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Research Article

Evaluating the teratogenic effect of Eugenol in the development of the chick embryos.

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Abstract

The aim of the present study is to investigate the effects of single Eugenol dose on chick embryo development. Fertilized treated eggs of *Gallus gallus domesticus* injected with a single volume of 0.1, 0.2, 0.3, 0.4 and 0.5 µl Eugenol to the egg air chamber and control egg group were incubated at 37ºC in the humidified incubator for 3, 6, 12 and 18 days. A visible damage effect caused by increasing of Eugenol doses on the vascular development with the cause of angiogenesis and brain acetychlorinesterase (AchE) activity suppression, incomplete embryonic development with the increasing of malformation and the mortality rate of the chick embryo. This work demonstrated that the treatment of the chick embryo in the early stages with Eugenol may be considered as teratogenic agents at the dose levels utilized in our laboratory conditions between days 3 to day 18 of incubation.

Keywords: Eugenol; Chick Embryo; teratology; brain acetychlorinesterase; malformations.

Introduction

The chick embryo's model organism offers several advantages for teratology screening research (Smith et al., 2012), as it provides an excellent, sensitive, cheap and rapid model system for studying the development of higher vertebrates wherein growth accompanies morphogenesis (Brent, 1999) and allows screening for any toxicity (Kotwani, 1998). The rapid development of the cardiovascular system in the chick is convenient for the investigation of the initial events that drive both cardiogenesis and the formation of the vasculature. Within the first 24-30 hours of incubation, blood and vascular development is initiated both in the form of extra-embryonic blood islands and intra-embryonic endothelial cell specification and differentiation (Pardanaud et al., 1996). The first major blood vessels of the embryo, the paired dorsal aorta, have formed by 30-35 hrs of development (Garriock et al., 2010, Pardanaud et al., 1996) and a beating heart is apparent by 38-42 hrs of development (Patten and Kramer, 1933). Furthermore, the embryo remains largely transparent, as many of the primary organ rudiments, including the heart, liver, lung, kidney, eye, brain and spinal cord, initiate their morphological development, making

observation of organ specific vascular formation possible with both simple and advanced microscopy techniques.

Eugenol has been applied in cooking, food processing and industry for its antiseptic properties and appetite, digestion stimulant (Burt, 2004), pharmacy, perfume, cosmetics. It used in dental care, as an antiseptic against oral bacteria (Cai and Wu, 1996) and analgesic (Cortés-Rojas et al., 2014). It is effective against a large number of other bacteria (Burt and Reinders, 2003, Fichi et al., 2007), virus (Lee and Shibamoto, 2001) and fungi (Deans et al., 1995). Previous Studies have reported Eugenols with anticarcinogenic and antiallergic (Dwivedi et al., 2011) effect, antimutagenic activity (Miyazawa and Hisama, 2003), anti-inflammatory and antioxidant (Lee and Shibamoto, 2001, Dragland et al., 2003) properties. A recommendation from a studies of Mohammed et al. (2016) to use the Eugenol during pregnancy in very small dose as it had a cardiotoxic effect. There is a lack of scientific evidence on the teratogenic effect of Eugenol and for this determination, this study is transmitted away to examine the actions of Eugenol in the developing chick embryo as similar patterns of human teratogens.

Materials and methods

Test chemicals

Eugenol was obtained from Sigma-Aldrich Co. LLC Packaging (E51791) with a purity of 99 % and molecular weight 164.20.

Test animals

Fertilized and specific pathogen free *Gallus gallus domesticus* (Linnaeus) (Al-Nasser et al., 2007) eggs purchased from Faculty of Agriculture, Suez Canal University farm weighing about 40 gm with a standard deviation of ±5 gm. All the unfertilized eggs were discarded from the experiment. Chickens raised on this farm ingest a standardized diet causing a minimal difference in yolk composition from generation to generation. Animal care complied with the (Council, 2010).

