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Maternal curcumin exposure causes fetal gross morphological anomalies and skeletal malformations in mouse

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Abstract

Curcumin is a phenolic compound extracted from the rhizome of turmeric (*Curcuma longa* L.). Although declared as safe for human consumption, curcumin has been found to be embryotoxic in some organisms indicating its potential as a teratogen. In this study, the teratogenic effect of maternal curcumin exposure in mouse fetuses was evaluated. Three experimental groups of pregnant mice were treated with 1.05, 1.52, and 2.0 mg/g body weight/day 95% curcumin, respectively, from gestation day (GD) 6 to 15. A fourth group without curcumin exposure served as a control. At GD18, the mice were sacrificed and the total number of implanted embryos including resorbed, dead, and live fetuses were counted for litter analysis. Extracted fetuses were also analyzed for gross morphological anomalies and subsequently have undergone alizarin staining for the visualization of skeletal malformations. Results showed an increased resorption rate in the 2.0 mg/g treatment ($p<0.001$). There is also a reduction of fetal weight ($p<0.001$) and crown-rump length ($p<0.001$) in a dose-dependent manner. Gross morphological analysis shows cranio-facial malformations such as flattened nose bridge ($p<0.05$) and micrognathia ($p<0.05$) in 2.0 mg/g treatment. Skeletal malformations such as large anterior fontanelle ($p<0.001$), misaligned ossification centers in the sternum ($p<0.001$), and delayed ossification in the forepaws, hind paws, and caudal vertebrae ($p<0.001$) were also observed at 2.0 mg/g treatment. Meanwhile, the presence of supernumerary ribs is not statistically different in the four groups. The results indicate that curcumin is teratogenic in mouse fetuses due to observed gross morphological anomalies and skeletal malformations.

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Introduction

Curcumin is a polyphenol derived from the rhizome of turmeric (*Curcuma longa* L.). As a widely used herb particularly in many Asian countries, curcumin has been studied extensively for its medicinal use. Among its therapeutic and pharmacological properties include antioxidant, anti-inflammatory, anti-carcinogenic, antimicrobial, antidepressant, antidiabetic, neuroprotective, and hepatoprotective effects (Mahmood *et al.*, 2015; Nelson *et al.*, 2017; Alafiatayo *et al.*, 2019).

Aside from the benefits of curcumin, previous studies also reported its toxicity *in vitro* and *in vivo*. These include dose and time-dependent induction of chromosomal aberrations in mammalian cell lines (Goodpasture and Arrighi, 1976) and damage to mitochondrial and nuclear DNA in human hepatoma cells (Cao *et al.*, 2006). Moreover, exposure led to various types of cancer in rats fed with curcumin for two years (National Toxicology Program, 1993) or lung cancer in mice (Dance-Barnes *et al.*, 2010).

Although there are existing reports regarding curcumin toxicity, a recent review of the European Food Safety Authority (EFSA) regarding E 100 (dicinnamoylmethane), a yellow dye derived from curcumin, indicated its safety at 3 mg/kg body weight/day acceptable daily intake (ADI). Together with the Joint FAO/WHO Expert Committee on Food Additives (JECFA), curcumin is reported as non-carcinogenic even with *in vitro* and *in vivo* genotoxic reports (EFSA Journal, 2010). Moreover, a reproductive toxicity study in rats showed no gross or microscopic changes in organs and no reproductive parameters were affected. However, there was a decrease in body weight gain in the F2 generation observed at the highest dose used (*Ganiger et al.*, 2007; EFSA Journal, 2010).

Despite its safety, limited studies exist regarding the embryotoxicity and teratogenicity of curcumin. One study indicated the negative effects of curcumin in zebrafish embryo which include bent or hook-like tails, spinal column curving, edema in pericardial sac, retarded yolk sac resorption, and shorter body length (Wu *et al.*, 2007). In a study published in 2012, exposure to 24 µM curcumin causes adverse effects on ICR mouse embryos in the early post-implantation stage including reduction of nuclei per outgrowth of the blastocysts and increased percentage of trophoblastic giant cells (Huang *et al.*, 2013). Another study reported that curcumin causes injurious effects on oocyte maturation, fertilization, and embryonic development. Exposure to curcumin during *in vitro* maturation of embryos resulted to increased resorption of implanted embryos and reduced body weight in the resulting fetuses. Moreover, adult mice exposed to 40 µM resulted to a decrease in oocyte maturation, *in vitro* fertilization of resulting oocytes, and implantation of the resulting embryos (Chen and Chan, 2012).

