The Teratogenic Effects of Caffeine on the Development of Chick Embryos

Jantima Roongruangchai, D.D.S., Ph.D.*, Yadaridee Viravud, D.D.S., M.Sc.*, Vasana Plakornkul, D.V.M., M.Sc.*, Kesorn Sripaoraya, M.Sc.*, Wuttisan Saengkeaw, M.Sc.*, Suphanut Lumpikanon, M.Sc.*, Kosol Roongruangchai, M.Sc., M.D.**

*Department of Anatomy, **Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok10700, Thailand.*

ABSTRACT

Objective: This study aimed to investigate the potential teratogenic effects of caffeine by using chick embryo as an animal model.

Methods: The fertilized white leghorn hen eggs were randomly divided into 4 groups and were incubated for 24 hours. They were injected with different concentrations of caffeine at 30, 40, 50 and 60 mg/ml to the yolk sac. After day 3, 6 and 10 of incubation the embryos were recorded for the mortality and survival rates, recorded the malformations and further studied by total mount and serial section.

Results: The mortality rate was very high especially in higher dose groups and older incubation day. The few living embryos showed both growth retardation and abnormality. The abnormalities comprised anencephaly, anophthalmia and microphthalmia. The retardation comprised retardation of CNS, several visceral organs and ossification. The brain of the day 3 embryo comprised only three primary brain vesicles, the optic cup and lens were smaller than the control, lesser number of branchial arch and underdevelopment of limb bud and somite. The living day 6 embryos showed smaller of the body size and the living day 10 showed lesser in ossification.

Conclusion: Excess caffeine concentration can cause embryonic dead, growth retardation and malformations of the chick embryo and may cause the same effects to human. Pregnant women should be cautioned about consuming excessive caffeine, because it may lead to premature abortion, intrauterine growth retardation and abnormality of the baby.

Keywords: Caffiene; teratogen; chick embryo (Siriraj Med J 2019;71:(Suppl 1): S298-S305)

INTRODUCTION

Caffeine (1, 3, 7-trimethylxanthine) is a white crystal xanthine alkaloid, a bitter substance found in many kinds of food, such as coffee beans, tea, soft drinks, chocolate, kola nuts and drugs. It has many effects on the body's metabolism and stimulation of the central nervous system (increases the activity of the sympathetic nervous system and decreases parasympathetic nervous system activity), diuresis, increasing heart rate and/or blood pressure after consumption.[1] Caffeine consumption can boost up of energy to the consumers, increase wakefulness, alleviate fatigue, and improve concentration and focus. On the other hand tachycardia, bradycardia, arrhythmia, hypertension, hypotension, vomiting, convulsions, coma and even death can occur in case of overdose consumption.[2] Caffeine can also the normal fetal development following overdose maternal consumption. Chick embryos were commonly used in developmental biological studies because of their similarity to early stage human embryos with many comparative data, simplicity and easy manipulate. Moreover, the previous studies reported that the relatively of early chick embryos were sensitive to external chemical compounds such as caffeine.[3] Many studies have used chick embryos to test the effects of caffeine and showed

Correspondence to: Jantima Roongruangchai
E-mail: jantima.roo@mahidol.ac.th

that overdose caffeine exposure can cause morphological malformation of embryonic eyes and affected neural crest cell migration around the embryonic optic cup.[4]

In 2008, Xiaoping et al., examined the maternal caffeine consumption during pregnancy and the risk of miscarriage in a population based prospective cohort study. In this report, the increasing of caffeine daily dose consumption in maternal was associated with increasing of risk of miscarriage when compared with maternal who does not consumed caffeine.[5] In 2015, Hitomi et al., studied maternal total caffeine intake, mainly from Japanese and Chinese tea, during pregnancy was associated with risk of preterm birth in 858 Japanese women by evaluated self- administered diet history questionnaire. They found that caffeine intake of maternal pregnancy was associated with increased risk of preterm birth.[6] In 2003, Cem et al., examined high dose of caffeine administered to pregnant rats caused histological changes on newborn rat cornea.[7]

In 2012, Xiao et al., studied caffeine affected embryonic development through over stimulating serotonergic system in chicken embryo. Caffeine reduced embryos viability and elevated abnormalities including incomplete formation of abdomen and organs. Moreover, the embryos were failure of neural tube closure. Caffeine content in the brain of chick embryos increased significantly with the increasing of caffeine dosage and incubation time. The development of serotonergic system was influenced with caffeine. Therefore, maternal caffeine consumptions increased the risk of birth defects, and lead to congenital malformation.[8]

