



## การเปลี่ยนแปลงด้านสัณฐานวิทยาของตัวอ่อนไก่อายุ 3 วันหลังจากได้รับขมิ้นชัน

### Morphologic Changes of the 3-day-old Chick Embryos after Curcumin Exposure

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#### บทคัดย่อ

ขมิ้นชันเป็นพืชสมุนไพร มีคุณสมบัติตามบัญชียาหลักแห่งชาติ (ฉบับที่ 2) พ.ศ. 2555 คือ บรรเทาอาการแน่นจุกเสียด ท้องอืด ท้องเฟ้อ ซึ่งเป็นอาการที่มักพบในหญิงตั้งครรภ์ระยะไตรมาสที่ 1 จึง มักนิยมใช้ขมิ้นชันเป็นทางเลือกในการรักษาแทนการใช้ยาแผนปัจจุบัน การศึกษาเชิงทดลองนี้มี วัตถุประสงค์เพื่อศึกษาความผิดปกติทางด้านสัณฐานวิทยาของตัวอ่อนไก่อายุ 3 วันหลังจากได้รับ สารละลายขมิ้นชันที่ความเข้มข้น 20, 40 และ 60 มก./ลิตร โดยใช้ไข่ไก่มีเชื้อพันธุ์โรดไทย จำนวน 94 ฟอง แบ่งเป็น 5 กลุ่ม ได้แก่ กลุ่มที่ไม่ได้รับสารใด ๆ กลุ่มที่ได้รับน้ำมันข้าวโพด กลุ่มที่ได้รับสารละลาย ขมิ้นชันที่ความเข้มข้น 20, 40, และ 60 มก./ลิตร โดยฉีดสารเข้าทางไข่แดง แล้วนำไปฟักที่อุณหภูมิ 37±0.5 องศาเซลเซียส เมื่อครบ 3 วันนำตัวอ่อนไก่มาศึกษาการเปลี่ยนแปลงทางด้านสัณฐานวิทยา บริเวณส่วน cephalic region ulyangky caudal region และลำตัว ใช้สถิติ Fisher's Exact test เพื่อทดสอบผล ของความเข้มข้นของสารละลายขมิ้นชันต่อสัณฐานวิทยาของตัวอ่อนไก่ ผลการศึกษาพบว่าความเข้มข้น ของสารละลายขมิ้นชันทั้ง 3 ระดับไม่มีผลต่อจำนวนการตายของตัวอ่อนไก่ และความผิดปกติทางด้าน สัณฐานวิทยาของตัวอ่อนไก่ ซึ่งในกลุ่มที่ได้รับสารละลายขมิ้นชันที่ความเข้มข้น 20 มก./ลิตร พบความ ผิดปกติของลำตัว กลุ่มที่ได้รับสารละลายขมิ้นชันที่ความเข้มข้น 40 มก./ลิตร พบความผิดปกติของ สมองส่วนหน้าและ neural tube ส่วนกลุ่มที่ใช้สารละลายขมิ้นชันที่ความเข้มข้น 60 มก./ลิตร พบว่า สมองส่วนหน้ามีการเจริญเพียงบางส่วน และไม่มีการเจริญของรยางค์ขา

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## Abstract

Curcumin is a medicinal plant enlisted in the National List of Essential Medicines (Issue 2) B.E.2012, that has been used to treat flatulence, which is common in pregnancy especially during the first trimester. The aim of this study was to monitor morphological changes of the embryos in 3-day-old chicks exposed to curcumin 20, 40, and 60 mg/l. A total of 94 fertilized Rhode Thai eggs were divided into 5 groups: non-injection group, corn oil group, Cur-20, Cur-40 and Cur-60 received 0, 20, 40 and 60 mg/l of curcumin respectively. All eggs were injected solutions into yolk sac and incubated at a temperature of  $37\pm 0.5^{\circ}\text{C}$ . On day 3 of development, all eggs were then opened to observed morphological changes including cephalic region, limbs, caudal region and trunk. The data were analyzed by using Fisher's Exact test to compare between group. The mortality percentage in chick embryos that exposed to curcumin 20, 40, and 60 mg/l were not statistically different when compared to non-injection group. The chick embryos exposed to curcumin 20 mg/l showed abnormal development of body curvature. The chick embryos exposed to curcumin 40 mg/l found abnormal development of cephalic region and closure failure of neural tube. Partial development of cephalic region and limb buds absence were observed in chick embryos exposed to 60 mg/l.