In this study, we report the teratogenic effect of curcumin in mouse fetus particularly gross morphological anomalies and skeletal malformations.

Materials and methods

Animals and Ethics

The procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Benguet State University, Philippines. A permit (reference no. AR-2018-119) from the Bureau of Animal Industry (BAI) of the Department of Agriculture, Philippines was also obtained for research purposes.

A total of 16 acclimated 6-week old female ICR mice from the College of Veterinary Medicine, Benguet State University, Philippines were utilized in this study. All mice were individually housed in cages and supplied with standard diet and distilled water *ad libitum*. The mice were housed in an animal room with standard rearing conditions according to IACUC guidelines.

After the acclimation period, mice were subjected to random timed mating (Heyne *et al.*, 2015) using harem system (1:2 male/female ratio). The date and time during which the vaginal plug was observed to

be present was designated as gestation day (GD) 0. After successful mating, the impregnated females or dams were separated from the males and were housed in individual cages. The dams were then randomly sorted into four treatment groups with four mice each.

Treatment and Dosages

Four experimental groups were designated as control (0.00 mg/g body weight/day) and treatment groups (1.05, 1.52, and 2.00 mg/g body weight/day curcumin) where the No Adverse Effect Level (NOAEL) is 1.05 mg/g (Ganiger *et al.*, 2007) and the Lethal Dose 50 (LD_{50}) of curcumin is 2.00 mg/g (Dadhaniya *et al.*, 2011). The desired concentration of 95% curcumin (CAS No. 458-37-7, Tokyo Chemical Industry Co., Tokyo, Japan) was dissolved in 0.2 mL of commercial olive oil to increase its bioavailability (Chang *et al.*, 2013). Oral administration began on GD6 until GD15 once a day to target the organogenesis of the developing fetuses. The control group received only 0.2 mL commercial olive oil. After the 9-day treatment, the dams were maintained until GD18.

Litter analysis

At GD18, dams were sacrificed. The uteri were extracted and dissected to determine the total number of implantations. The total number of implantations includes all formed and resorbed fetuses (Bolon, 2015). Resorbed fetuses were identified as small masses in the uterus usually between two normal fetuses. Formed fetuses were characterized as either alive or dead by their ability to breathe upon extraction from the uterus (Marsden and Leroy, 2013). The resorption and mortality rates were computed based on the data gathered. The fetuses were also weighed and were measured from the crown to the rump to determine the crown-rump length (Mu *et al.*, 2008).

Teratogenicity analysis

Freshly extracted fetuses were examined under a digital microscope to observe the gross morphology and common external malformations such as flattened nose bridge and micrognathia (Wang *et al.* 2012; Etemad *et al.* 2013; de Araújo Costa *et al.* 2016; Gholami *et al.* 2016).

For the analysis of skeletal malformations, half of the fetuses in each litter underwent staining using alizarin stain set (CAS No. 72-48-0) as previously described (Reynaud and Jocteur-Monrozier, 2013). The fetuses were then examined under a stereomicroscope to locate the following bones; skull, vertebrae, ribs, sternebrae, thoracic girdle, pelvic girdle, forelimb bones (humerus, radius, ulna, carpals, metacarpals, phalanges), and hind limb bones (femur, tibia, fibula, tarsals, metatarsals, phalanges).

Statistical analysis

The fetal body weight, crown-rump length, and litter size were analyzed using one-way analysis of variance (ANOVA) with post-hoc Tukey HSD test with p-value set to $\alpha = 0.05$. Resorbed fetuses, resorption rate, live fetuses, dead fetuses, mortality rate, flattened nose bridge, micrognathia, large anterior fontanelle, misaligned ossification centers in the sternum, supernumerary ribs, and delayed ossification at forepaws, hind paws, and caudal vertebrae were evaluated using Pearson Chi-square test with p-value set to $\alpha = 0.05$. All statistical analyses were done using SPSS Statistics®.

Results

Litter analysis

No mouse died in all the experimental groups during the 9-day treatment period so the data for the litter analysis were obtained consistently. Table 1 shows the litter characteristic of dams in the four experimental groups. The total number of implantations (resorbed, dead, and live fetuses) and the mean litter size are not significantly different between the groups ($p>0.05$). There is a 25.4% resorption rate of implanted fetuses (Fig. 1) in the 2.00 mg/g treatment ($p<0.001$) which is significantly higher than the other groups. Moreover, a 1.72% mortality rate was observed in the 1.52 mg/g treatment but it is not considered significant ($p>0.05$).

Table 1. Litter characteristic of the dams at gestation day 18 (GD18).

