The Teratogenic Effects of Dichlorvos on the Development of Chick Embryos

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ABSTRACT

Objective: The purpose of this study was to elucidate the teratogenic effects of dichlorvos on developing chick embryos.

Methods: The fertilized Leghorn hen eggs were divided into two groups: the experimental group which was injected with 0.1 ml of 0.5% and 1% dichlorvos in normal saline and the control group which was injected with 0.1 ml of normal saline after 21 h of incubation. On day 3, 6, and 11, the embryos were collected for studying embryonic dead and abnormalities.

Results: The results showed that the mortality rate increased with the increasing concentration of dichlorvos and time of incubation. The total mount of day 3 had only three primary brain vesicles, small and retarded primordial eye, dilated U-shaped heart looping, bifurcation of spinal cord and trunk when compared with the control. The results in the serial section of day 3 and 6 showed several abnormalities especially the retardation of eye and heart. Day 11 embryo revealed morphological anomalies including hematoma and bone deformation.

Conclusion: Dichlorvos caused congenital abnormalities in chick embryos in 3 categories, the growth retardation, the malformations and the embryonic death which were predicted to cause the same results in contaminated humans. Dichlorvos exposure increases the risk of malformations and embryonic death. The present study revealed that dichlorvos was a powerful teratogenic compound and therefore its use should be limited and pregnant women should avoid contamination with dichlorvos especially in the first trimester.

Keywords: Dichlorvos; organophosphate; teratogen; chick embryo (Siriraj Med J 2018;70: 44-52)

INTRODUCTION

Currently, pesticides are more benefit in everyday life for preventing, destroying, repelling or eliminating pests. They include herbicides, fungicides, insecticides and various other substances used to control pests (U.S.EPA2012).[1] Insecticide dichlorvos, an organophosphate compound produces toxicity primarily by irreversible inhibition of the acetylcholinesterase (AChE) enzyme that stops the function of acetylcholine (ACh), a neurotransmitter, at the synapses. Moreover, the anti-AChE insecticides are known to affect the reproductive system in both female and male. The persons who are exposed to these insecticides can be affected on pregnancy, mentality and health (Gupta RC, 2011).[2] At present, because of the claimed property of rapidly degradation and less remaining in the nature,organophosphate insecticide is the most commonly used around the world although it is highly toxic (Pesticides News 1995).[3]

In 2002, Asmatullah, studied embryotixicity of methylparathion, anorganophosphate insecticide, in developing chick. There were severe gross malformations such as microcephaly, anophthalmia, micromelia, twisted spinal cord and ectopia cordis (Asmatullah, Salah E 2002).[4] In 2004, Szabo *et al* studied the effects of dichlorvos in the chick embryos which were injected directly into the air chamber on day 0 and evaluated on day 19 of incubation.

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The rate of mortality was significantly increased with doses.[3] Dichlorvos has many adverse effects to various species, including human, especially a pregnant women, as dichlorvos can cause the adverse effects to baby such as malformations, or spontaneous abortion (Adhikari J, 2011).[5] However, there is no any study to confirm the teratogenicity, toxicity and also the pattern of disability produced by dichlorvos. This study was conducted to demonstrate the teratogenic effects of dichlorvos to the embryo by using chick embryo as an animal model and compared the results with the age related stages of human embryo. Chick embryos are the most suitable animal models for teratogenic screening study as its development stages were proved to be comparable to humans (Hamburger V, Hamilton HL, 1992)[5] and there were plenty of databases of comparison. Moreover, chicken eggs are easily available, chick embryos have short gestation and are also inexpensive and easily manipulated. Animal studies are important as they can lead to the mechanisms of teratogenicity and because when such an agent causes similar patterns of anomalies in several species, human teratogens should also be suspected.

MATERIALS AND METHODS

Ethics statement

According to German animal care guidelines, no IACUC approval was necessary to perform this experiment. According to the local guidelines, only experiment with chick embryos of E18 and more need IACUC approval. However, this study used earlier stages of chick embryos, day 3-day 11.

ACUCGuideline

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 11.

Experiment protocol

150 fertilized white leghorn hen (*Gallus domesticus*) eggs were purchased from the Institute of Suwanvajokkasikij Animal Research and Development, Department of Animal Science, Faculty of Agriculture, Kasetsart University. The eggs were randomly divided into 5 groups, 30 each and treated with 0.1 ml of 0.5%. 1% and normal saline at 21 h of incubation into the yolk sac and further incubated until day 3, 6 and 11 of development. All embryos from each stage were recorded the number of deaths and survivals as well as the normal and the abnormal eggs that could be visible externally. The viability was indicated by the heart beating and the blood circulation. The day 3 embryos were processed for total mount and serial section, the day 6 embryos were processed for the serial section and the day 11 embryos were processed for the cartilage and bone staining.

