *Gallus gallus* after exposure to copper sulphate.

Materials and Methods

Selection and collection of animal model:

The experimental animal *Gallus gallus* is a type of domestic fowl and an most common source of food (meat and egg). Hen eggs have typically bright white to shade of brown. The shape is elliptical and oval. Morphologically it is telolecithal type of egg with a large and dense yolk spread throughout the egg, but separated from pole of developing embryo.

The required number of eggs were collected from Poultry Farm located at the Vadange, Tal-Karveer, Kolhapur. The rearing house was constructed using bamboo to maintain natural ventilation. After collection, healthy and almost same sized eggs of *Gallus gallus* were selected by considering following parameters as-- colour- light brown; shape- small and oval; size- average 5 cm; weight- average 35 to 40 g.

Toxicant and preparation of stock solution:

Copper sulphate - $CuSO_4.5H_2O$ (molecular weight 159.602 g/mol), often called as blue vitriol available as crystalline substance blue in colour and soluble in water was used as a toxic substance.

For the present investigation about 1000 mg of copper sulphate was dissolved in 1000 ml of distilled water so as to prepare a solution of 1 mg/ml. The salt was completely dissolved and kept at room temperature and used to expose developing embryo in the eggs which were incubated at 37±1 C for different exposure period.

Experimental design and induction of dose:

Eggs were selected and their weight were recorded prior to the start of experiment. For the present experiment 2 sets were prepared, first as control group having 10 eggs and second set having three groups (each having 10 eggs) intoxicated with copper sulphate solution after 48 h of incubation of eggs. Group A was incubated up to 72 h; group B up to 96 h and group C up to 120 h. All the eggs were kept in Egg Hatcher incubator (MSW-233) (Fig. 1) which was initially cleaned, sterilised using 70% alcohol to maintain the aseptic and made it free from germ and micro-organisms.

Fig. 1: Egg hatcher incubator (MSW-233)

The incubator was pre-started to maintain 37 C temperature which is essential for the development of chick embryo. After 48 h of incubation, eggs were again cleaned with 70% alcohol and under sterilized and aseptic condition the eggs were treated with 0.1 ml stock solution of copper sulphate (Fig. 2). After microinjection eggs with developing embryo were re-sealed with adhesive sterile tapes. Again all the control and experimental eggs were kept in incubator for further embryonic development (Fig. 3). The eggs were weighed and embryos were observed at 72 (24 h after microinjection), 96 (48 h after microinjection) and 120 h (72 h after microinjection).

Fig. 2: Microinjection of copper sulphate into egg

Fig. 3: Treated eggs in Incubator

Results and Discussion

Present study focused on intoxication effect of $CuSO_4$ against development of chick embryo. During this study embryo showed some changes as compared to control embryonic development considering egg weight and angiogenetic pattern of embryo of *Gallus gallus* after intoxication with 0.1 ml of $CuSO_4$ solution.

Egg weight:

There is an insignificant reduction in egg weight at 72, 96 and 120 h after treatment with copper sulphate (Table 1).

Table 1: Weight (g) of control and copper sulphate treated eggs of *Gallus gallus*

Sr. No.	Exposure period (h)	Control	Treated
1.	72	34.61±2	34.28±4
2.	96	35.91±3	35.76±2
3.	120	37.06±6	36.24±5

Changes in angiogenesis:

After 72 h (i.e. 24 h after copper sulphate exposure) and 96 h (i.e. 48 h after copper sulphate exposure) of incubation the treated embryos showed no change in primary blood vessels and secondary blood vessels (Table 1). Treated embryos showed reduced number of tertiary blood vessels after 72 h (control - 23 and treated – 12) (Figs. 4, 5) and 96 h (control – 27 and treated – 24) (Figs. 6, 7). After 120 h (i.e. 72 h after copper sulphate exposure) of incubation reduced number of primary blood vessels (control – 8 and treated – 6) , secondary blood vessels (control – 13 and treated – 8) and tertiary blood vessels (control – 52 and treated – 41) were noticed (Figs. 8, 9; Table 2).

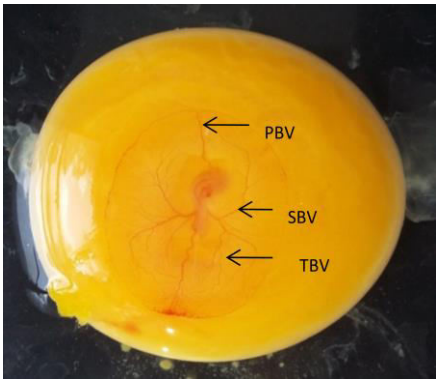

Fig. 4: 72 h control embryo showing normal development with control angiogenic pattern of *Gallus gallus.* PBV – Primary Blood Vessel, SBV – Secondary Blood Vessel, TBV – Tertiary Blood Vessel.

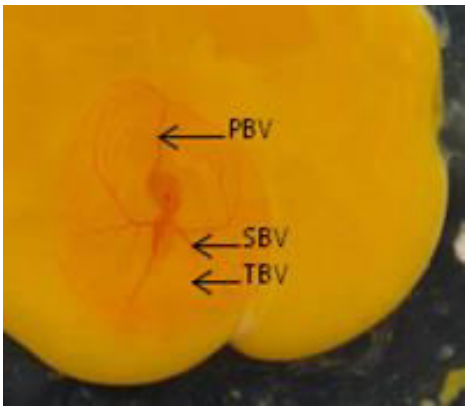

Fig. 5: 72 h treated embryo poorly branching of blood vessels indicating vestigial embryonic development. PBV – Primary Blood Vessel, SBV – Secondary Blood Vessel, TBV – Tertiary Blood Vessel.