Litter characteristic	Curcumin Concentration (mg/g body weight/day)			
	0.00 (n = 4)	1.05 (n = 4)	1.52 (n = 4)	2.00 (n = 4)
1. Total number of implantations, n	58	59	48	63
2. Litter size, n (mean ± SD)	14.5 ± 1.73	14.75 ± 2.06	12 ± 2.45	15.75 ± 4.79
3. Total resorbed fetuses, n (%)	0	0	0	16 (25.4)***
4. Total live fetuses, n	58	59	47	47
5. Total dead fetuses, n (%)	0	0	1 (1.72)	0

***p<0.001 compared with control.

Gross morphological analysis

At GD18, fetuses extracted from the dams were analyzed for gross morphology. Table 2 shows the comparison of fetal characteristics that were examined. A significant decrease in body weight (1.09 ± 0.25 g) was observed in the 2.00 mg/g treatment as compared to the untreated group (1.37 ± 0.19 g), p<0.001, while a significant increase was observed in 1.52 mg/g treatment (1.58 ± 0.11 g) relative to the untreated group (p<0.001). For the crown-rump length shown in Fig. 2, there was a decrease in the 2.00 mg/g treatment (22.04 ± 2.62 mm) relative to the untreated group (25.80 ± 1.48 mm), p<0.001.

Meanwhile, there was an increase in crown-rump length in the 1.52 mg/g treatment (26.76 ± 1.15 mm) when compared with the untreated group (25.80 ± 1.48 mm), p<0.001.

Table 2. Gross morphology of fetuses extracted from dams at GD18.

Fetal characteristic	Curcumin Concentration (mg/g body weight/day)			
	0.00 (n = 58)	1.05 (n = 59)	1.52 (n = 47)	2.00 (n = 47)
1. Mean ± SD of body weight in grams (g)	1.37 ± 0.19	1.44 ± 0.10	1.58 ± 0.11***	1.09 ± 0.25***
2. Mean ± SD of crown-rump length in millimeters (mm)	25.80 ± 1.48	25.64 ± 1.41	26.76 ± 1.15***	22.04 ± 2.62***
3. Flattened nose bridge, n (%)	0	0	0	3 (6.38)*
4. Micrognathia, n (%)	0	0	0	4 (8.51)*

***p<0.001 and *p<0.05 compared with control.

Cranio-facial malformations were also observed in the 2.00 mg/g treatment, as shown in Fig. 3. Flattened nose bridge was documented in 6.38% of the fetuses (p<0.05) and 8.51% showed micrognathia (p<0.05).

Skeletal malformation analysis

As shown in Table 3, the skeletons of alizarin-stained fetuses were compared. Large anterior fontanelle (Fig. 4) was observed in the curcumin treatments but it was significantly high (p<0.001) in the 2.00 mg/g treatment because 80% of the fetuses exhibited the malformation. All treatment groups also showed misaligned ossification centers in the sternum (Fig. 5) but the highest frequency was observed in the 2.00 mg/g treatment where 100% of the fetuses had the malformation (p<0.001). For supernumerary ribs (Fig. 6), there is a dose-dependent increase in occurrence but it is not statistically different in the groups (p>0.05).

Meanwhile, delayed ossification in the limbs and tail was observed frequently in the 2.00 mg/g treatment. There was delayed ossification in the forepaws (Fig. 7A) of 24% of fetuses (p<0.001) and delayed ossification in the hind paws of 100% of fetuses (p<0.001) in the 2.00 mg/g treatment (Fig. 7B).

Table 3. Skeletal malformation analysis of fetuses extracted from curcumin-exposed dams.

	Curcumin Concentration (mg/g body weight/day).			
Skeletal malformation	0.00 (n = 30)	1.05 (n = 31)	1.52 (n = 24)	2.00 (n = 25)
1. Large anterior fontanelle, n (%)	0	3 (9.68)	3 (12.5)	20 (80.0)***
2. Misaligned ossification centers in the sternum, n (%)	7 (23.3)	11 (35.5)	15 (62.5)	25 (100)***
3. Supernumerary ribs, n (%)	6 (20.0)	10 (32.3)	7 (29.2)	10 (40.0)
4. Delayed ossification at;				
a. forepaws, n (%)	0	0	0	6 (24.0)***
b. hind paws, n (%)	0	0	0	25 (100)***
c. caudal vertebrae, n (%)	0	0	2 (8.3)	25 (100)***

***p<0.001 compared with control.

Moreover, delayed ossification in the caudal vertebrae was seen in 1.52 and 2.00 mg/g treatments (Fig. 7B) but it was significantly high in the latter concentration since 100% of the fetuses exhibited the malformation (p<0.001). All other bones not reported were normal or present.