The total mount of day 3 embryos were stained with Mayer's Camine for 30 min and dehydrated with graded series of alcohol for 5 mins each change. Then the specimens were cleared and mounted. The serial sections of day 3 and day 6 were stained with hematoxylin and eosin stains. The cartilage and bone staining of the day 11 were performed by removing the skin and visceral organs and fixed in 4% paraformaldehyde before washing and dehydration with absolute alcohol then transferred to acetone for 24 hr before staining with alcian blue and alizarin red for 3-4 days each at 37°C, then rinsed and transferred to 1% KOH and glycerol. The crown-rump length (CRL), beak, mandible, and other long bones were measured by the red color of the alizarin red, then these were compared with the control and tested statistically using Statistical Package for the Social Science (SPSS) by one way analysis of Variance (ANOVA), the data represented by mean ± SD with significant difference at $p<0.05$.

RESULTS

The effects of dichlorvos on days 3, 6 and 11 chick embryos

The mortality rate

The mortality and survival rates of day 3, 6 and 11 chick embryos were observed by the heart beating and blood circulation. These stages can be compared to the 4th, 5th and 8th weeks of human embryo after fertilization, respectively. All control groups showed 100% survival while the experiment groups showed increases in mortality rates as the concentrations of dichlorvos increased and also corresponding to the advancing age. The highest mortality rate was 74.43% of day 11 in the 1% concentration group.

TABLE 1. Mortality and survival rates of the day 3 chick embryo after treated with different concentrations of dichlorvos and compared with the control which was treated with NSS.

Concentration (%) Control (NSS) (n=7)	Mortality rate (%) 0 (n=0)	Survival rate (%) 100 (n=7)
0.5 (n=14)	28.57 (n=4)	71.43 (n=10)
1 (n=13)	38.46 (n=5)	61.54 (n=8)

*NSS is normal saline solution

TABLE 2. Mortality and survival rates of the day 6 chick embryo after treated with different concentrations of dichlorvos and compared with the control.

Concentration (%) Control (NSS) (n=5)	Mortality rate (%) 0 (n=0)	Survival rate (%) 100 (n=5)
0.5 (n=12)	41.67 (n=5)	58.33 (n=7)
1 (n=13)	46.15 (n=6)	53.85 (n=7)

TABLE 3. Mortality and survival rates of the day 11 chick embryo after treated with different concentrations of dichlorvos and compared with control.

Concentration (%) Control (NSS) (n=4)	Mortality rate (%) 0 (n=0)	Survival rate (%) 100 (n=4)
0.5 (n=7)	57.14 (n=4)	42.86 (n=3)
1 (n=7)	71.43 (n=5)	28.57 (n=2)

Statistic analysis of the body measurement data of the day 6 chick embryo

Measurements of the day 6 chick embryo included body weight, crown rump length (CRL), width of head, width of eye, upper limb and lower limb. All parameters were processed for statistical analysis shown in Table 4.

TABLE 4. Statistic analysis of the body measurements of the day 6 chick embryos after treated with different concentrations of dichlorvos and compared with control.

Group	Control	0.5%	1%
Body weight (g±SE)	0.29±0.02	0.23±0.02	0.22±0.03*
CRL (mm±SE)	12.85±0.29	11.59±0.44	7.69±2.03*
Width of head (mm±SE)	6.60±0.28	5.73±0.29	3.64±0.98*
Width of eye (mm±SE)	3.52±0.07	3.31±0.17	1.55±0.48*
Upper limb (mm±SE)	3.42±0.08	3.25±0.11	1.69±0.50*
Lower limb (mm±SE)	3.83±0.05	3.42±0.13	1.81±0.55*

Data are the mean±standard error. *Statistic mean difference showed significant ($p<0.05$) by one way ANOVAs.

Statistical analysis data of the day 6 chick embryo showed the body weight, the CRL, the width of head, the width of eye, the upper limb and the lower limb of the 1% dichlorvos-treated groups were significantly lower than the control group while 0.5% dichlorvos-treated groups showed no significant difference when compared with the control.

Total mount of the day 3 chick embryo

The effects of dichlorvos on day 3 chick embryo were examined and compared with the control. In the control, the total mount of the day 3 chick embryo can be referred to as Hamburger-Hammilton stage 18 which had approximately 36 somites. The brain showed 5 secondary brain vesicles which comprised telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. The eye comprised optic cup and lens vesicle at the diencephalic region and the otocyst at the myelencephalon. Anterior and posterior limb buds were presented (Fig 1A). Figs 1B and 1C were 0.5% and 1% dichlorvos treated groups which showed several abnormalities and growth retardation. The brain showed three primary brain vesicles with only cephalic flexure with very small primordial eye which is referred to as microphthalmia. The heart loops were looser with distended heart chambers. Branchial arches showed only one arch. Neural tube appeared as two dense lines which were not parallel. The somites appeared only on the upper part of the body. There was absence of the limb bud and tail fold with the opening of the posterior neuropores. The 1% dichlorvos group showed caudal degeneration. One of the 0.5% and 1% of dichlorvos treated groups showed similar abnormalities, conjoined twins, appeared as the lower part of the body bifurcated (Fig 1D and 1E) and also showed brain retardation and abnormality of eye (microphthalmia and anophthalmia) and heart development. All primordial eyes were extremely small and the heart loop showed abnormal looping, and the brain was under developed.