Experimental design

A total of 288 Eggs cleaned with distilled water and then they were divided into control and five treated groups. Control eggs were not receiving an injection. Treated eggs were injected with a single volume of 0.1, 0.2, 0.3, 0.4 and 0.5 µml Eugenol applied through the egg airspace into the yolk sac during the first days of development as the maximum volume enter the used eggs air space is 0.5 µml. All fertilized eggs were incubated in a forced air incubator at 37.5°C and turned every 4 hours with relative humidity ranging from 80 to 90%. The injection hole was sealed with melted paraffin. The control and treated eggs were opened after 3, 6, 12 and 18 days of incubation according to Hamburger and Hamilton (1992) with theoretical stage 20, 29, 35, 38 and 44. For each set of treatment and time 12 eggs were used. The number of mortality was recorded for each group by the absence of beating heart. Vessels formation was also observed after incubation of eggs and photos were also taken using a Digital Camera and classified to one of the categories according to (Giles and Bannigan, 1999): (1) Normal: blood vessel development was comparable to control. (2) Disturbed pattern: vessels had formed and

communicated with the intraembryonic circulation, but one or more of the major trunks (vitelline veins, sinus terminals, omphalomesenteric vessels) were either absent, retarded or abnormally branched. (3) Avascular: a live embryo with active heartbeat, blood islands had formed, but there was no organized extraembryonic vasculature. (4) Dead: total arrest of all development, including embryo death. Embryos were dissected out and washed with Phosphate buffer saline solution (PBS). After 3 and 6 days incubation, the eggs were open and the average value of three records heartbeats (beats/ min.) for 5 min. intervals of living embryos were measured at constant temperature . Observations were made for the body weight. After different incubation time morphological abnormalities of treated and untreated embryos was examined according to (Bhaskar et al., 2014).

Histological studies

Six days chick embryos were fixed in 10 % neutral formaldehyde for 24 hrs. then, processed using routine histological techniques, consisting of dehydration in increasing ethanol series (70 to 100%), clearing in Xylene, and embedded in paraffin to obtain 5-µm sections stained with Hematoxylin-Eosin (HE).

Skeltal and neural embryonic defect studies by determination Acetylcholinesterase (ACHE) activity (µmol./min./g) in the brain tissues (Hoffman and Sileo, 1984, Bacchetta et al., 2008)

Six embryonic brains from each group were incubated for 6 days after injection with different doses of Eugenol and then homogenized in pH 8.0 phosphate buffer centrifuged, then the supernatant was cleared for assay of ACHE activity (µmol./min./g) in the brain tissues by the colorimetric method of Ellman et al. (1961).

Statistical analysis

The results were described as the mean ± standard error of the mean (SEM). *P* values <0.05 were considered significant against controls. Statistical analysis was performed using Student "t" test used for the analyzing of the data. The Mortality rate was compared by Mann- Whitney "U" test.

Result

The mortality rate, the heart beats and the vascular development

The cumulative mortality rates of embryos for Eugenol injected groups were predominantly higher than control groups (Table 1). These data indicated that the percentage of mortality increase correlated with the increasing of Eugenol volumes. Almost all the abnormal treated embryos were dead. Besides the average heart beat of the treated live embryo with 0.3 and 0.4 µl Eugenol and incubated for three days showed a significant increase compared to the control while the treated group incubated six days showed a nonsignificant difference compared to the control (Table 2).

Table 1. shows the mortality rate of control and treated chick embryos at different days of development.

Treatment	Duration of incubation							
	3 days		6 days		12 days		18 days	
Control	0	0%	0	0%	0	0%	0	0%
0.1 µl Eugenol	0	0%	2	16.6%*	3	25%*	1	8.3%*
0.2 µl Eugenol	0	0%	4	33.33%*	2	16.6%*	2	16.6%*
0.3 µl Eugenol	4	33.3%*	6	50%	3	25%*	4	33.3%*
0.4 µl Eugenol	8	66.6%*	7	58.3%	6	50%*	8	66.6%*
0.5 µl Eugenol	8	66.6%*	8	66.6%*	9	75%*	10	83.3%*

The data represent the average number and the percentage of dead embryos for 12 embryos in different incubation. *$P \leq 0.005$ significantly different from treated and controls.

Table 2. shows the average rate of heart beat (number of beats per minute) of control and treated chick embryos after 3 and 6 days of incubation.

Treatment	Duration of incubation	
	3 days	6 days
Control	98.83±6.2	137.3±14.7
0.1 µl Eugenol	110±5.52	121.1±18.2
0.2 µl Eugenol	113.3±0.8	109.3±17.6
0.3 µl Eugenol	125±1.9*	146.3±5.1
0.4 µl Eugenol	103±0.6	123.3±13.48
0.5 µl Eugenol	130±2.4*	122.1±10.05

(*) Data represented on average of twelve embryos± SEM is highly significant $p \leq 0.05$ when compared to control embryo. Student T-test.