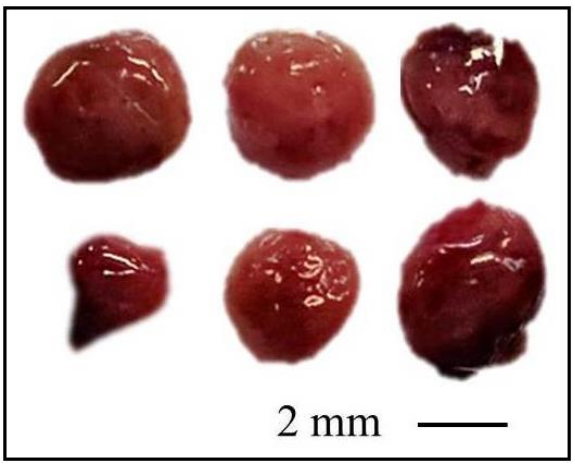

Fig. 1. Resorbed fetuses in the 2.0 mg/g curcumin treatment.

Discussion

Litter analysis

The results show that prior to the treatment of curcumin, the number of implanted embryos for all groups do not vary. The mean litter sizes of all groups are also not different from each other and the means are close to mean litter size of commercial ICR (Shin *et al.*, 2017). Therefore, the experiment utilized a good population of mice. The 9-day treatment of curcumin resulted to a significant increase in resorbed fetuses in the 2.00 mg/g concentration. This can be attributed to the apoptotic potential of curcumin which lowers the expression of Bcl-2 gene in mice as observed by Chen *et al.* (2010). In another study, *in vitro* exposure of ICR mouse blastocysts to 24 μM curcumin induced resorption of post-implanted embryos (Chen and Chan, 2012). The documented resorption indicates that curcumin was able to cross the blood-placenta barrier as reported by Guo *et al.* (2017) but this warrants further investigation. Curcumin treatment did not cause lethal toxicity in the developing fetuses since there is an insignificant mortality rate observed.

Gross morphological analysis

Curcumin also results to a decrease in fetal weight and crown-rump length at 2.00 mg/g concentration. This study supports the observations that curcumin exposure affects body weight. A study by Ganiger *et al.* (2007) reported that high curcumin dose resulted to a small reduction in body weight gain of the F2 pups in Wistar rats. The observed reduction in crown-rump length is similar to the observation of shortened body length in zebrafish larvae exposed to curcumin (Wu *et al., 2007)*. In contrast, a significant increase in fetal weight and crown-rump length were observed in the 1.52 mg/g treatment relative to the untreated group. Here, a positive effect on fetuses was caused by curcumin exposure.

The exact mechanism on how curcumin reduces body weight and crown-rump length is not clear but possible reason can be attributed to the anti-

angiogenic and anti-adipogenic properties of curcumin reported in mice (Ejaz *et al.*, 2009). Moreover, curcumin inhibits nitric oxide production in endothelial cells resulting to improper vasodilation (Dewar *et al.*, 2011). Limited blood flow can result to low oxygen and nutrient supply in fetuses therefore causing a reduction in weight and crown-rump length (Sagar *et al.*, 2006; Dewar *et al.*, 2011). It can also be due its pro-apoptotic effect (Chen *et al.*, 2010). As for the beneficial effects of curcumin, lower doses result to vasodilation which could have increased blood flow to the placenta thereby promoting growth (Dewar *et al.*, 2011). Further studies are needed to investigate these observations.