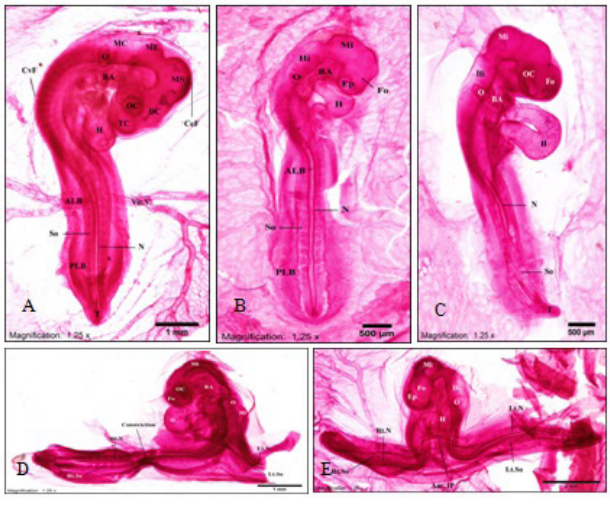

Fig 1. Total mount of the control (A), 0.5% (B) and 1% (C) dichlorvos treated groups, showed normal development in the control and growth retardation in all the experiment groups with severe underdevelopment of brain and the flexures. The eyes were smaller than the control which referred to as microphthalmia. The heart loops were looser U-shaped and distended. The somites were not extend to the caudal end with the absent of limb bud and tail fold. The 1% dichlorvos treated group showed caudal degeneration from the middle of the body. ID and 1E showed similar interesting congenital abnormality, bifurcation of the body or referred to as the conjoined twins, and also showed the retardation of brain formation, microphthalmia, anophthalmia, looser of heart looping (TC) telencephalon, (DC) diencephalon, (MS) mesencephalon, (ME) metencephalon, (MC) myelencephalon, (OC) optic cup, (O) otocyst, (BA) branchial arch, (H) heart, (CeF) cephalic flexure, (CvF) cervical flexure, (So) somite, (N) neural tube, (ALB) anterior limb bud, (PLB) posterior limb bud, (T) tail fold, and (VitV) vitelline vein.

The serial section of the day 3 and day 6 chick embryo

The abnormalities of the eye

The serial section of the day 3 chick embryo in 0.5% (2B) and 1% (2C) dichlorvos treated group (Fig 2) showed severe retardation of the eye when compared with control group (2A). In the control (2A) the optic cup comprised inner nervous layer and outer pigment layer which were closely contacted to each other. The lens placode was completely fused and detached from the surface ectoderm. The lens comprised the anterior lens epithelium and posterior lens fiber which was elongated to obliterate the lens cavity. In the experimental groups, the lens vesicle presented clearly the lens pit (2B and 2C) which indicated that the lens placode did not undergo a process of double fusion to become the lens vesicle. Posterior lens fiber showed no elongation and the lens cavity was widely opened. The optic cup was smaller than the control and showed severe retardation, the intraretinal space was widely separated when compared with the control.

The effects to the eye of the day 6 chick embryo (Fig 2D, 2E and 2F), the optic cup and lens of the control group illustrated in Fig 2D showed oval-shaped double-walled optic cup comprised of 2 layers; outer pigment layer and inner nervous layer. Both layers were outer thin layer and inner thick layer separated by obliterated intraretinal space. The lens vesicle was an oval-shaped, and comprised anterior lens epithelium which was a cuboidal epithelium and posterior lens fiber which was extremely elongated as columnar epithelium. The lens cavity was obliterated in the day 6 chick embryos. The

cornea developed from the mesenchyme and surface ectoderm superficial to the lens. The anterior chamber located between cornea and lens while the vitreous chamber located posterior to the lens. Most of the day 6 chick embryos showed more or less retardation of eye development such as smaller, irregular-shaped optic cup and lens vesicle when compared with the controls. There were wide separation of the intraretinal space and underdeveloped lens (Fig 2E).

One of the 1% dichlorvos group showed severe abnormal eye development, which represented only primitive eye primordial, optic vesicle, and there was no invagination to form double walled optic cup. However, distal part of optic vesicle induced the surface ectoderm to form small lens detached from the surface ectoderm (Fig 2F). This optic primordium originated from the diencephalon and the distal end expanded to form optic vesicle. The right optic vesicle induced the surface ectoderm to form successively a small lens before the development ceased (Fig 2F). The left optic vesicle failed to induce the surface ectoderm to form lens (Fig 4A).