The vascular development

After a three day incubation the extraembryonic vasculature of the control (Fig. 1-A) showed no effect on survival and vascular development an extensive plexus of large and small vessels was seen radiating from the sinus terminals towards the embryo. The Blood was pumping away from the embryo through the omphalomesenteric arteries towards the sinus terminalis and returned through the anterior and posterior vitelline veins. At the same time, the intraembryonic vasculature had been established with dorsal aorta, branchial arch and cephalic arteries. The

vitelline arteries were extensively branched, ending at the sinus terminalis. A network of veins had now developed which appeared to be superimposed on the major arteries. Also, the allantois and limb buds were well developed. The embryonic vascular development disturbed by Eugenol (Fig. 1) did not undergo confluence leading to vascular stasis and complete suppression of extraembryonic vasculogenesis with high lethality which is dose dependent. The embryo of high dose Eugenol was greatly retarded in development compared with controls and had no visible intraembryonic blood vessels (Fig. 1C to I).

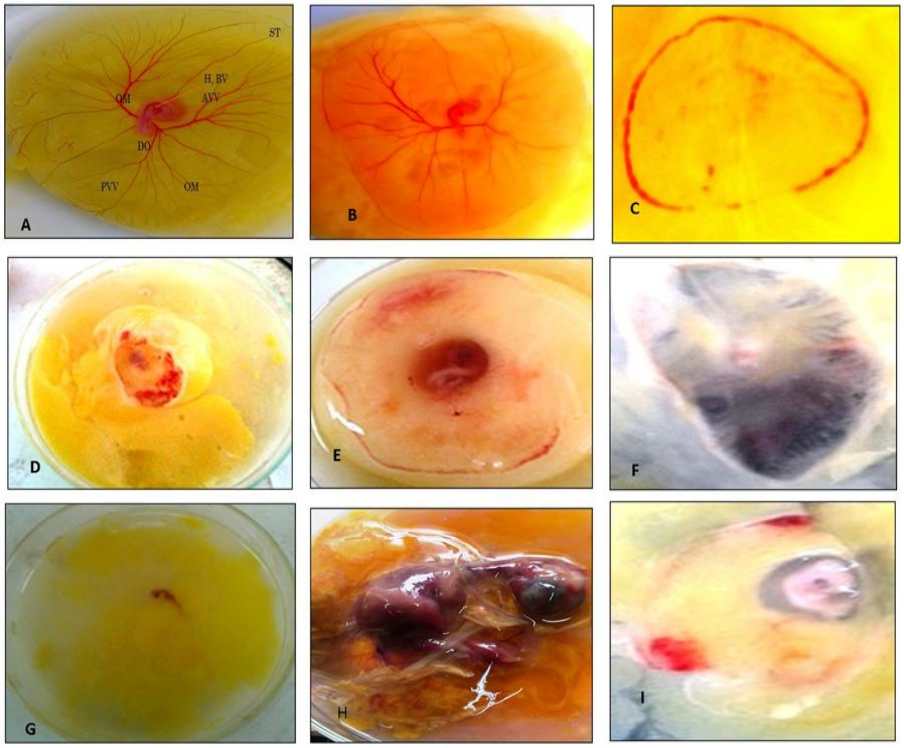

Figure 1. Representative picture of chorioallantoic membrane of chick embryo at different incubation time. (A) 3 days incubation of control chick embryo showing chorioallantoic membrane with normal vascular development, Abbreviation: sinus terminals (ST), anterior and posterior vitelline veins (Vv), omphalomesenteric vessels (OM), dorsal aorta (DA), branchial vessels (Bv), heart (H). (B) 3 day incubation of treated embryo with 0.1 µl Eugenol showing chorioallantoic membrane with normal vascular development (c) 3 day incubation of treated embryo with 0.5 µl Eugenol showing chorioallantoic membrane with the total arrest of all the vascular development with a dead embryo. (D) 6 days incubation of treated embryo with 0.1 µl Eugenol showing chorioallantoic membrane with avascular development. (E) 6 days incubation of treated embryo with 0.3 µl Eugenolshowing chorioallantoic membrane with Avascular development (F) 6 day incubation of treated embryo with 0. 4 µl Eugenol showing chorioallantoic membrane with the total arrest of all the vascular development with a dead embryo. (G-I) 12 day incubation of treated embryo with (0. 5, 0.3 and 0.4) µl Eugenolrespectively, showing chorioallantoic membrane with the total arrest of all the vascular development with embryonic death.