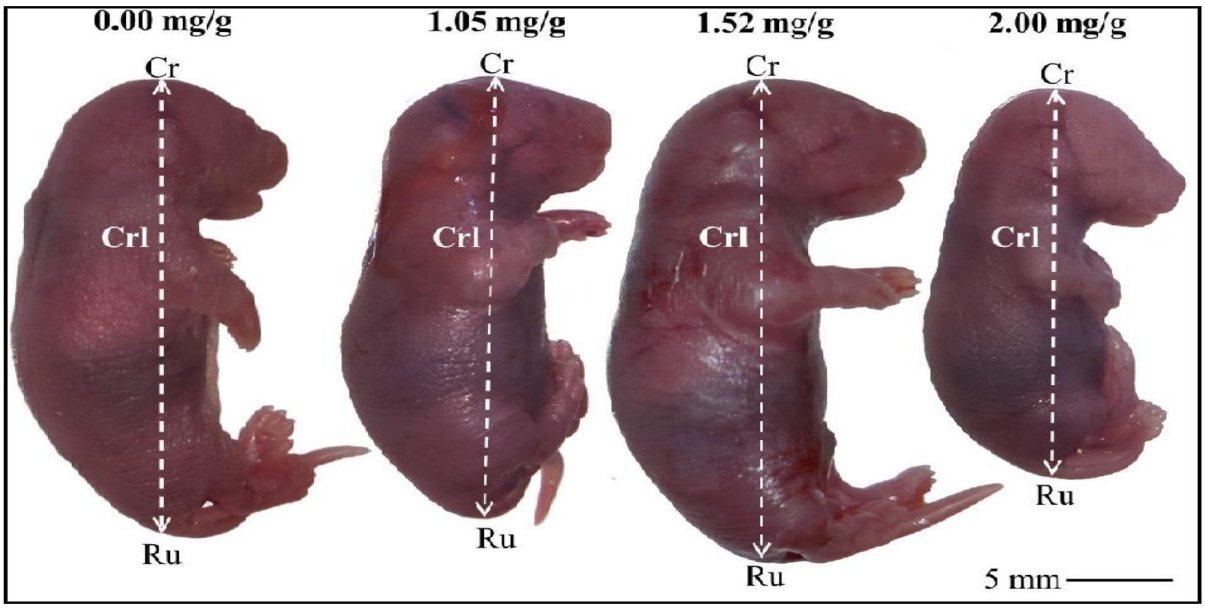

Fig. 2. Lateral view of the crown-rump length (Crl) of fetuses measured from the crown (Cr) to the rump (Ru). A significant increase in Crl is observed in 1.52 mg/g treatment while a significant decrease in the 2.00 mg/g treatment.

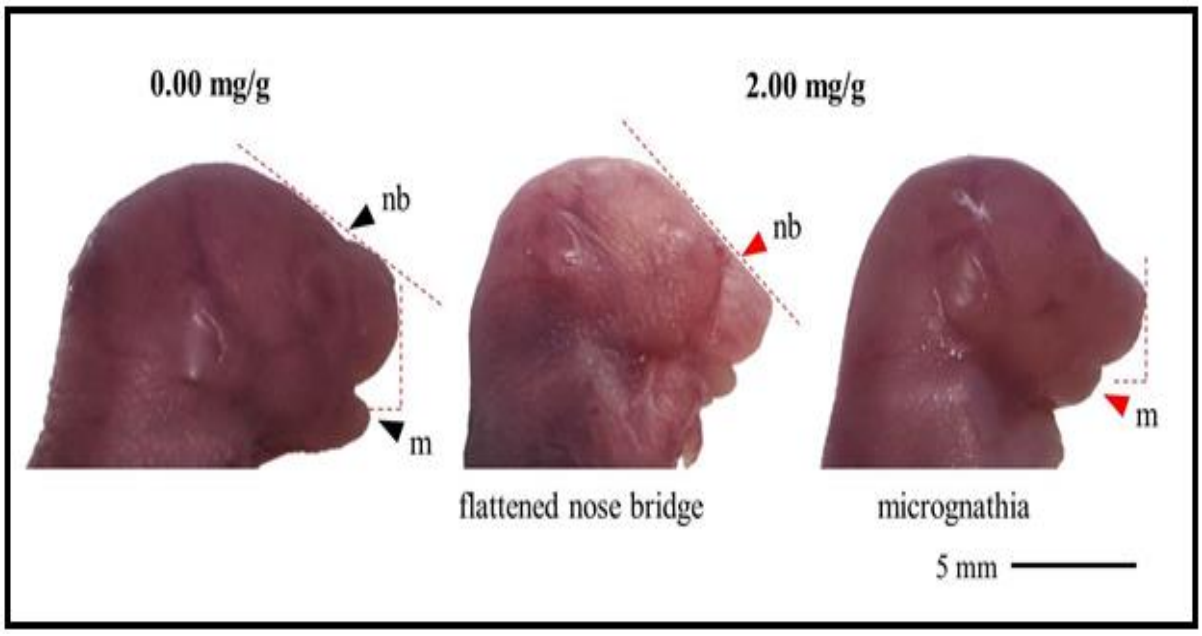

Fig. 3. Cranio-facial malformations of fetuses in the 2.00 mg/g treatment. Flattened nose bridge (nb, red arrowhead) and micrognathia (m, red arrowhead) were significantly higher in frequency when compared with the control group.

