TERATOGENIC EFFECTS OF CHLOROQUINE ON DEVELOPING CHICK EMBRYOS

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ABSTRACT

Chloroquine is used to prevent and treat malaria, rheumatoid arthritis and systemic lupus erythematosus (SLE) due to its anti-inflammatory effect. Treatment of rheumatoid arthritis requires long-term medication in all ages including pregnant women. Pregnant women with rheumatoid arthritis and on chloroquine medications may effect to the embryo or fetus. This study was conducted to study the teratogenic effects of chloroquine by using chick embryos as an animal model and compared to the comparing stage of human embryo. Fertilized white leghorn hen eggs were injected *in ovo* with three concentrations of chloroquine, which were 0.2g/ml, 0.4 g/ml and 0.6 g/ml in 0.9% NSS at equal volume of 0.1 ml to the yolk sac of 24 hours incubation before injection. After injection, they were further incubated until 72 hours. After that they were sacrificed and processed for total mount and serial section. The present study showed the mortality rate of different concentrations of chloroquine. The total mount of day 3 showed growth retardation and major abnormalities of the brain vesicle and eye such as retardation of brain vesicle. The serial section of day 3 showed growth retardation of eye development (wide intraretinal space), looser of heart looping and also atrium and bulbus cordis malformations. At 0.6 g/ml chloroquine has the most effects to the growth of day 3 of chick embryos. In conclusion, chloroquine cause embryonic death and abnormalities in day 3 of chick embryos. Which the risk is indicated when chloroquine was used with higher doses than the recommended dose. Nevertheless, more extensive investigation showed be carried out in other animal models to confirm its teratogenicity.

Keywords: Chloroquine, teratogen, chick embryos.

1. Introduction

Chloroquine drug is used to prevent and treat malaria. It also used in the treatment of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). It is an alkylated 4-aminoquinolines, identified chemically as amphiphilic weak based on 2-aromatic ring, conjugated double bonds. Mechanism of chloroquine to plasmodium is accumulation in food vacuole of parasites, infected RBCs. It is toxic to the parasites, which inhibit heme polymerase activity (Lek-utha, 2015). For rheumatoid arthritis, the mechanism is a consecration of a small rise in the pH of cells, it called lysosomotropic action. This activate cell malfunction including protein processing. It has

consequences, especially on the adaptive immune response, which substantiate negative effects on a of proinflammatory cytokines (S.J., 2012). The dose recommended by the World Health Organization is 25 mg/kg independently of the age of the subject (Añez, 2016) and chloroquine can be cross the placenta which was categorized in drug category C.

This drug is reported to have significant side effects visually with patients taking this drug for a long time may cause temporary or permanent blindness. It is believed that the drug is accumulated in the retina of the eye which causes bull's eye maculopathy (Marmor et al., 2016). Chloroquine, this antimalarial has been extensively used in pregnancy but use for treatment of *P. falciparum* malaria is considerably restricted. Chloroquine was reported to associate with abnormality of rats at 1000 mg/kg dose had embryonic in 27%, anophthalmia, microphthalmia in 47% of the surviving fetuses and growth retardation skeletal dysmorphism. Rat embryo culture also had malformations such as (1) Inhibited cranial neural tube (2) Microophthalmia (3) Altered morphology of (4) Cranial neural crest cells (5) Accumulation in the eyes in early gestation and eyes and ears in later pregnancy. (Nosten et al., 2006)

Animal studies are so important for teratogenesis study. Mammalian models are regularly used for preclinical assessment of a new drug due to closely accompanying to humans. In addition the mammalian models are difficult to evaluate and assemble. It's expensive and more time consuming projects. The chick embryo is an excellent model system, which many benefit to use chick embryos because of available all year and inexpensive, it can be purchased in any specified quantity. There are enough of databases of developmental stage and similar and can be compared to humans (Darnell, 2000). Chick embryo is an excellent model system for screening the teratogenic effects of new drugs before further studies are carried out in other mammalian models. The researcher selected chicken embryos in this study.