The precise mechanisms and consequences of caffeine-induced toxicity are still ambiguity. This study was conducted to use early chick embryos to evaluate changes of caffeine-treated embryos compared with the control during chick embryogenesis. Although we can handle the amounts of caffeine we feed our own body, but embryos cannot and because caffeine can cross placental membrane, embryos cannot fully metabolize the caffeine. This experiment in the chick embryo may demonstrate that caffeine is a potential teratogen. If so the pregnant woman should avoid consumption of caffeine in every form during pregnancy.

MATERIALS AND METHODS

Ethics Statement

According to German animal care guidelines, no IACUC approval was necessary to perform the embryo experiments. According to the local guidelines, only experiments with chick embryos E18 and older need IACUC approval. However, the embryos used in this study were all in early stages of embryonic development (between E1 and E11).[8]

ACUC Guideline

The Use and Euthanasia Procedures of Chicken/ Avian Embryos Avian embryos are not considered live animals under PHS policy. However, there is a consensus in the scientific community that at a certain point in development, avian embryos can experience pain. Because that exact point is not known for chicken embryos, chicken use and euthanasia guidelines differ across institutions. Cal Poly Pomona has chosen to adopt a guideline with the belief that pain occurs on or after gestation day 13, in anticipation of reviewing protocols including them. However, this study used earlier stages of chick embryos, day 3-day 10.[9]

Fertilized white leghorn (*Gallus gallus domesticus*) eggs were obtained from Suwanvajok-kasikit Research Station, Department of Animal Sciences, Faculty of Agriculture, Kasetsart University. They were divided into 2 groups; experimental group and control group. Control group: Group I was treated with normal saline solution 0.9%. Experimental group: Group II, III, IV and V were treated with 30 mg/ml, 40 mg/ml, 50 mg/ml and 60 mg/ml caffeine, respectively. 25 embryos of 24 hours incubation of each group were injected with caffeine of different concentrations at the equal volume of 0.15 ml to the yolk sac by sterile needle and returned to the incubator until day 3, 6 and 10. The day 3 embryos of each group were recorded for the mortality and survival rates and fixed in Dietrich's FAA solution for 4 hours and processed for total mount and serial section. The day 6 were recorded for the mortality rate and washed in normal saline solution before fixed in Dietrich's FAA solution before processing for the serial section. The remaining eggs were allowed to develop until day 10. After recording of the mortality rate, the skin and visceral organs were removed before transferred to Dietrich's FAA solution for 24 hours. The embryos were stained with alcian blue and alizarin red for observing the bone formation.

RESULTS

The effect of caffeine on mortality of the day 3 chick embryos

The survival and mortality rates of day 3 chick embryos was determined by the heart beating and blood circulation then compared with the control, the result showed in Table 1.

The survival rate of the day 3 chick embryos of the control group was 100% and the experimental groups which were exposed to 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml concentration of caffeine were 75%, 60%, 56.67%, 60%, respectively.

The effect of caffeine on the mortality of the day 6 chick embryos

The survival rate of the day 6 chick embryos in the control group was 100% and the experimental groups exposed to 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml were 60%, 45%, 32%, 24% , respectively. The mortality rates increased with the higher concentration of caffeine.

The effect of caffeine on the mortality of the day 10 chick embryos

The survival rate of the day 10 chick embryos in the control group was 100% and the experimental groups exposed to 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml of caffeine were 35%, 0%, 25%, 15% , respectively.

TABLE 1. The percentage of mortality and survival rates of the day 3 of experimental groups compared with control.

Group (mg/ml)	n	Survival rate n (%)	Mortality rate n (%)
Control	20	20 (100)	0 (0)
30	20	15 (75)	5 (15)
40	25	15 (60)	10 (40)
50	30	17 (56.67)	13 (43.33)
60	20	12 (60)	8 (40)
Total	115	79 (68.70)	36 (31.3)

TABLE 2. The percentage of survival and mortality rates of the day 6 chick embryos of the control and the experimental groups.