The observed cranio-facial malformations such as flattened nose bridge and micrognathia are due to exposure to 2.00 mg/g curcumin. Possible explanation for this can be attributed to the modulation of expression of *Ednra* gene in response to curcumin exposure (Shen *et al.*, 2006). Gene disorder involving *Ednra* can cause micrognathia and the thickening of the malar bones which may lead to an observed flattened nose bridge (Gordon *et al.*, 2015). Furthermore, curcumin coupled with small molecule inhibitors inhibit the expression of the *Fgfr1* gene causing facial malformations such as a flattened nose bridge in mice (Catela *et al.*, 2009; Lin *et al.*, 2012). The exact molecular mechanisms for these observations can be further investigated.

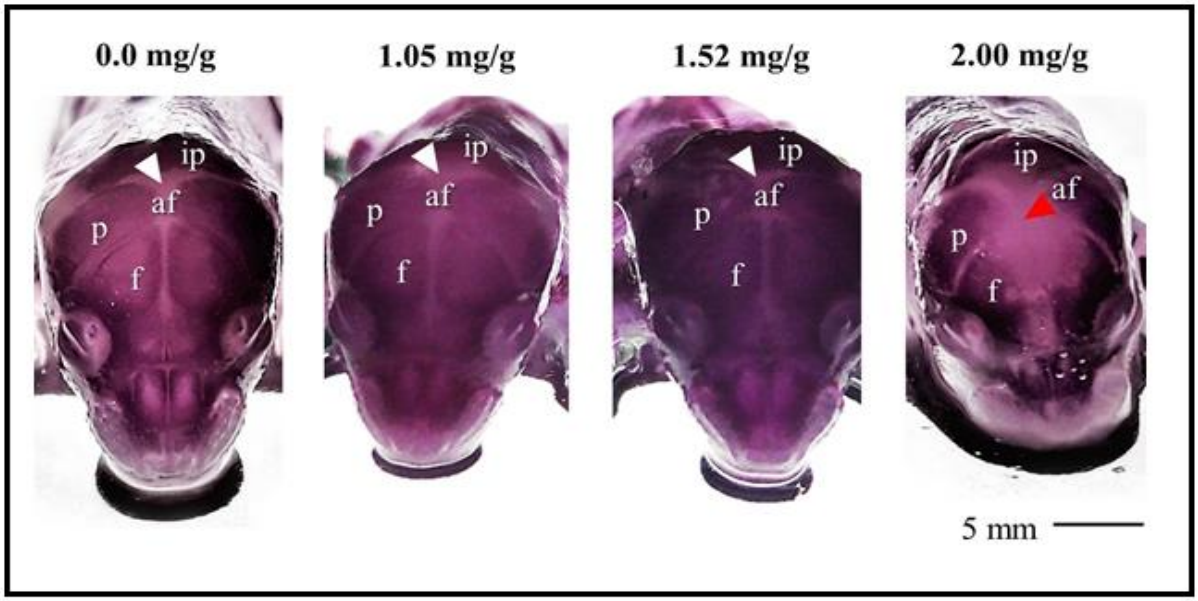

Fig. 4. Skull bones; frontal (f), parietal (p), and interparietal (ip) surrounding a normal anterior fontanelle (af, white arrowheads). A large anterior fontanelle (af, red arrowhead) which indicates delayed ossification is frequently observed in the 2.00 mg/g curcumin treatment.