**Keywords:** Curcumin, Chick embryo, Teratogenic effect

## Introduction

The use of herbal medicine has increased by 60% in many developed countries whereas the prevalence of the herbal medicine uses for common symptoms such as nausea, vomiting and reflux has grown considerably worldwide amongst pregnant women (Laelago, 2018; Ahmed, Hwang, Choi, & Han, 2017). Studies showed that they believed herbal products are natural and have less adverse effects, thus considered safer compared with other conventional medicines. (Ahmed et al, 2017; Forster, Denning, Wills, Bolger, & McCarthy, 2006). The most common herbal supplements used during pregnancy were ginger, raspberry leaves, chamomile, peppermint, garlic, and curcumin (Laelago, 2018). Curcumin is a hydrophobic polyphenol compound derived from rhizome of *Curcuma longa*. Curcumins has also been used in cosmetics, wound healing, treatment of hepatitis and urinary tract disease. It has also been recorded for its anti-inflammatory, anti-carcinogenic and anti-infection properties (Chen, Hsieh, Hsuuw, Huang, & Chan, 2010; Wu, Lin, Lin, Ken, & Wen,



2007; Huang et al., 2013). Curcumin is classified as class ‘C’ by Food and Drug Administration (FDA) and recorded as a precaution in pregnancy by the National List of Essential Medicines 2019 (National drug system development committee, 2019). In vivo animal and human studies confirmed that dietary curcumin was very safe and did not have hazardous effects at high dose (Chen et al., 2010; Huang et al., 2013). The embryotoxic effect of curcumin showed spinal column curved, pericardial sac edema, and hook-like tails in zebrafish embryos at concentration of 5 $\mu$ M (Wu et al., 2007). Curcumin was also reported to greatly induce apoptosis in mouse embryos at blastocyst stage (Chen et al., 2010). However, there are a few studies investigating the teratogenicity of curcumin. This study was conducted to demonstrate the teratogenic effect of curcumin; morphological changes in the chick embryos. The main advantage of the chick embryos as a model has been the ease with its short gestation and similarity to mammalian systems and easy to manipulate (Hamburger & Hamilton, 1992; Roongruangchai et al., 2018).

## Objective

To study 1) the morphological changes in 3-day-old chick embryos exposed to curcumin solution at concentration of 20 mg/l, 40 mg/l, 60 mg/l, and 2) the mortality percentage and morphological changes when compared to the control group.

## Research methodology

### 1. Ethics statement

For planning purposes, the 3-day-old chick embryo is advantageous. Since experimentation on chick embryos younger than 14 days does not require approval from an animal ethics committee (Institutional Animal Care and Use Committee [IACUC], 2019).

### 2. Experiment protocol

#### 2.1 Animals

A total of 94 fertilized Rhode Thai eggs, weighing 45-50 g, were taken same breeder and stall from Bann Rai hatchery.



## 2.2 Chemicals

The curcumin was purchased from a local supermarket. The curcumin solutions were from curcumin capsule (350 mg/capsule, dry power), dissolved in corn oil to increase solubility and to make three different concentrations (20 mg/l, 40 mg/l, 60 mg/l). Because of no NOEL of curcumin in chick, the curcumin concentrations were chosen based on NOEL of 250-320 mg/kg of body weight per day in rat (JECFA, 2019).