Group (mg/ml)	n	Survival rate n (%)	Mortality rate n (%)
Control	25	25 (100)	0 (0)
30	20	12 (60)	8 (40)
40	20	9 (45)	11 (55)
50	25	8 (32)	17 (68)
60	25	6 (24)	19 (76)

TABLE 3. The percentage of survival and mortality rates of the day 10 chick embryos of the control and experimental groups.

Group (mg/ml)	n	Survival rate (%)	Mortality rate (%)
Control	25	25 (100)	0 (0)
30	20	7 (35)	13 (65)
40	20	0 (0)	20 (100)
50	22	5 (25)	17 (75)
60	22	3 (15)	19 (85)

The total mount of the day 3 chick embryo

The control group

The total mount of the day 3 chick embryos were classified as HH stage 18 (Hamburger and Hamilton Stage 18 of normal chick development).[9] The 2 flexures of chick embryo were cervical flexure and cephalic flexure, the cervical flexure presented between the myelencephalon and spinal cord, cephalic flexure was at the mesencephalon. There were limb buds and tail-bud. The 30-36 somites extended to the level of leg bud. 4 pharyngeal arches were visible. The chick embryos had 5 secondary brain vesicles telencephalon, diencephalon (forebrain), mesencephalon (midbrain), metencephalon and myelencephalon (hindbrain). The optic cups situated at the diencephalon were large size of horse shoes-shaped and lens at the middle and otocyst at the myelencephalon level. The heart was an S-shaped loop. The neural tube appeared as 2 dense lines parallel to each other (Fig 1A).

The total mount of the day 3 chick embryo that exposed 30 mg/ml of caffeine showed abnormalities and growth retardation. The body was flexing to ventral side at the point caudal to the heart. The heart was dilated with U- shaped heart looping. In addition, the brain was composed of primary brain vesicles (prosencephalon, mesencephalon, rhombencephalon) and showed no cervical flexure, the cephalic flexure was visible at level of mesencephalon. The optic cup and lens at level of prosencephalon presented irregular shaped and smaller in size when compared with the control group. The limb bud and tail fold were absent. Somites were not extend to the caudal end but can be found only at cephalic ¾ of the body. The unclear neural tube presented as 2 dense lines unevenly and incomplete (Fig 1B). The 40 mg/ml of caffeine concentration showed abnormalities and growth retardation similar to the 30mg/ml group but the brain showed more retarded with incomplete separation of the brain part. The optic cup and lens was inconspicuous (Fig 1C). The 50 mg/ml of caffeine showed severe abnormality, the anterior neuropore was still opened (Fig 1D). The 60 mg/ml group was similar to the 30 and 40 mg/ml groups moreover it showed sign of caudal degeneration (Fig 1E).

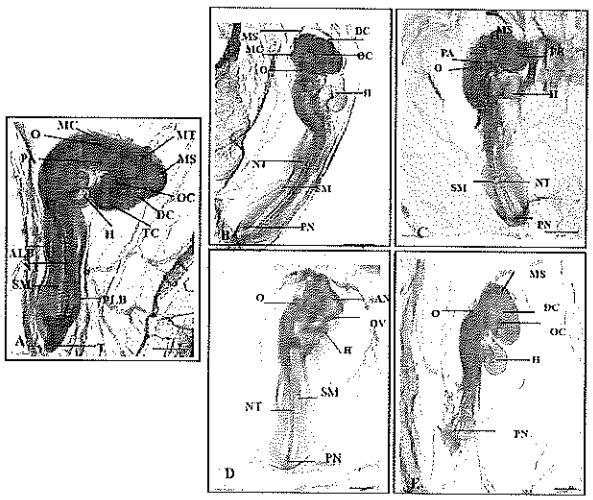

Fig 1. (1A) showed total mount of the 3rd day chick embryo of the control group, (1B), (1C), (1D) and (1E) showed total mount of the 3rd day chick embryos exposed to 30, 40, 50 and 60 mg/ml of caffeine, respectively. (1A) showed normal development of the day 3 embryo as stage 18 classified by Hamburger and Hamilton, while the experimental groups showed both retardation and abnormal. TC =Telenchephalon, DC=Diencephalon, OC=Optic cup, MS=Mesencephalon, MT=Metencephalon, MC=Myelencephalon, O=Otocyst, PA=Pharyngal arch, H=Heart, ALB=Anterior limb bud, SM= Somite, PLB=Posterior limb bud, T= tail fold, AN=Anterior neuropore, PN=Posterior neuropore. (1B), (1C) and (1E) showed retardation of brain development, (1C) was more severe with the absent of eye primordia. The hearts of all treated groups were all looser in looping with dilated lumen and thinned heart wall. All showed retardation of somite formation and opened of posterior neuropore in (1D) and (1E). (1D) showed opening of anterior neuropore (anencephaly).