The growth parameters

Treatments with Eugenol affected the wet embryo weight change in a dose -dependent fashion. The treated chick embryo weight after incubation three days significantly ($P<0.05$) with the highest treatment compared to control embryo (table 3), while after an incubation period increase the chick embryo weight showed no significant difference compared to the control.

Table 3. shows the weight (g) of control and treated chick embryos at different days of development.

Treatment	Duration of incubation		
	3 days	6 days	18 days
Control	0.4287±0.004	0.8230±0.11	29.565±0.98
0.1 µl Eugenol	0.4090±0.008	0.6263±0.10	34.728±3.00
0.2 µl Eugenol	0.3650±0.00	0.6763±0.04	32.45±3.180
0.3 µl Eugenol	0.3640±0.026	0.6910±0.08	37.38±2.615
0.4 µl Eugenol	0.3988±0.024	0.6337±0.06	35.11±1.88
0.5 µl Eugenol	0.3240±0.044*	0.6883±0.10	32.58±2.43

(*) Data represented on average of six embryos± SEM is highly significant $p\leq0.05$ when compared to control embryo. Student T-test.

The morphological malformations

All the dead embryos are with morphological malformations. Among the total 240 treated embryos, there were 95 cases (39.5 %) morphological abnormal compared to the control table (4). The malformations were observed periodically in all treated groups. No specific pattern of malformations Fig. (2, 3, 4 and 5) was observed among the treated embryos, with absent of dependency of the dose with certain malformation. The control embryos group were well developed and showed the absence of malformations, while those treated with various concentrations of Eugenol appeared with anophthalmia is the absence of one or both eyes, meromelia the lacking of a part, but not all, of one or more limbs in a shrunken and deformed extremity, ectopiacordis in which the heart was visible as a lump of muscle abnormally located either partially or totally outside the thorax , beak deformities in the form of micrognathia (parrot beaks) is a condition in which the embryo has a very small lower jaw, ectopia viscera in the form of Omphalocele is abdominal wall defect in which the intestines, liver, and occasionally other organs remain outside of the abdomen in a sac because of failure of normal return of intestines and other contents back to abdominal cavity during its development and exencephaly, the absence of development the skull roof and general growth retardation a small size and poorly feather, Gastroschisis which is the protruding of intestine shortened, thickened, and covered with a fibrous peel. The prominent features of live treated embryos on day 18 were exteriorization or interiorization of yolk.