## 2.3 Experimental design

The fertilized eggs were randomly divided into five groups: NI (non-injection) group, Corn oil group, Cur-20 group, Cur-40 group, and Cur-60 group. All eggs were incubated at temperature of  $37\pm 0.5^{\circ}\text{C}$  with 70% humidity. At 21h of incubation, all eggs were injected with corn oil and curcumin at concentration of 20, 40, and 60 mg/l into yolk sac at volume of 0.1 ml each. After injection, the eggs were sealed with adhesive tape. Incubation continued until day 3 of development. All embryos were opened. The surviving chick embryos were fixed in the Dietrich's solution and processed for total mount technique. The fixed embryos for evaluation of morphology were then stained with Mayer's carmine for 10 minutes and dehydrated with a series of alcohol for 20 minutes in each change. Then were cleared with xylene for 30 minutes in each change before mounting the specimens (Roongruangchai et al., 2018; Mishrikoti & Kulkarni, 2018).

## 2.4 Morphological parameters

The fixed chick embryos were evaluated morphology according to Hamburger and Hamilton stage (Hamburger, & Hamilton, 1992), including brain vesicles, limb buds, tail bud, and body curvature.

## 2.5 Statistical procedures

The data were analyzed by using Fisher's Exact Test to compare between groups by SPSS version 26 software.  $P < 0.05$  was considered statistically significant.

## Results

### 1. The mortality percentage

The Survival and mortality rates were observed by the heart beating. The survival and mortality rates in chick embryos treated with corn oil, curcumin 20 mg/l, curcumin 40 mg/l and



curcumin 60 mg/l were not statistically different when compared to non-injection group by Fisher's Exact Test at  $p < 0.05$  (Table 1).

**Table 1** The mortality percentage of chick embryos exposed to curcumin in different concentrations

Condition	Experimental groups					Fisher's Exact Test (Sig.)
	NI	Corn oil	Cur-20	Cur-40	Cur-60	
Number of eggs	10	10	25	25	24	2.29
Survival (%)	100	100	88	96	95.83	(0.664)
	(n = 10)	(n = 10)	(n = 22)	(n = 24)	(n = 3)	
Mortality (%)	0 (n = 0)	0 (n = 0)	12 (n = 3)	4 (n = 1)	4.17 (n = 1)	

NI; not-injected, Cur-20; curcumin 20 mg/l, Cur-40; curcumin 40 mg/l, Cur-60; curcumin 60 mg/l

## 2. Morphological changes

The morphological change in chick embryos treated with corn oil, curcumin 20 mg/l, 40 mg/l, and 60 mg/l were not statistically different when compared to non-injection group by Fisher's Exact Test at  $p < 0.05$  (Table 2).

In non-injection and corn oil groups showed normal development of secondary brain vesicles as stage HH 18, including telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon and the cephalic flexure showed acute angle at the mesencephalon (Figure 1A-B). In cephalic region, the brain vesicles were decreased in size (Figure 1F, K, and L) and absence of brain vesicles (Figure 1J).

The closure of neural tube was normal development in all groups, except one chick embryo exposed to curcumin 40 mg/l was closure failure in neural tube (Figure 1J). The development of somite was parallel blocks and extended to the caudal end with the tail fold in all groups, except some chick embryos exposed to corn oil, curcumin 60 mg/l did not extend to the caudal end.

In normal chick embryo as stage HH 18 was found wing and leg buds. The development of wing buds was normal in all groups. The development of leg buds was normal in all groups, except some chick embryos exposed to corn oil, curcumin 20 mg/l, 40 mg/l, and 60 mg/l were partial (Figure 1E-F, K) and absence of leg buds (Figure 1G-I, L).