Eye development observed by serial section of the day 3 chick embryos

The eye development of the day 3 chick embryos were studied by serial section at the level of diencephalon. In the control, the optic cup and lens vesicle were as HH-stage18, the optic cup comprised the thinner outer pigment layer and the thicker inner nervous layer. The two layers were closed to each other and obliterated the intraretinal space. The lens vesicle situated in the middle of the cavity of the optic cup, comprised the anterior lens epithelium and the posterior lens fiber. The anterior lens epithelium was simple cuboidal epithelium with mitotic figures of the cell while the posterior lens fiber elongated to be the columnar epithelium. The lens cavity existed. All experiment groups showed retardation of eye development. The optic cup was smaller than the control group and showed irregular shape. The outer pigment layer and the inner nervous layer of optic cup were widely separated by the intraretinal space. The lens placodes did not undergo process of double fusion but leaving a hole known as the lens pit on the outer surface of the face.

The day 6 chick embryo

The normal feature of day 6 chick embryos was around stage 29 of Hamburger Hamilton stages of chick embryos.[9] The beak was more prominent than the previous stage but showed no development of the egg-tooth. There were grooves between three first digits. The mandibular process fused with 2nd arch and auditory

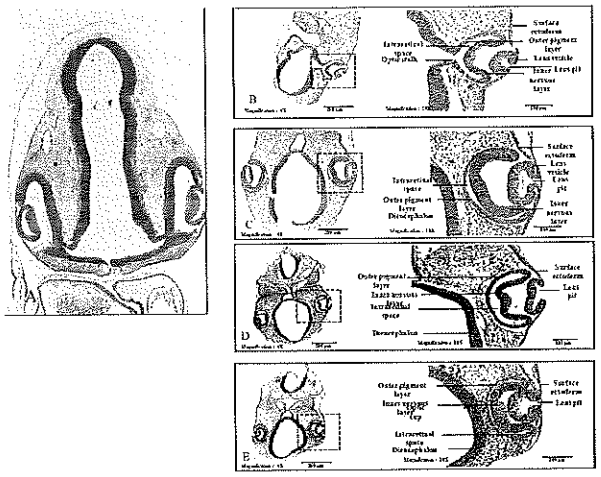

Fig 2. (2A) showed the serial section of the 3rd day chick embryo at the level of eye development of the control group, (2B), (2C), (2D) and (2E) were 30, 40, 50 and 60 mg/ml of caffeine treated group. All treated groups showed retardation of eye development, the eye primordia were smaller than the control and showed persistent of intraretinal space and lens pit.

meatus presented at the end of fusion. The eyes were large and round shaped. The heart located at thoracic level. The upper and lower limbs can be observed. There were distinctive the 5 brain vesicles.

The embryos exposed to 30 mg/ml of caffeine showed retardation of several areas such as small body size and abnormal body twisting, asymmetry and small eyes, small upper and lower limbs, the brain delayed development and some showed absent of eyes, anophthalmia (Fig 3B and C).The embryos exposed to 40 mg/ml of caffeine showed more retardation of development than the 30 mg/ml of caffeine group (Fig 3D). The body size and eyes were smaller than the control.

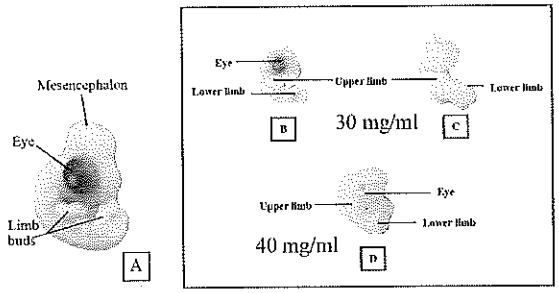

Fig 3. (3A) showed normal day 6 embryo of the control group, (3B), (3C) were the 30 mg/ml caffeine treated group and (3D) was the 40 mg/ml caffeine treated group. The control group showed prominent mesencephalon, eye and limb buds. The experiment groups showed smaller size of head and body and inconspicuous limbs and mesencephalon. The eyes were abnormal, microphthalmia in (3B) and (3D) while (3C) showed anophthalmia.