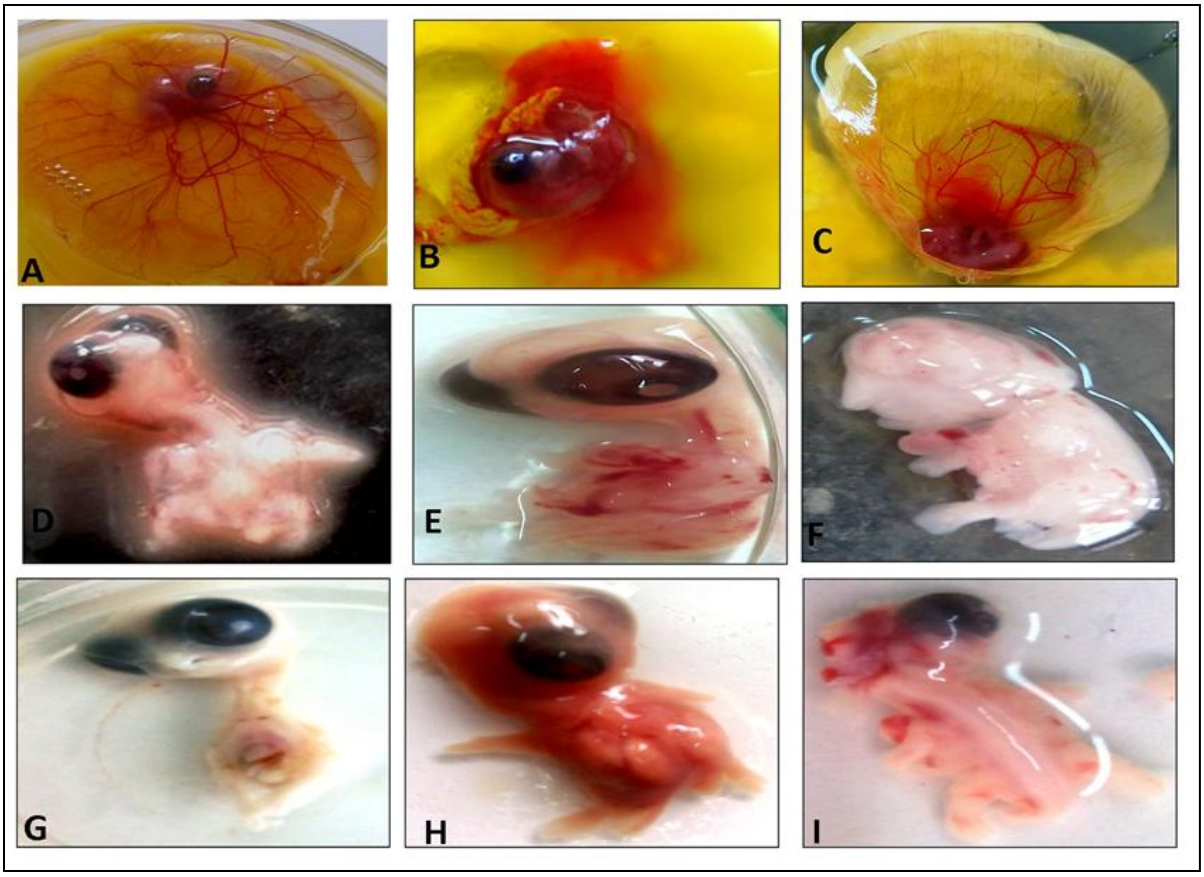

Figure 2. (A) 6-days treated control untreated chick embryo showing normal allantois and vitalline with normal vein. (D) 6-days treated control untreated chick embryo showing the normalities in the formation of beak and eye. (B) 6-days treated chick embryo with 0.3 µl of Eugenolshowing the damage of allantois vein and vitalline vein. (C) 6-days treated chick embryo with 0.3 µl of Eugenol showing the damage of allantois and decrease of vitalline vein branching. (E) 6-days treated (0.5 µl of clove oil) embryo showing growth retardation, the heart is the outside and parrot beak. (F) 6-day treated (0.5µl of clove oil) chick embryo showing absence of eyes, undeveloped brain. (G) 6-day treated (0.2 µl of clove oil) chick embryo showing the heart outside. (H) 6-days treated (0.4 µl of clove oil) chickembryo showing general visceral outside and parrot beak. (I) 6-days treated (0.5 µl of clove oil) chick embryo showing the present of only one eye.

Table 4. Type and number of malformation in the treated groups after 6, 12 and 18 days of incubation.

Type of malformations	Dose of treatment with Eugenol				
	0.1 µl	0.2 µl	0.3 µl	0.4 µl	0.5 µl
Anophthalmia	1	1	1	2	2
Meromelia			1	2	2
Ectopiacordis	1	1	1	2	2
Parrot beaks	1	1	1	2	2
Ectopia viscera	1	1	1	1	2
Omphalocele		1	1	1	2
The absence of development the skull roof			1	1	2
Growth retardation	1	1	1	1	2
Gastroschisis			1	1	1
Exteriorization of yolk	1	2	4	8	10

The data represent the average number of malformation embryo of total 12 embryos.