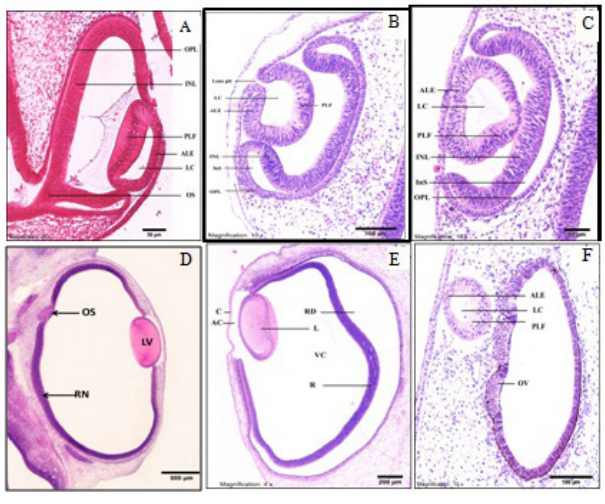

Fig 2. The serial section of the day 3 chick embryo in the control (2A) and the 0.5% (2B) and the 1% (2C) of dichlorvos treated groups: In the control (2A) the optic cup comprised outer pigment layer and inner nervous layer which contacted each other so the intraretinal space was obliterated while in 2B and 2C there were wide separation of intraretinal space. The lens of the control was completely fused and the posterior lens fiber was elongated while the lens placodes of 2B and 2C were incompletely fused left the opening of lens pit. (OPL) outer pigment layer, (INL) inner nervous layer, (PLF) posterior lens fiber, (ALE) anterior lens epithelium, (LC) lens cavity, and (OS) optic stalk, (OC) optic cup, (LV) lens vesicle, (DC) diencephalon, (OPL) outer pigment layer, (InS) Intraretinal space, (INL) inner nervous layer, (PLF) posterior lens fiber, (ALE) anterior lens epithelium, and (LC) lens cavity. The optic cup and lens of the day 6 chick embryos of the control (2D), 0.5% (2E) and 1% (2F) of dichlorvos treated groups, 2E showed retardation of eye development i.e. smaller optic cup, the wide separation of intraretinal space. 2F showed malformation of eye development, no optic cup formed and there was only rudimentary structure of eye primordial. C, cornea, AC, anterior chamber, L lens, RD, retinal detachment, VC, vitreous chamber, R, retina, ALE, anterior lens epithelium, PLF, posterior Lens fiber, LC, lens cavity, OV, optic vesicle.

The multiple abnormalities

Several embryos of both treated groups showed multiple abnormalities. The one with brain anomaly might show anomaly of the internal ear and eye together with the heart and viscerae. The 2 figures below (Figs 3 and Fig 4) showed two types of multiple abnormalities of brain, ear, eye, heart, liver, mesonephros and body wall with trunk degeneration (Figs 3 and 4).