2. Objectives of the study

This study was conducted to know about the teratogenic effects of chloroquine by using chick embryos as an animal model and the results were compared to the embryo of human of the same stage.

3. Materials and methods

Fertilized white leghorn hen eggs *(Gallus gallus domesticus)* was purchased from Department of Animal Science, Faculty of Agriculture, Kasetsart University were used as animal model. Eggs were divided randomly into 4 groups were (1) Group 1: Control group, treated with normal saline (2) Group 2: chloroquine treated group, treated 0.2 g/ml of chloroquine (3) Group 3: chloroquine treated group, treated 0.4 g/ml of chloroquine. (4) Group 4: Chloroquine treated group, treated 0.6 g/ml of chloroquine. For group 3, this doses is used because we are testing LD_{50} is one way to measure the short-term poisoning potential (acute toxicity) of a material. This make use of pilot testing drug is LD_{50} before the experiments.

Incubation and injection

All fertilized eggs were rinsed with 70 % ethanol and predefined number of each group. They were incubated at 37.5 ± 0.5 °C and 70–80 % relative humidity. The eggs must be placed in a horizontal position in the incubator tray and repositioned on its axis every two hours. The Fertilized egg were incubated until 24 hours before injection. The eggs shell were cleaned with 70 % ethanol and drilled at the blunt of the air cavity by dental driller. All eggs were injected at equal volume of 0.1 ml to yolk sac on 24 hrs incubation. The hole was sealed by sterile tape after injection and continued incubated until day 72 of development. The eggs from all groups were sacrificed after processed for total mount by stained with mayer's camine techniques and the serial section of day 3 were stained with hematoxylin and eosin.

4. Results

The effects of choloquine on days 3 chick embryos

4.1 The survival and mortality rates

The survival and mortality rates of day 3 was observed by the blood circulation and heart beat. This stages can be compared to the 4th weeks of human embryo after fertilization. The experiment groups was investigated of the teratogenic effects of chloroquine phosphate which were exposed to the developing chick embryos on embryonic day 3 compared with the normal development. **Table 1** showed the mortality rate of day 3 embryos were 0%, 78.57%, 56.00% and 60.87% in the control group, 0.2 g/ml, 0.4 g/ml and 0.6 g/ml of chloroquine, respectively.

Table 1 Percentage of survival and mortality of the day 3 chick embryos of the control and the experiment group

Group (g/ml)	n (%)	Survival n (%)	Mortality n (%)
Control groups	10 (100)	10 (100)	0 (0)
0.2	28 (100)	22 (78.57)	6(21.49)
0.4	25 (100)	14 (56.00)	11(44.00)
0.6	23 (100)	14 (60.87)	9(39.13)
Total	86 (100)	60 (69.77)	26(30.23)

4.2 The total mount of the day 3 chick embryo

The effects of chloroquine on day 3 chick embryo were examined and compared with the control. In the control group showed normal development (Figure1A), the day 3 chick embryo was categorized as Hamburger

Hamilton stage 18 and approximately 36 somites. The development of the five brain vesicles were completed including telencephalon, diencephalon, metencephalon, mesencephalon and myencephalon. The two flexures were distanced difference bent to the right, cephalic flexure and cervical flexure. At the cervical flexure, the axis of the medulla forms approximately a right angle to the axis of the posterior trunk, cephalic flexure at the level of mesencephalon and cervical flexure was between the myelencephalon (hindbrain) and spinal cord. Pharyngeal arches distinct characteristics and maxillary process and fourth visceral cleft were absent or inconspicuous. The optic vesicle located at the level of the side of the myelencephalon, the optic cup was large horseshoe- shaped located at the diencephalon and the lens located at middle to optic cup. Limb buds enlarged leg-buds slightly larger than wing-buds (Hamburger Hamilton, 1951). The heart tube was S-shaped looping, it is completed the proximal two-thirds of the primitive conus shifts toward its definitive position ventral to the right atrium. (Manner, 2000).