The effects of 40 mg/ml of caffeine to the 6th day chick embryos

One of the 40 mg/ml group showed severe abnormal of the brain development which showed widely opening of the anterior neuropore together with small optic cup and lens vesicle (microphthalmia). The optic cup showed widely opening of the intraretinal space. In the same section showed the small and underdeveloped heart at the dorsal aspect (Fig 4).

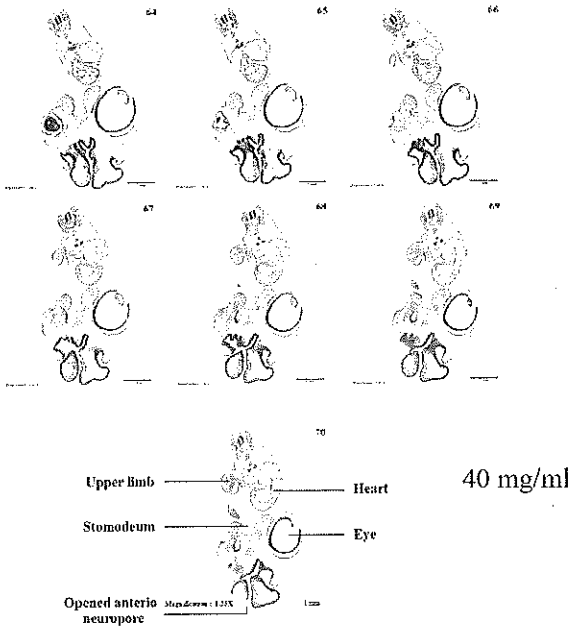

Fig 4. Serial section of the day 6 chick embryo of the 40 mg/ml caffeine treated group at the level of developing eye to show the opening of the anterior neuropore. In this case there was widely opening of the whole brain (anencephaly). The eyes were much smaller than the control (microphthalmia) and showed persistent of intraretinal space and the small lens.

The effects of 30 mg/ml of caffeine to the 6th day chick embryos

Another set of serial section of the day 6 chick embryo of the 30 mg/ml caffeine treated group showed widely opening of the anterior neuropore with bilateral anophthalmia or absent of both eyes (Fig 5).

The day 10 chick embryos

The 10th day chick embryo was the stage 36 of Hamburger and Hamilton stage of chick embryos. The statistical analysis of the body parameter measurement of the 10th day chick embryos by measuring of the body weight, crow rump length (CRL), head diameter (distance between eye to eye), eye diameter, lengthening of the upper limbs, proximal lower limb (distance between hip to knee) and distal lower limb (distance between

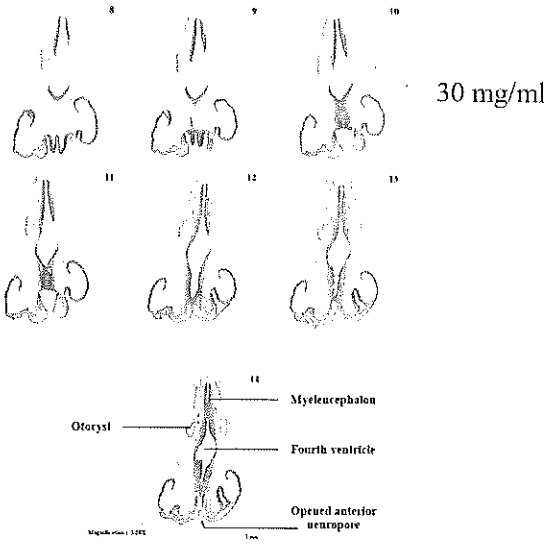

Fig 5. Another set of serial section of the day 6 chick embryo of the 30 mg/ml caffeine treated group at the level of developing internal ear or the myelencephalon to show the opening of the anterior neuropore (section no. 9-14). In this case there was widely opening of the whole brain (anencephaly) and absent of both eyes (anophthalmia).

knee to feet). This statistical analysis was computed by Independent samples Kruskal-Wallis Test using SPSS version 18 to compare the data between the control group and experimental groups. The statistics showed different significances in the crow rump length (CRL) of 50 mg/ml (23.44±1.30) caffeine and the parameter of beak of 60 mg/ml (1.92±0.54) caffeine when compared with the control group. The other parameters showed no significant difference in all of body parameter both of the control group and the experimental groups.