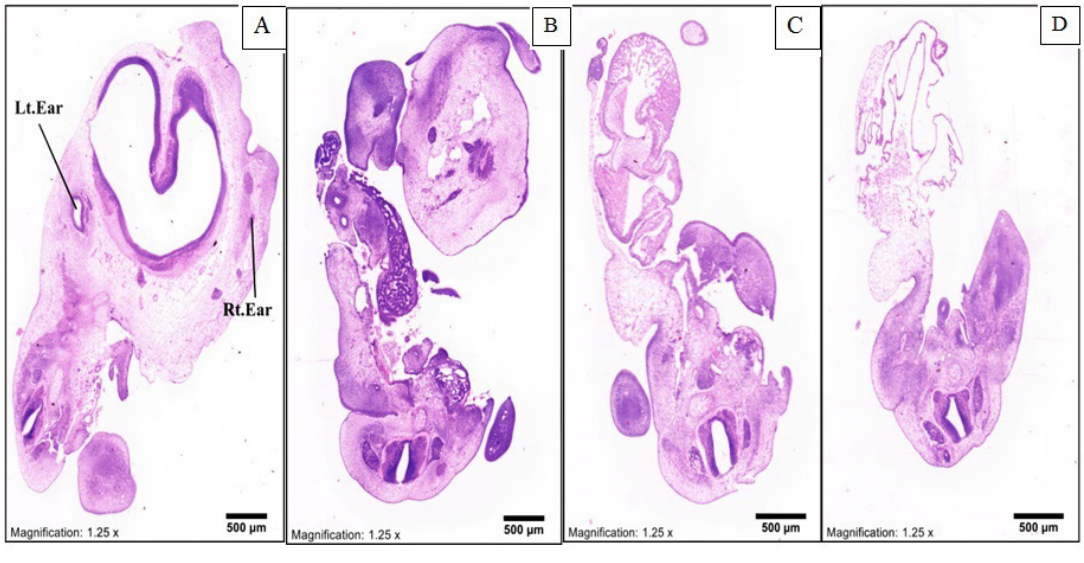

Fig 3. The series of serial section of one of the 6 chick embryo of 1% dichlorvos-treated group, showed very severe retardation and abnormality of the development. The structures in the embryo were dislocated and underdeveloped such as heart, liver, and urogenital system. The ear was developed only at the left side. At the right side, the ear was obliterated and ceased. The eye did not develop in this embryo bilaterally as a result in the bilateral anophthalmia (A). B showed small and underdeveloped liver (L) situated outside the body (ectopia viscerae), C showed heart (H) with ectopia cordis, D showed degenerated trunk. Me, mesonephros.

The 1% of dichlorvos treated group showed multiple abnormalities in the same embryo. Figs 3 and 4 were 2 sets of serial section of 2 embryos of the 1% dichlorvos treated group. Fig 3A cut through the level of myelencephalon where the otocyst formed. This embryo showed twisted and irregular brain and there was an absence of left side optic primordium, while the right otocyst developed adjacent to the myelencephalon. This embryo also showed absence of both primordial eyes as well as unidentified diencephalon and this was the so call bilateral anophthalmia. When tracing caudally to the trunk, this embryo showed a large opening of thoracoabdominal wall which caused the liver and all gut structures to be situated outside the body which was called ectopia viscerae. The mesonephros was rudimentarily situated at the posterior body wall. Fig 3C showed the open body wall and the heart situated outside the body cavity which was called ectopia cordis. The spine showed irregular-shape with the widening of the roof plate. Fig 3D showed degeneration of the lower trunk. This embryo of 1% dichlorvos treated showed absence of eyes and ears with irregularities of brain and spinal cord, ectopia viscerae with small liver and short digestive tract, ectopia cordis and small mesonephros.

Fig 4A showed normal development of otocyst at the level of myelencephalon, while ventrally showed abnormality of both eyes. Abnormality of both optic vesicles failed to form double walled optic cups, and the left side induced the surface ectoderm to form a small lens while the right side failed to form lens (bilateral anophthalmia). Fig 4B showed the heart situated outside the thoracic cavity (ectopia cordis). Figs 4C and 4D showed small and underdeveloped liver and mesonephros.