The effects of chloroquine treated 0.2 g/ml (Figure 1B) were growth retardation and major abnormalities of the brain vesicle, the branchial arches were divided into three and a half arch from all four arch. The limb buds were absent. Heart loop was looser than the control.

The effects of chloroquine treated 0.4 g/ml (Figure 1C) on day 3 chick embryo showed growth retardation and major abnormalities of the brain vesicle. The eyes showed microphthalmia and looser of heart looping with thinned wall and constricted to u-shaped loop. The branchial arch were divided into three arch from all four arch. At the limb buds were lesser than the control, the somite does not extend to caudal ends.

The effects of chloroquine treated 0.6 g/ml (Figure 1D) on day 3 chick embryo were growth retardation and retardation of development of brain vesicle. The cephalic flexure and cervical flexure were absent. The eyes showed microphthalmia and looser of heart looping with thinned wall and constricted to u-shaped loop. At branchiales arch were fewer number than the control. The limb buds were absent. The somites do not extend to caudal ends. The neural tube were two dense lines which were not parallel, the neural tube does not close completely. At the tail fold was absent.The posterior neuropores was open.

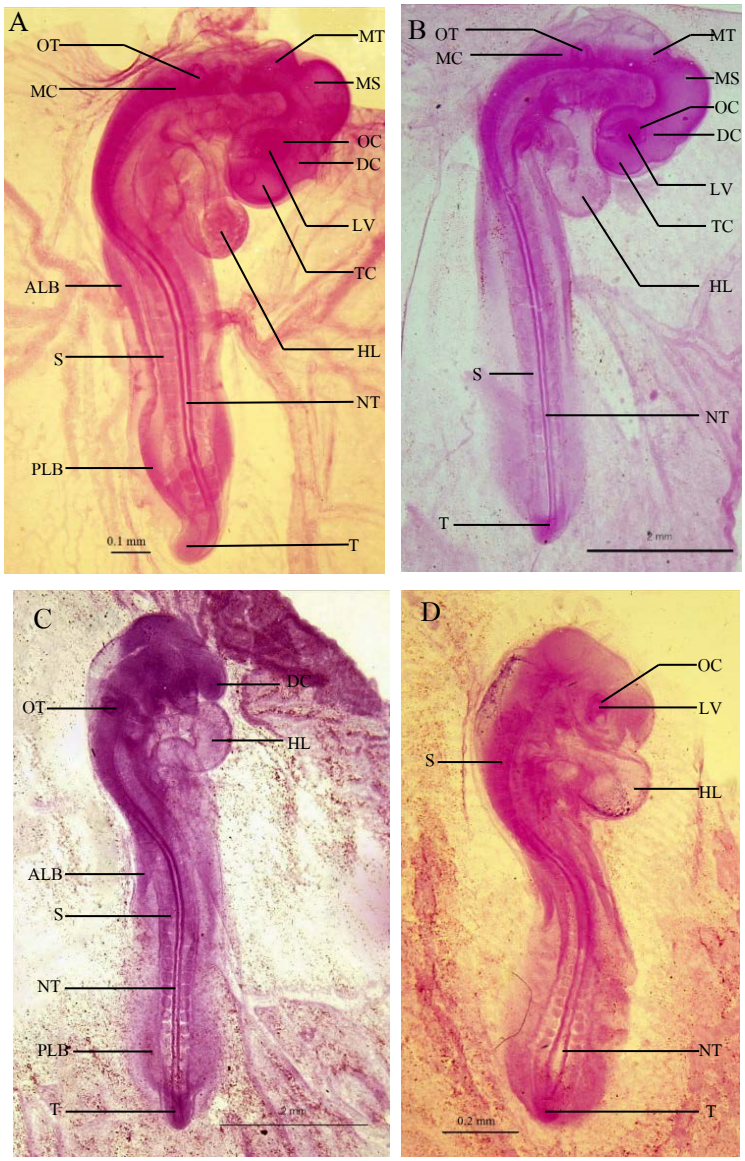

Figure 1 The total mount of day 3 chick embryos, A. control, B. 0.2 g/ml, C 0.4 g/ml and D 0.6 g/ml of chloroquine treated groups. (TC = telencephalon, DC = diencephalon, MT = metencephalon, MS = mesencephalon MC = myencephalon, OC = Optic cups, LV= lens vesicle, OT = otocyte, HL = heart loop, ALB = anterior limb bud, N = neural tube, S = somite, PLB = posterior limb bud, T = tail fold)

4.3 The serial section of the day 3 chick embryo

The control group showed normal development (Figure 2A) of the eyes, which emarginated from the diencephalon. The optic vesicle grew up and touched ectoderm, induced ectoderm in this area thicker and became lens placode. Later on distal end part in optic vesicle expanded and curved side called optic cup, which included 2 walls, outer pigmented layers