Table 3 showed the very high mortality rate of the day 10 experiment groups especially in the 40 mg/ml group which showed that all embryos died before day 10. The survived embryos mostly showed normal development except for smaller in size which showed no significant different when compared with the control. The embryos of the experimental groups which died before day 10 showed in Fig 6B, C and D. They were smaller than the control and showed more or less abnormalities such as the microphthalmia and ectopia viscerae, abnormal and rudimentary limbs and some showed subcutaneous hemorrhage.

TABLE 4. The data, statistical analysis of the body measurement of the 10th day chick embryo (Mean ± Standard deviation).

Group / Parameter	Control	30 mg/ml	50 mg/ml	60 mg/ml
Body weight (g ± SD)	2.02±0.14	2.05±0.30	1.95±0.21	1.73±0.09
Beak (mm ± SD)	3.82±0.68	3.40±0.98	2.46±0.78	1.92±0.54*
CRL (mm ± SD)	27.10±0.94	26.07±1.76	23.44±1.30*	25.55±0.33
Width of head (mm ± SD)	12.44±0.54	12.43±0.87	10.46±2.33	11.59±0.47
Eye Diameter (mm ± SD)	7.43±0.45	6.94±0.64	5.25±2.33	6.22±0.82
Upper Limbs (mm ± SD)	8.83±0.88	8.78±0.25	8.45±1.33	8.45±0.20
Proximal Lower Limbs (mm ± SD)	7.53±0.53	7.94±1.36	7.84±0.81	7.25±0.12
Distal Lower Limbs (mm ± SD)	8.63±0.90	8.43±1.49	6.60±1.62	7.45±0.80

Significant difference was computed by Independent samples Kruskal-Wallis Test uses SPSS version 18* $P \leq 0.05$

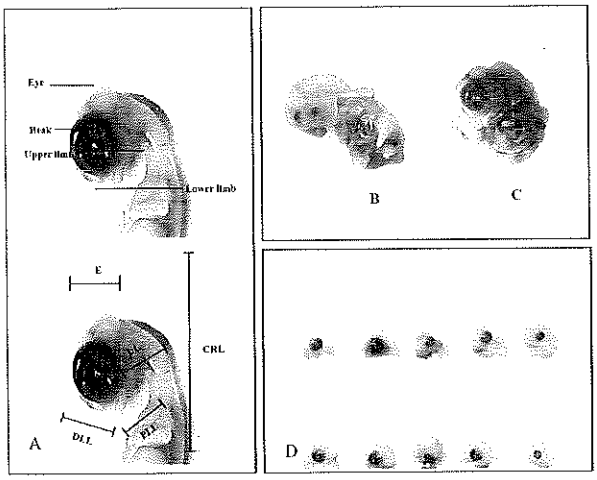

Fig 6. (A) showed normal general features of the day 10 chick embryo of the control group. (E = Eye diameter, B = Beak, UL = Upper limb, PLL = Proximal lower limb, DLL = Distal lower limb, CRT = Crown rump length). (B) and (C) showed general feature of the day 10 chick embryo exposed to 50 mg/ml of caffeine. (D) showed the dead embryos of the day 10 which died earlier. They exhibited many abnormal structures such as bleeding in the eyes, smaller eye size (microphthalmia), ectopia viscera, and abnormal body shaped.

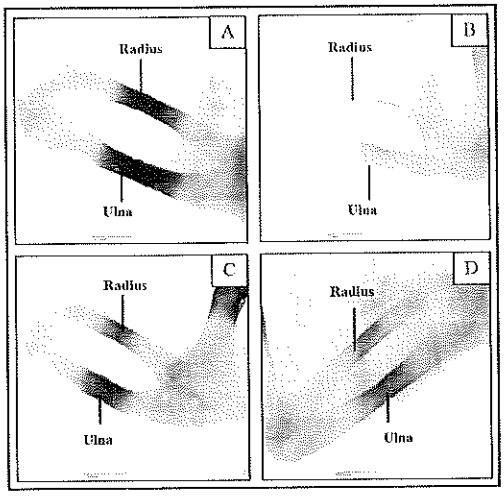

Fig 7. showed the alcian blue and alizarin red staining of the cartilage and bone of the control (A), the 30 mg/ml (B), the 50 mg/ml (C) and the 60 mg/ml (D).