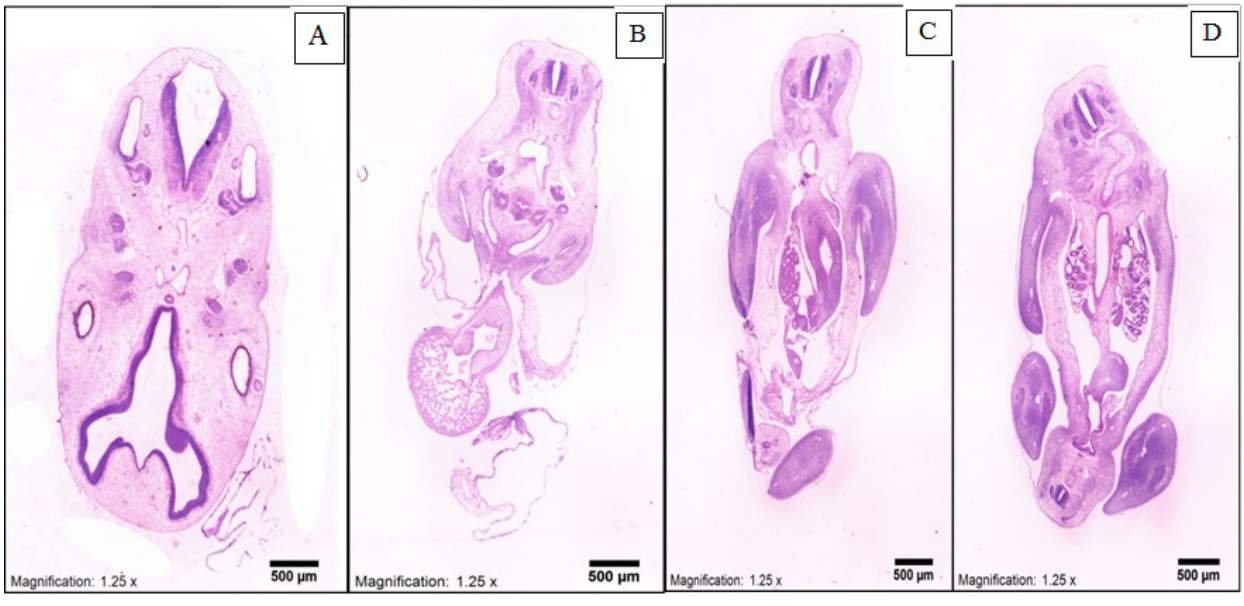

Fig 4. Another series of serial section of the day 6 chick embryo of 1% dichlorvos-treated group showed very severe retardation and abnormality of the development. The structures in this embryo were dislocated and underdeveloped such as heart, liver, and urogenital system. The eye did not develop to the final stage but ceased at the stage of optic vesicle (A), result in the bilateral microphthalmia. Body wall was absent with ectopia cordis (B) while other viscerae developed inside the body but showed small and underdeveloped liver (C) and mesonephros (D). D, diencephalon, OC, optic cup, H, heart, L, liver, Me, mesonephros.

The effects of dichlorvos on the day 11 chick embryo

General features of the 11th day chick embryo

The 11th day chick embryo of the control group shown in Fig 5A was about the Hamburger Hamilton stage 35. Length of beak from anterior edge of nostril to tip of bill could be measured.The length of the third toe was measured to represent the length of digit of the foot. Scales were overlapping on inferior as well as superior surfaces of leg. The feather was well developed all over the body. Fig 5B was the 0.5% of dichlorvos treated group showed smaller and under developed external appearance. It showed shorter beak and smaller eye, shorter upper and lower limbs, and no feather formation. Fig 5C was the 1% of dichlorvos treated group which showed smallest of the three with lower limb and trunk degenerated. Anterior body opened and the viscerae laid outside the body cavity.

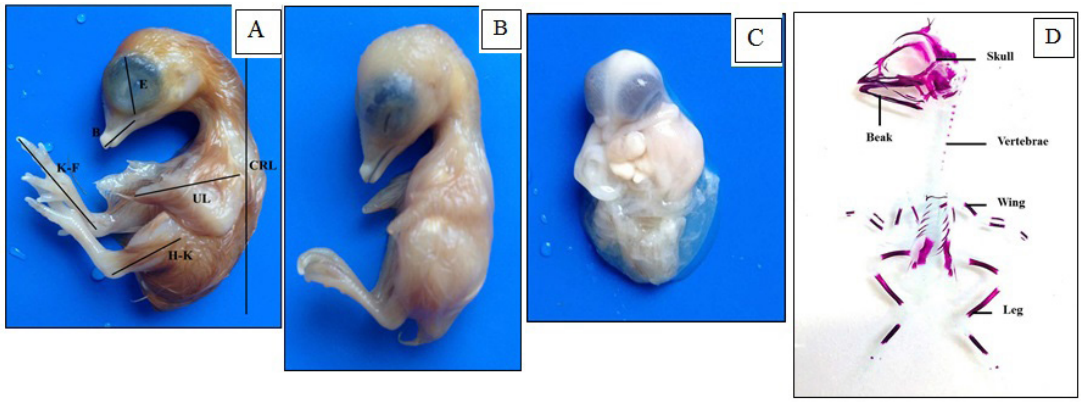

Fig 5. The external appearance of the day 11 chick embryos of the control (A), 0.5% (B) and 1% (C) of dichlovos treated groups, A showed normal development. B showed smaller and no feather germ. C were severely small with under developped limbs and tail. C also showed no anterior body wall with ectopia viscerae. E, eye, B, beak, CRL, crown rump length, UL, upper limb, HK, hip knee, KF. Knee foot, EV, ectopia viscerae. D was the alcian blue and alizali red staining of the cartilages and bones. The red area of bone were measured and test statistically in Table 5.

The alcian blue and alizarin red staining

This procedure was performed to observe the skeletal malformation. The experimental group of the day 11 showed a striking mortality rate in which very few embryos could survive up to 11 days. The survival embryos showed normal appearances when compared with the control and when they were processed for alcian blue and alizarin red staining, the bone and cartilage showed no significant difference as shown in Fig 5D. The ossification centers were stained red while cartilage stained blue. The lengths of the ossification center of each bone were measured by the stereomicroscope, the data were then evaluated for statistical difference between the control, 0.5% and 1% dichlorvos treated groups by mean-+standard error at $p<0.05$ by one way ANOVAs as shown in Table 5.

TABLE 5. Statistic analysis of the bone measurement data of the 11th day chick embryo after treated with different concentrations of dichlorvos and compared with the control.