and inner nervous layer that separated by intraretinal space. Inside cavity of optic cup had lens vesicle grew from lens placode curved and double fusion. The lens vesicle had thin front wall and had many mitotic called anterior lens epithelium. The back wall was elongated cells which called anterior lens fiber and optic cup that attached diencephalon with optic stalk, grew up to become optic nerve and in proximal part will became narrower.(Roongruangchai , 2016)

The control group showed normal development (Figure 3A) of the heart. It is single tube but surround curve. Curve in left-right direction due to gene affect. Bulbus cordis is in the right and above to ventral aorta which aortic arch linked with dorsal aorta, next to bulbus cordis below to ventral and caudal. The biggest part of heart is ventricle, from ventricle to the left next to the bulbus cordis is atrium which at the back is sinus venosus. Sinus Venosus getting blood from vitelline vien that notable Chick Embryo flow from wall of york sac vitelline vein from both left and right. It will flow together at anterior intestinal portal and anastomose, the new blood vessels called portal vien and most of blood vessels will flow into sinus venosus. (Roongruangchai, 2016)

The effects of chloroquine were studied by serial section of day 3. It was stained with hematoxylin and eosin techniques to study abnormalities in the experimental groups compared with the control group for following abnormalities (Figure 2 B, C, D) such as retardation and small of eyes, wide separation of the intraretinal space, the posterior lens fiber not-elongated and (Figure 3 B, C, D) looser of heart looping and malformations atrium and bulbus cordis when compared with control group.

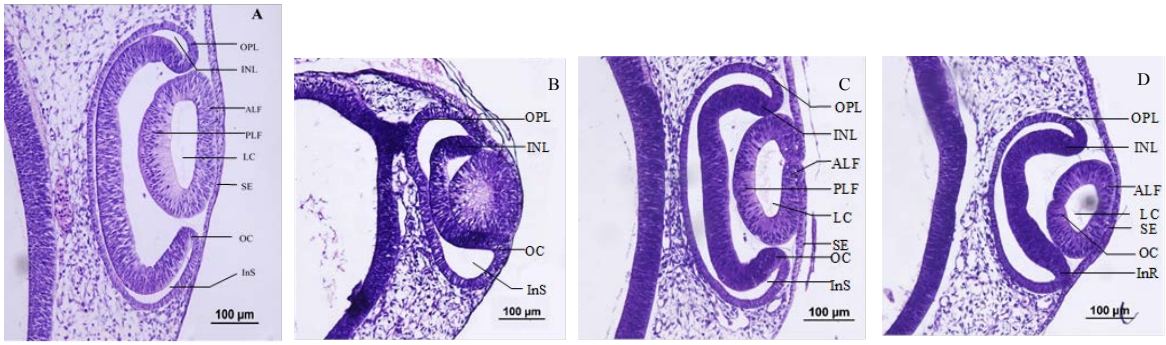

Figure 2 The micrograph of day 3 embryo at eye development by serial section, A. control, B. 0.2 g/ml, C 0.4 g/ml and D 0.6 g/ml of Chloroquine treated groups. (OPL = Outer pigment layer, INL = Inner nervous layer, ALF= Anterior lens fiber, PLF= Posterior lens fiber, LC = Lens cavity, SE = Surface ectoderm, OC = Optic cups, InS = Intraretinal space)