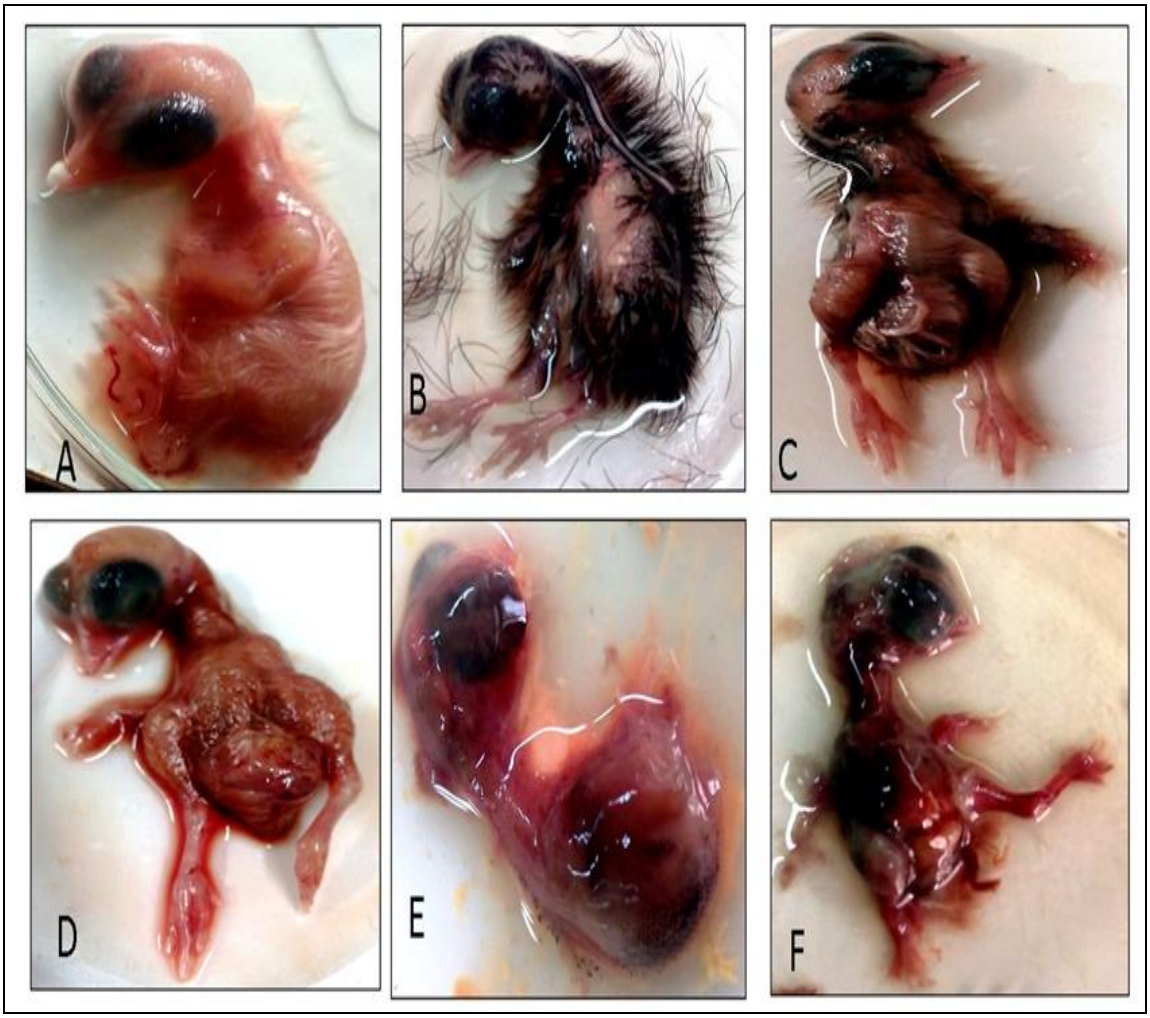

Figure 3. A) 12-days control chick embryo. (B) 12-day treated (0.1 µl of Eugenol) chick embryo showing growth retardation (C) 12-day treated (0.2 µl of Eugenol) chick embryo showing Omphalocele and general growth retardation. (D) 12-day treated (0.3 µl of Eugenol) chick embryo showing Omphalocele and general growth retardation and hemorrhage under the skin. (E) 12-day treated (0.4 µl of Eugenol) chick embryo showing general growth retardation and hemorrhage under the skin. (F) 12-day treated (0.5 µl of Eugenol) chick embryo showing general growth retardation and abnormal growth of hair, hemorrhage under the skin and Omphalocele .