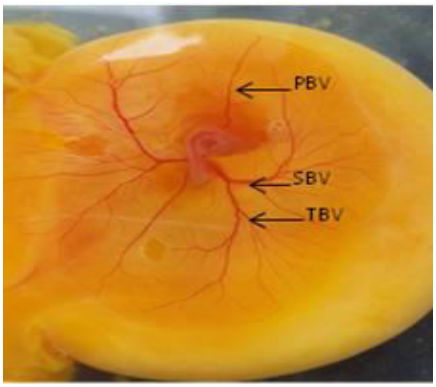

Fig. 6: Regular pattern of embryo development after 96 h. PBV – Primary Blood Vessel, SBV-Secondary Blood Vessel, TBV – Tertiary Blood Vessel.

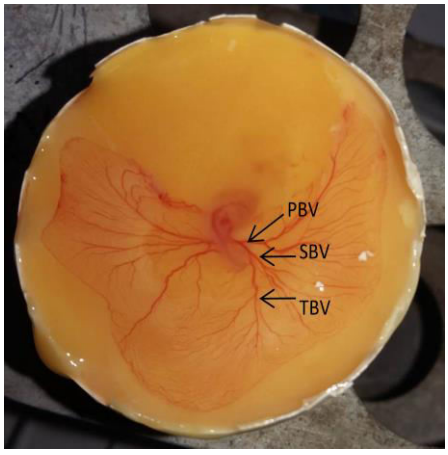

Fig. 7: 96 h embryo treated with copper sulphate has thin vascularization with stunted growth of embryo. PBV – Primary Blood Vessel, SBV – Secondary Blood Vessel, TBV – Tertiary Blood Vessel.

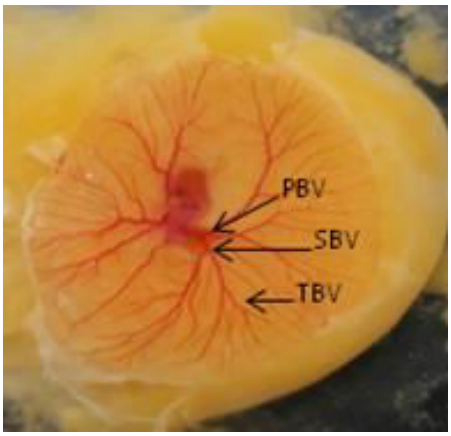

Fig. 8: Normal embryo after 120 h. PBV – Primary Blood Vessel, SBV – Secondary Blood Vessel, TBV – Tertiary Blood Vessel.

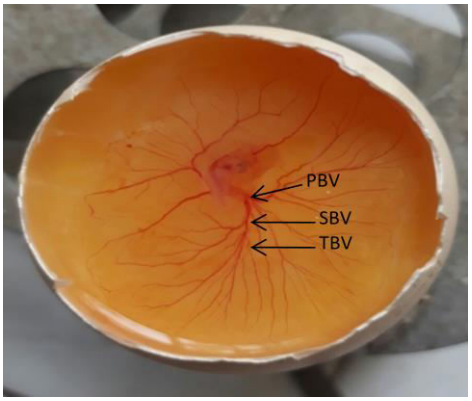

Fig. 9: Unequal vascularization in the embryo, with underdeveloped organization after intoxication of copper sulphate. PBV – Primary Blood Vessel, SBV – Secondary Blood Vessel, TBV – Tertiary Blood Vessel.

Table 2: Number of blood vessels in control and treated embryos

Hours	No. of Blood Vessels					
	Control			Treated		
	P	S	T	P	S	T
72	3	6	23±4	3	5	12±2
96	4	7	27±3	4	6	24±3
120	8	13	52±5	6	8	41±5

P – Primary Blood Vessel, S – Secondary Blood Vessel, T – Tertiary Blood Vessel.

Schlueter and Brand (2013) noticed that pro-epicardial cells were derived from the somatic mesoderm in the chick embryo during the different developmental stages. Katz *et al.* (2012) observed that distinct compartments of the pro-epicardial organ gave rise to coronary vascular endothelial cells which can be disturbed due to toxicity impact. Jayachitra *et al.* (2017) documented sequential morphological development including cardiovascular system of chick embryos on Namakkal variety of chicken. Szabo *et al.* (2011) assessed toxicity of s-metolachlor containing formulation and

heavy metals to chicken embryos and documented major inflammation of circulatory system.

Rashidi and Sottile (2009) documented that chick embryo found as suitable hatching model for contemporary scientific and biomedical research. The body weight of embryo was disturbed by treatment of $CuSO_4$ stock solution (Lehel *et al.*, 2014). The angiogenesis patterns get disturbed by $CuSO_4$ treatment. The supplementation of $CuSO_4$ in the eggs leads to decreased level of cholesterol and triglycerides synthesis in blood (Payvastegan *et al.* 2013). Gamakarange *et al.* (2011) noticed a significant drop in haemoglobin level which would have been contributed by the copper induced haemolysis. There was a significant reduction in the viscosity and thickness in the nutrient content of embryo i.e. albumin. $CuSO_4$ causes the reduction in the protein concentration present in embryo. It can be concluded that copper sulphate can induce toxic interaction which can highly reduce the viability of the embryo.

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