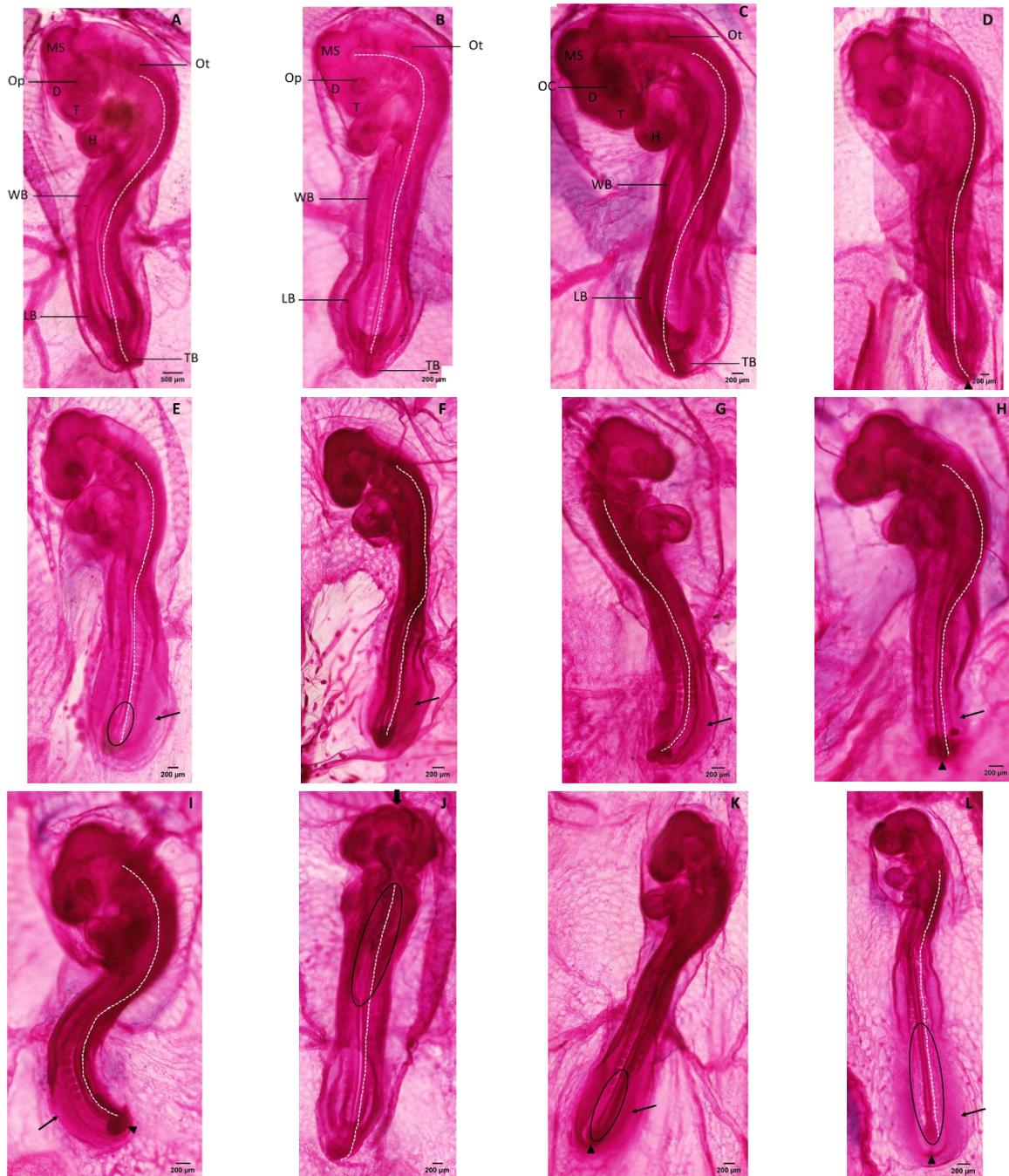
The abnormal of cervical curvature (Figure 1J) and trunk curvature (Figure 1F, G, I) were observed in chick embryos exposed to corn oil, curcumin 20 mg/l, and 40 mg/l. The caudal region was normal development in all groups, except some chick embryos exposed to corn oil, curcumin 40 mg/l, and 60 mg/l.