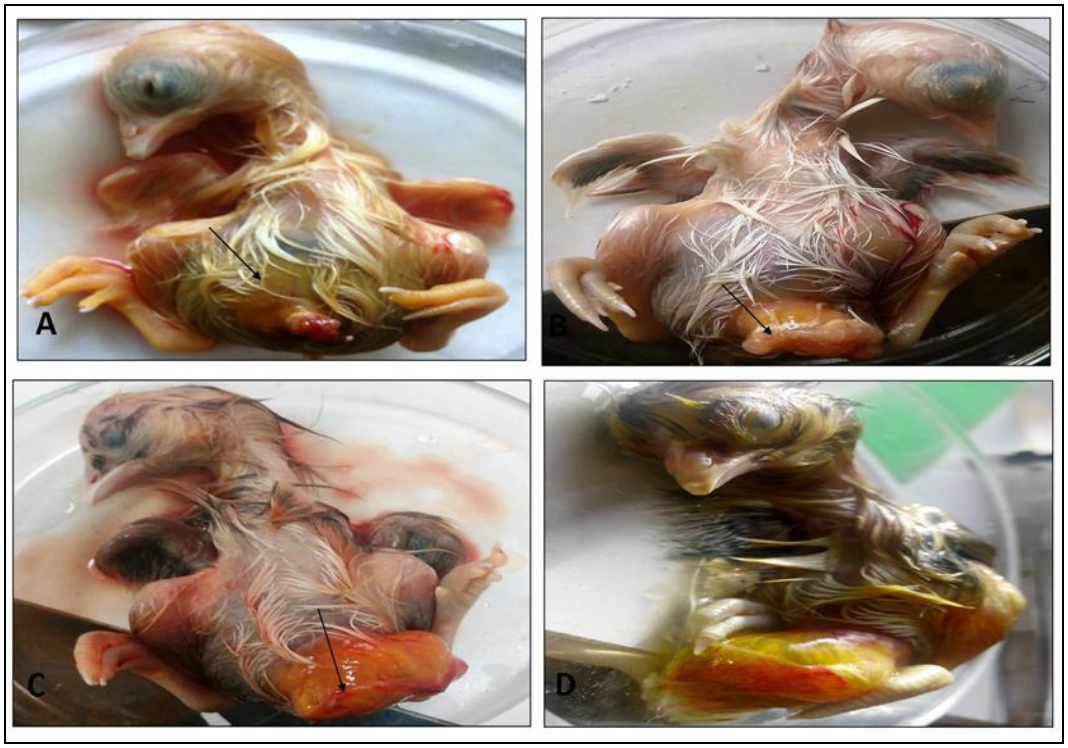

Figure 4. (A) 18-days control chick embryo normal yolk (arrow). (B) 18-days treated (0.1 µl of Eugenol) chick embryo showing an increase of yolk outside (arrow). (C) 18-days treated (0.3 µl of Eugenol) chick embryo showing an increase of yolk outside (arrow). (D) 18-days treated (0.5 µl of Eugenol) chick embryo showing of yolk outside (arrow).

Histological studies

The sagittal section of control chick embryo showed of well-developed heart with a complete interventricular septum, somites, mesonophric, dorsal aorta and coelom. The treated chick embryo wih Eugenol showed atrophy in somites, dilation of coelom, degeneration of dorsal aorta and the mesenopheric tissues fig. (5).

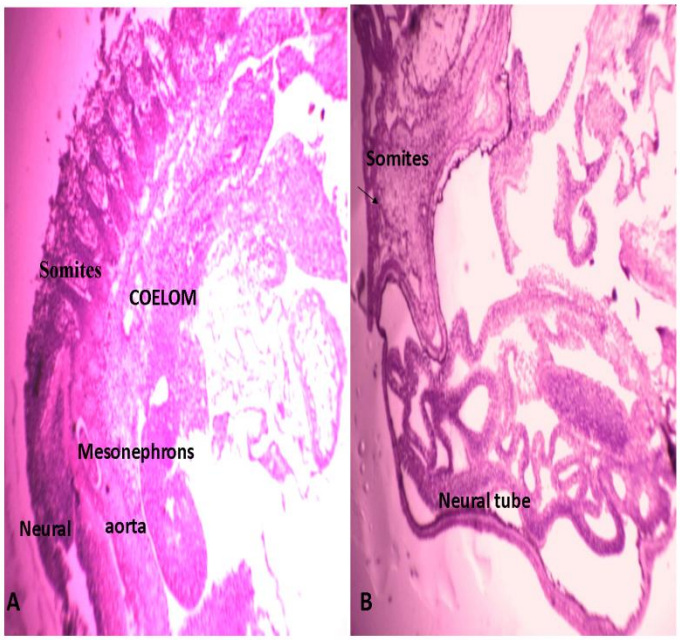

Figure 5. Sagittal sections stained with hematoxylin and eosin of control 6-days chick embryo (A) showed normal histological structure and treated 6-days chick embryo with Eugenol 0.4 µl (B) showed atrophy of somites and degeneration of aorta (X10).

Determination of AChE activities

The AChE activities showed significant decrease in the Eugenol-treated embryos in comparison to controls by increasing Eugenol volume (Table 5).

Table 5. shows the AChE activities (µmol/min./g) of control and treated chick embryos (*Gallus domesticus*) after 6 days of incubation.

Treatment	Duration of incubation
	6 days
Control	0.5611±0.08
0.1 µl Eugenol	0.455±0.03*
0.2 µl Eugenol	0.332±0.008*
0.3 µl Eugenol	0.175±0.02*
0.4 µl Eugenol	0.166±0.006*
0.5 µl Eugenol	0.146±0.008*

(*) Data represented on average of six embryos± SEM is highly significant $p \leq 0.05$ when compared to control embryo. Student T-test.