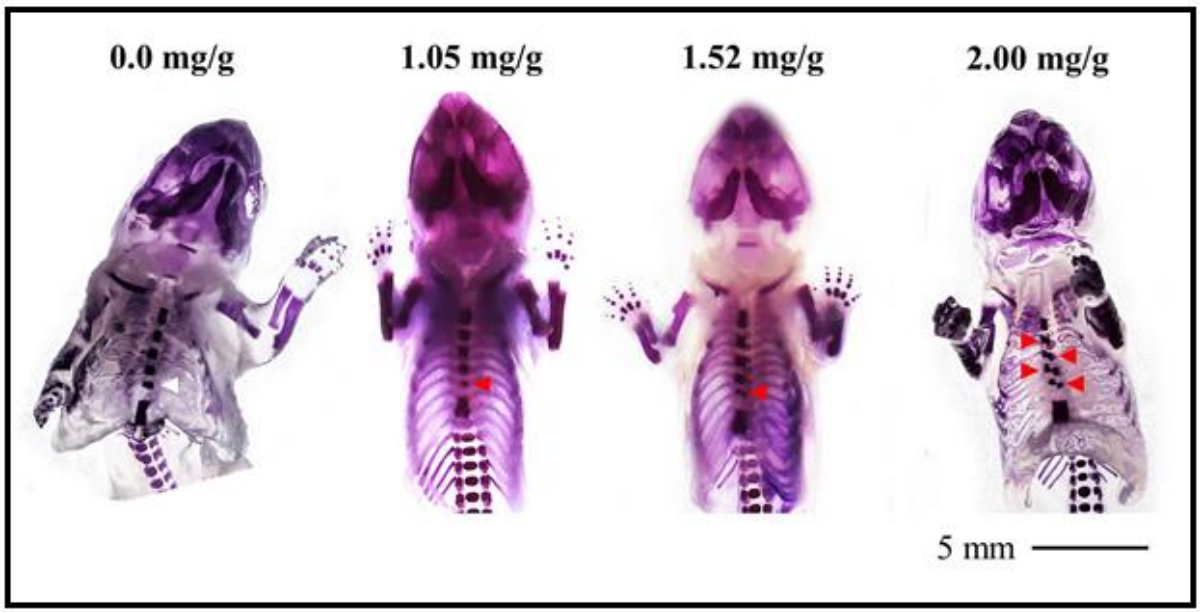

Fig. 5. Normal 5thsternebra (white arrowhead) and misaligned ossification center of 5thsternebra (red arrowheads). The 2nd, 3rd, and 4th sternebrae (red arrowheads) are also misaligned in the 2.0 mg/g curcumin treatment.

Skeletal malformation analysis

Evident skeletal malformations occur in the skull, sternum, limbs, and tail with a general increase in the frequency of these malformations in the 2.00 mg/g treatment. This may be due to curcumin's effect on the apoptotic pathway and enzymes such as the matrix metallopeptidase-9 (MMP-9) which is needed in bone degradation for bone remodeling and bone resorption (Chen *et al.*, 2010; He *et al.*, 2013; Gordon *et al.*, 2015). In a study which utilized mice with glucocorticoid-induced osteoporosis, curcumin was able to inhibit the activity of MMP-9 (Li *et al.*, 2015).

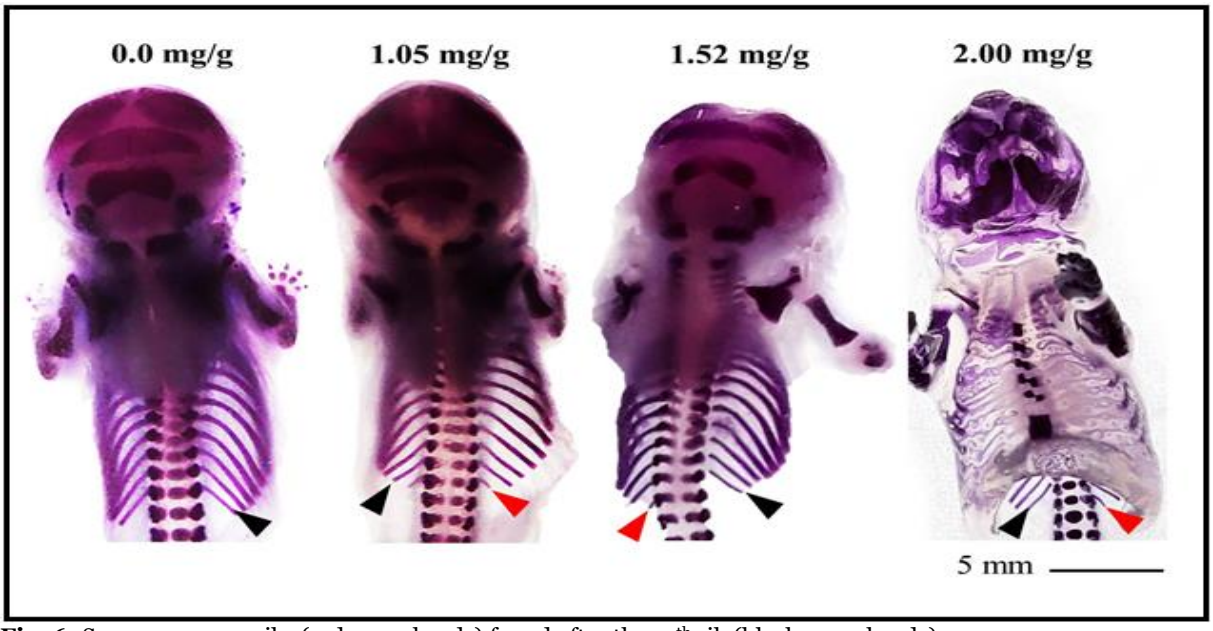

Fig. 6. Supernumerary ribs (red arrowheads) found after the 13th rib (black arrowheads).