Group	Control	0.5%	1%
CRL (mm±SE)	45.11±3.20	39.19±3.46	39.51±2.19
Beak (mm±SE)	7.87±0.10	4.73±0.29*	5.54±0.20*
Mandible (mm±SE)	14.58±0.04	12.90±0.17	13.41±0.25
A-P (mm±SE)	21.88±0.13	19.11±0.40*	18.58±0.18*
Lt. humerus (mm±SE)	4.83±0.16	4.26±0.41	4.00±0.23
Rt. humerus (mm±SE)	5.09±0.03	3.94±0.32*	4.04±0.00*
Lt. radius (mm±SE)	4.87±0.09	4.00±0.24	3.64±0.25*
Rt. radius (mm±SE)	5.41±0.03	3.89±0.12*	3.72±0.13*
Lt. ulna (mm±SE)	4.80±0.06	4.13±0.21	3.81±0.16*
Rt. ulna (mm±SE)	4.82±0.05	3.96±0.20*	3.85±0.00*
Lt. metacarpus (mm±SE)	3.33±0.02	2.59±0.16*	2.48±0.02*
Rt. metacarpus (mm±SE)	3.29±0.14	2.66±0.10*	2.69±0.14*
Lt. femur (mm±SE)	7.81±0.03	6.29±0.36	6.06±0.08
Rt. femur (mm±SE)	7.77±0.03	6.07±0.31*	5.84±0.25*
Lt. tibia (mm±SE)	10.44±0.01	8.18±0.40*	7.90±0.02*
Rt. tibia (mm±SE)	10.29±0.04	8.25±0.51*	7.92±0.19*
Lt. metatarsus (mm±SE)	7.26±0.06	5.67±0.33*	5.01±0.44*
Rt. metatarsus (mm±SE)	7.12±0.10	5.41±0.45*	5.02±0.45*
Lt. digit of foot (mm±SE)	10.07±0.05	8.39±0.12	6.92±1.41*
Rt. digit of foot (mm±SE)	10.08±0.04	7.62±0.65	7.80±0.29

Data were the mean±standard error. *Statistic mean difference shows significant ($p<0.05$) by one way ANOVAs.

Statistical analysis of the length of ossification centers of bones which were measured by stereomicroscopy indicated that ossification centers of the experimental group showed significantly lesser bone formation except for the length of mandible, humerus, femur and right digit of foot which showed slightly less, but non- significant when compared with the control. Moreover the 0.5% dichlorvos treated group showed non-significant difference of the length of the left ulna, left radius and left digit when compared with the control group (Table 5).

DISCUSSION

The majority of pesticides do not specifically target the pest only and during their application they also affect non-targetted plants and animals, such as wild bees, butterflies, birds, frogs, fishes, etcetera. Repeated application leads to loss of biodiversity. Many pesticides are not easily degradable, they persist in soil, leach to groundwater and surface water and contaminate the wider environment. Depending on their chemical properties they can enter the organism, bioaccumulate in food chains and consequently influence also human health. For this reason, no one can ensure that such chemical residues were the major causes of the congenital abnormalities which occurred in the babies born in the pesticide treated area.

Dichlorvos (DDVP), the organophosphate (OP) insecticide, is widely used to control pests. Several studies reported that dichlorvos irreversibly inhibited the function of the acetylcholinesterase (AChE), and acetylcholine (ACh) was then uncontrolled and accumulated at the synapse, resulting in increase of the cholinergic receptor activity.[6] The cholinergic receptors comprised muscarinic and nicotinic receptors. The ACh affected the muscarinic receptors of the eye, heart, GI tract, glands, and respiratory system and the nicotinic receptor of the muscle cell. Moreover, the acetylcholine affected to the non-neuronal cell activity[7,8] which presents on the epithelial cell[9] endothelial cell,[10] and tendons[11] including those involved with the cell proliferation and differentiation.[12] In addition, acetylcholinesterase activity is very important to the embryonic development especially the gastrulation and neurulation steps.[13,14,15] Furthermore, the effects of abnormal AChE activity were similar to the effects of the abnormal ACh activity especially cell proliferation, cell migration and differentiation.[16] The abnormality of the embryo could be observed when the function of the acetylcholinesterase was irregular. Although, DDVP is known to be used for several functions, it is very dangerous for the embryo such as anomaly of morphology, visceral abnormality, and the severe congenital malformation.

This result indicated that the mortality rates increased when the concentration of the dichlorvos increased, and also the duration of exposure was longer. This result supported the previous study which reported the adverse effect of dichlorvos to the embryo which depended on the size and species of animal, the concentration of dichlorvos and duration of exposure.[16,17]

Dichlorvos caused congenital abnormalities in chick embryos in 3 categories, the growth retardation, the malformations and the embryonic death which were predicted to cause the same results in contaminated humans. The mortality resembled spontaneous abortion, and growth retardation indicated the intrauterine growth retardation in the pregnant woman and the malformation indicated the human congenital abnormalities. This study illustrated the abnormal development of brain, internal ear, anophthalmia, microphthalmia, irregular heart looping, ectopia cordis, ectopia viscerae, small and underdeveloped liver and mesonephros and caudal degeneration, some of which agree with the previous reports.[3-5]