Discussion

Eugenol is a flavouring substance authorized by the European Commission for use in foodstuffs, although no acceptable daily intake (ADI) has been calculated or no safe exposure limit could be established by the Commission. the ADI of Eugenol was revised by the International Programme on Chemical Safety in 1982 is 0–2.5 mg/kg bw and the estimatation of average human consumption of eugenol vary from 7 to 76 µg/day (Maralhas et al., 2006). No further evaluation has been performed by major health risk organizations. There exists only a small number of reports dealing with the embryotoxicity of Eugenol in different developmental stages (Mohammed et al., 2016, Chen et al., 2010).

Eugenol affects the survival rate of chick embryos by increasing the mortality rate which linked to the increasing Eugenol concentration. The average body weight of treated embryos in comparison to the control was not change and that may attributed to the absence of Eugenol effect to the metabolic processes and protein and DNA synthesis rate during the chick embryo development (Cavanagh, 1964, Pennington, 1990) and these results is agreement with Yang et al. (2010) which indicate that Eugenol may have limited potential growth rate improvement of cattle fed with high concentrate diets.

The average heart beats of control chick embryo incubated three and six days is 110 beats/min. and 137.3 beats/min and treated embryo with 0.3 and 0.4 µl Eugenol showed a significant increase compared to the control group and that may be resulted from the increased blood flow which increased the heart beats (Cohn & With the Assistance of Edith, 1925). These result is different from the observation of Cohn and With the Assistance of Edith (1925) who found the average heart beats of chick embryo after incubation three days was 185-210 beats per min. and after incubation six days was 200-235 beats per min and Sticht and Smith (1971) who observed no changes in dogs heart rate were after intravenous administration of diluted and undiluted eugenol solution.

The incidence of cardiovascular histological damage was low or absent found in different treated doses of Eugenol, and these results are in agreement with other studies (Mnafgui et al., 2016, Fouad and Yacoubi, 2011, Shukri et al., 2010, Rezk, 2013), who showed the protective role of eugenol against heart damage. Different concentrations of Eugenol induced a decrease in the number of CAMs' vessels and failed to induce angiogenesis on CAM after the exposition. The angiogenesis, is a process regulated by endogenous excitatory and inhibitory mediators characterized by branching and remodeling of primary capillary network in which larger vessels originate resulted in a complex structure (Buschmann and Schaper, 1999, Ribatti, 2016). Thus, this study suggests that Eugenol is harmful to blood vessels and chorioallantoic membranes which supply embryo with nutration and respiration at the early stages of development (Vargas et al., 2007). These results are not in agreement with Nangle et al (2006) who reported the antioxidant role of clove in preventing the vascular dysfunction of streptozotocin induced diabetic rats. Eugenol may be useful as an antiangiogenic agent in the treatment of cancer and used with cancer drugs, these results is in agreement with Jaganathan and Supriyanto (2012) who highlighted the antiproliferative activity and molecular mechanism of the eugenol in induction of apoptosis against the cancer cells and animal models and synergism in enhancing the chemotherapeutic potential of gemcitabine followed by induction of anticarcinogenic and anti-inflammatory activity in human cervical cancer cells (Hussain et al., 2011).

There is little literature on the effect of Eugenol on the development of chick embryos and its mechanism of action as a teratogenic agent. The Eugenol showed a strong negative developmental effect for all doses used. There were no specific malformations associated with Eugenol treatment in all set of the experiments which were not seen in the controls, and its frequency exceeds the 50%, which is considered background noise in the pre-hatching chicks. The embryonic mortality isnot clearly correlated with the only higher dose, but also the malformed embryos. Eugenol decreases the activity of AChE and that are inconsistent with studies of (Dalai et al., 2014). The AChE inhibition may cause paralysis of muscle (Karalliedde, 1999, Singh et al., 2013) or defect in the neural development and axial Skelton (Hoffman and Sileo, 1984, Bacchetta et al., 2008) and that explained the morphological defects and histological damage found in the chick embryo development.

Conclusions

In conclusion, this study demonstrates that Eugenol possesses a significant teratogenic potential, at least in the chick embryo and a further investigate is needed for the evaluation of the teratogenic potential of Eugenol to the mammals.

Conflict of interests

The authors declare that there are no conflicts of interest.

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