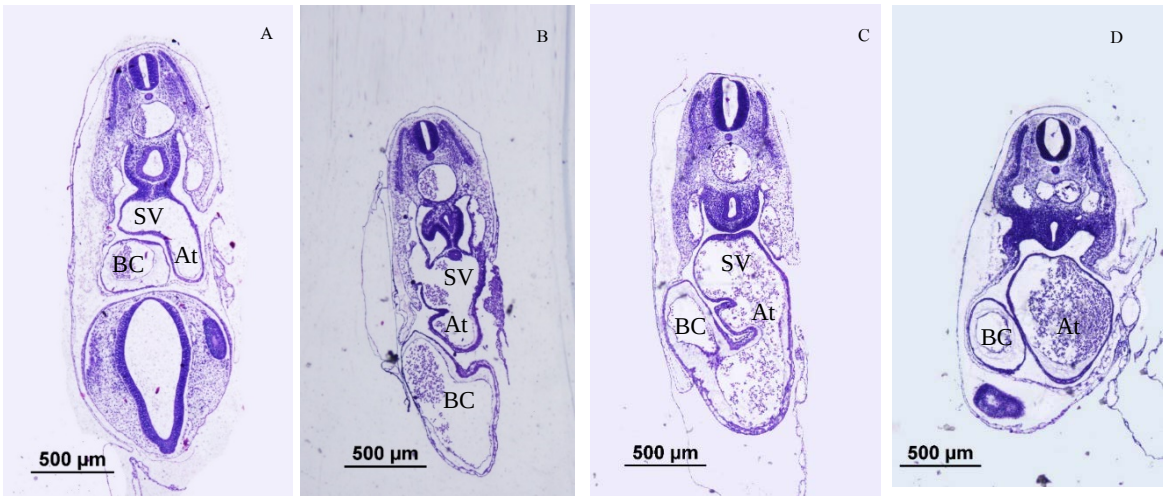

Figure 3 The micrograph of day 3 embryo at heart development by serial section, A. control, B. 0.2 g/ml, C 0.4 g/ml and D 0.6 g/ml of Chloroquine treated groups. (SV= sinus venosus, At = atrium, Bc = bulbus cordis)

5. Discussion

The result of the percentage of embryonic death in high doses and experimental group showed growth retardation, major abnormalities of brain vesicle and eyes, limb buds were absent and heart abnormality of day 3 chick embryo. This agreed with the report in rat, when the rat embryo were exposed to chloroquine at 1000 mg/kg dose showed embryonic death and anophthalmia, microphthalmia of the surviving embryo (Nosten, 2006).These previous reports showed that one of the primary effects of embryotoxicants which disrupt lysosomal function in the visceral yolk sac cause growth retardation in the embryo. Chloroquine were tested in animal model at the high dose tested 250 - 1,500 mg/kg cause abnormalities of eyes and internal ear which abnormalities of 45 % of animal model at 1000 mg/ kg. It is reported about abnormality of inner ears and other after treat with higher doses than the recommended dose of chloroquine at 500 mg/kg (Khamashta, 2006).

6. Conclusion

This study showed chloroquine cause embryonic death and abnormalities in day 3 of chick embryos. Which the risk is indicated when chloroquine was used with higher doses than the recommended dose. Nevertheless, more extensive investigation showed be carried out in other animal models to confirm its teratogenicity.

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