The bone and cartilage of the day 10 chick embryo

The skeletal system, bone and cartilage, can be observed by alizalin red and alcian blue staining, the cartilage models stained blue while bones stained red. Fig 7 showed the alcian blue and alizarin red staining of the cartilage and bone of the control (A), the 30 mg/ml (B), the 50 mg/ml (C) and the 60 mg/ml (D). The bones were radius and ulna bones, the red indicated the bone formation while the blue indicated the cartilage models. There was marked ossification at the shafts of the bones of the control and slightly in the 50 and 60 mg/ml caffeine treated groups while in the 30 mg/ml treated group showed no sign of ossification at all. This indicated that caffeine may interfere with the process of ossification.

DISCUSSION

The chick embryos in experimental groups showed higher mortality rate when compared with the control and the mortality rate tended to increase with the higher concentration of caffeine and the increasing embryonic day. The eyes development, showed abnormal and retardation including irregular shaped optic cup and lens, smaller in size (microphalmia), the widely opened interatinal spaced and lens cavity. The anophthalmia presented in 40 mg/ml group of the day 6 chick embryo. CemEverekliogluin 2003 found that caffeine caused vacuolated endothelial cells, endothelial cell agenesis, increased stromal mitotic activity and focal increase in corneal thickness of Wistar-Albino rat embryo.[11] Moreover, Monika Kujavwa – Hadryoein 2010, caffeine caused changing of layers and the total thickness of cornea development.[12] Caffeine may interfere PAX6 expression which PAX6 is the main control the formation of the eyes corresponding to Canto-Soler MV in 2006[13] and Zheng-lai Ma in 2014.[14]

The heart development showed retardation, looser in heart looping, thined heart wall with dilatation of the lumen when compare with the control. Corresponding to Momoi N in 2008 and Christopher C Wendlerin 2009 who studied mice embryos and found that caffeine affected cardiovascular function of mice's embryos.[14,15] Rana N in 2010 who found that caffeine affected on embryonic heart rate of zebrafish' embryo by inhibition of ether-a-go-go potassium channels.[1]

On the day 3 chick embryos in the experimental group showed retardation of brain (primary brain vesicles). The day 6 chick embryos in 30 mg/ml group showed opened anterior neuropore. This agreed with Stephane Marret in 1997 who found caffeine caused failure of neural tube closure. Rebecca J. Schmidt in 2009 who found that maternal consumption of caffeine increased risk of neural tube defects (anencephaly, spina bifida, or encephalocele).[16] Zheng-lai Ma in 2012 reported that caffeine caused malformations of the neural tube and induce other teratogenic effects on nervous system development.[3] The external morphology of the day 10 showed the corresponding results to Bruyere HJ Jr in 1983 who studied the external malformations in chick embryos which exposed to methylxanthines and beta-adrenomimetic agents. They found that caffeine and

theophylline induced beak malformations.[17] Wilkinson JM in 1994 and Momoi N in 2008 found that caffeine cause decreased significant of the crow rump length in rat embryo which exposed to caffeine when compared with the control.[18]

Bone staining of the 10th day chick embryos showed decreased significant in radius and ulna when compared with the control. Caffeine induced apoptosis in osteoblasts via a mitochondria dependent pathway. Caffeine down regulated some important events in osteogenesis and ultimately affected bone mass. Yang Tan in 2012 found that caffeine inhibit fetal skeletal development in signaling pathway of lower insulin-like growth factor (IGF)-1 activity.[18]

CONCLUSION

Caffeine caused several congenital abnormalities, growth retardation and embryonic death. The mortality rate was very high especially in the day 10 embryos. The small number of survived embryos were mostly normal indicated some can escape from the dangerous teratogen and developed normally. The dead embryos showed abnormalities such as anencephaly, anophthalmia, microphthalmia, ectopia cordis, ectopia viscerae and retardation of ossification. Caffeine consumption in high dose can caused embryonic death. Thus, the further study of teratogenic effect of caffeine should be carried on in mammalian models that closer to human than chick embryo and specific gene alteration when exposed to caffeine should be emphasized.

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