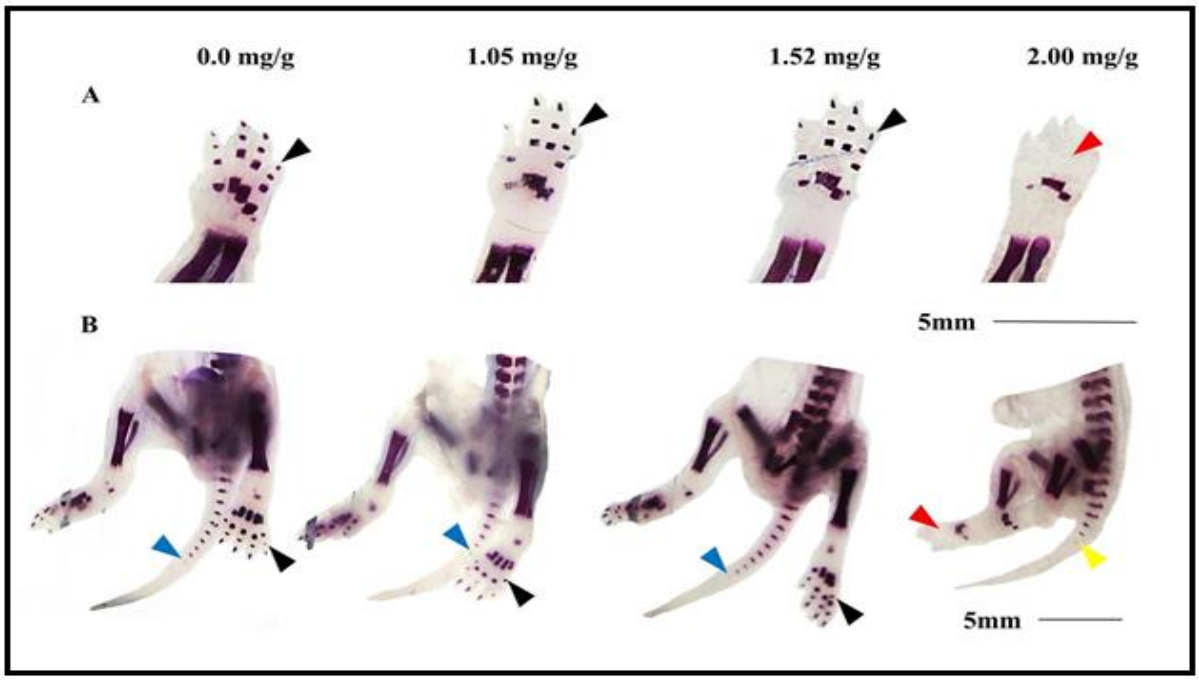

Fig. 7. A. Forepaw of normal fetuses showing formed phalanges (black arrowheads) and an abnormal forepaw (red arrowhead indicates missing phalanges). B. Hind paw of normal fetuses where phalanges are visible (black arrowheads) and an abnormal hind paw (red arrowhead indicates missing phalanges). Tail of normal fetuses (last caudal vertebra pointed by blue arrowheads) and an abnormal fetus (last caudal vertebra pointed by yellow arrowhead).

It is possible that inhibition of this enzyme or disrupting the apoptotic pathway in mice could lead to skeletal malformations such as delayed ossification in skull formation which results to a large anterior fontanelle, misaligned ossification centers in the sternum, and delayed ossification in the forepaw, hind paw, and caudal vertebrae. Supernumerary ribs, on the other hand, occur in all treatment groups without significant difference. It is normal that fetuses develop extra ribs in response to maternal stress but it can also be due to exposure to exogenous compounds (Chernoff and Rogers, 2004).

An interesting pattern was observed in the experiment where lower body malformations such as hind limb and tail malformations occur more frequently than forelimb malformations. This observation can be further investigated to elucidate the exact molecular mechanisms on how curcumin affects developmental pathways in animals.

Conclusion

It is likely that maternal curcumin exposure in mouse causes an increase in resorption of fetuses and a reduction in the fetal body weight and crown-rump length. Moreover, dose-dependent exposure results to cranio-facial malformations such as flattened nose bridge and micrognathia. Lastly, exposure to high concentrations of curcumin results to skeletal malformations such as large anterior fontanelle in the skull, misaligned ossification centers in the sternum, and abnormal ossification in the forepaws, hind paws, and tail. These findings suggest that curcumin is teratogenic in mouse fetuses and the information can be used to evaluate the safety of curcumin consumption or administration in pregnant women.

Declaration of Interest

The authors declare no conflicts of interest.

Acknowledgments

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