Additionally, in normal chick embryos as stage HH 18 was found the heart loop twisted as S-shaped which the ventricle laid caudally and ventrally on the right side to atrium. Some chick embryos exposed to curcumin 20 mg/l, 40 mg/l, 60 mg/l were heart loop distended and loose as a U-shaped appearance. The development of optic cups and lens vesicles were round shaped and located at the level of diencephalon in all groups, some chick embryos exposed to curcumin 40 mg/l and 60 mg/l were small size of optic cups and lens vesicles.

**Table 2** The morphological occurrence of the chick embryos exposed to curcumin in different concentrations

Abnormal development	Experimental groups					Fisher's Exact Test (Sig.)
	NI	Corn oil	Cur-20	Cur-40	Cur-60	
Number of eggs	5	5	12	12	12	
Cephalic region	0	0	0	1	0	1.000
Limb buds	0	0	0	2	2	0.733
Tail bud	0	0	0	1	2	0.834
Body curvature	0	0	2	2	1	0.784

NI; not-injected, Cur-20; curcumin 20 mg/l, Cur-40; curcumin 40 mg/l, Cur-60; curcumin 60 mg/l



**Figure 1** Morphology of 3-day-old chick embryos. A-B: non injection group with normal morphology as stage HH 18. C-E: Chick embryos exposed to corn oil. F-G: Chick embryos exposed to curcumin 20 mg/l. H-J: Chick embryos exposed to curcumin 40 mg/l. K-L: Chick embryos exposed to curcumin 60 mg/l. E, K, L: somites undeveloped (circle area). J: neural tube malformation (circle area) and cephalic region malformation (thicker arrow). D, H, I, K, L: caudal region changing (arrow head). E-L: alteration on limb buds (thinner arrow). F, G, I, J: alteration of body curvature (dashed line). MS: mesencephalon, D: diencephalon, T: telencephalon, H: heart, Op: optic vesicle, Ot: otic vesicle, WB: wing bud, LB: leg bud, TB: tail bud



## Discussion

This study used to chick embryos was exposed to a single dose of curcumin at 72h of incubation that compared to 4-5 weeks or 33-day-old human embryo (Hill, 2018). The critical period of organogenesis in human embryos begin fertilization to 8 weeks that corresponding to blastula to 72 h of incubation (Christensen, 2001).

The percentage of mortality in three Cur-treated groups was increased, compare to the control group, which supported the three previous studies (Chen et al., 2010; Wu et al., 2007; Huang, 2013). Wu (2007), those studies suggested that the death of curcumin treated zebrafish embryos may result from the curcumin inhibited SERCA (sarco/endoplasmic reticulum  $Ca^{2+}$  ATPase) which plays an important role of maintaining low  $Ca^{2+}$  concentration within cells. The inhibition of SERCA was the result of disrupting cardiac development, slower heartbeat, and weak heart contractility (Wu et al., 2007). In vitro mouse embryo, curcumin induced apoptotic injury effects and inhibited cell proliferation of blastocyst (Chen et al., 2010; Wu et al., 2007).

In this study, some chick embryos exposed to curcumin 40 mg/l and 60 mg/l were closure failure of anterior neuropore, deformation of body curvature, small size of eyes, and heart looping that corresponding to zebrafish embryos exposed to curcumin (Wu et al., 2007). Wu (2007) reported the curcumin induced abnormal development of brain, anophthalmia, microphthalmia, irregular heart looping, bent or hook-like tails, spinal column curving, pericardial sac edema, and shorter body length in zebrafish embryos which may result from the deregulation of the function of multiple genes.

## Conclusion

We have demonstrated the embryotoxic effects of curcumin on the 3-day-old chick embryos which affected the abnormal development of the cephalic region and neural tube and alteration of leg buds and body curvature but not showed statistically different when compared to the control group. The data analysis may be concluded that the curcumin did not relate to mortality percentage and morphological change.



### Suggestion

The future study will be providing the equal sample size for prevent bias and investigating the histological change and teratogenic effects in different stage of chick embryos.

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