There were bifurcations of the caudal part of the bodies in two dichlorvos-treated groups. From the previous study[18], they explained about the abnormality of the twinned embryo which produced duplicated posterior. The embryo was formed by bisecting of the primitive streak. Then, both primitive streaks developed and the anterior end was coiled as the head. The concept of the result in this study was adjusted from their idea and the idea from the previous study about the mechanism of dichlorvos toxicity. Dichlorvos is an organophosphate insecticide which inhibits the function of acetylcholinesterase permanently. The AChE activity began from the gastrula stage (H.H stage 5). At the gastrula stage, the AChE was located in the area opaca. During the first neurulation, most AChE reaction was found in the notochord and in the portion of the neural fold.[5-7] Thus, AChE was collected in the notochord. Dichlorvos affected on the notochord at the point caudal to anterior intestinal portal in the late gastrulation period at 21 hours of incubation. The notochord broke and the caudal part of the notochord bifurcated. Each notochord developed and induced neuroectoderm to be a neural tube. In the early stage, cephalic part of neural tube developed into primary brain vesicle which had only one in this embryo. Two caudal parts of neural tube which developed from the bifurcation of caudal part of the notochord developed into two spinal cords. Thus, this embryo had only one head and two bodies. This idea was explained by diagram as shown (Fig 6).

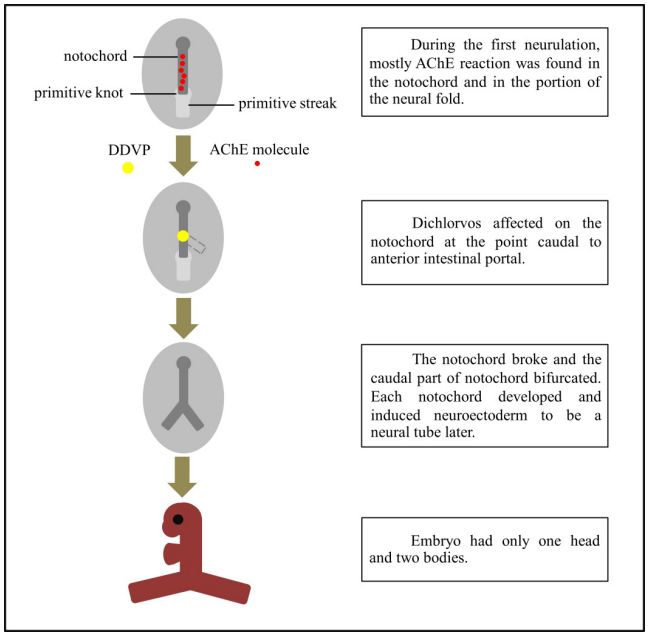

Fig 6. The idea of bifurcation at caudal part of notochord led to develop two bodies in the abnormal day 3 embryos of both experiment groups of this study.

CONCLUSION

Dichlorvos (DDVP) was reported in this experiment to cause several malformations in chick embryos which might cause the same effects to human embryos of the age related period. DDVP caused abnormalities to brain, ear, eye, heart, liver, mesonephros, gonad and anterior abdominal wall which might relate to multiple gene deformities. In the area using the pesticides, DDVP might produce harmful effects to humans in the same way as occurred in animal models. Therefore, there should be strong measure to prevent the toxicity such as the limit of using the chemical, the users themselves must have protective equipment and clothing and enough knowledge. In particular, pregnant women should avoid contamination with the chemicals especially in the first trimester of pregnancy.

REFERENCES

1. United States Environmental Protection Agency (U.S. EPA). Pesticides. 2012 Sep.[cited 2015 Jan. 7] Available at: http://www.epa.gov/pesticides/
2. Gupta RC, Malik JK, Milatovic D. Chapter 37- Organophosphate and carbamate pesticides, Reproductive and Developmental Toxicology (pp. 471-86). Kentucky, USA: Elsevier, 2011.
3. Asmatullah, Salah E, Andleeb S, Iqbal A. Embryotoxicity of methlyparathion in developing chick. Punjab University J Zoology 2002; 17: 28-34.
4. Szabo R, Keseru M, Fejes S, Budai P, Juhasz E, Pongracz A. Toxicity of a dichlorvos containing insecticide formulation and an atrazine containing herbicide formulation in chicken embryos after individual administration. Commun Agric Appl Biol Sci. 2004;69(4):811-4.
5. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. 1951. Dev Dyn. 1992;195(4): 231-72.
6. Wessler I, Kirkpatrick CJ. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br J Pharmacol. 2008; 154(8):1558-71.
7. Wessler I, Kilbinger H, Bittinger F, Unger R, Kirkpatrick CJ. The non-neuronal cholinergic system in humans: expression, function and pathophysiology. Life Sci. 2003;72(18-19):2055-61.
8. Klapproth H, Reinheimer T, Metzen J, Munch M, Bittinger F, Kirkpatrick CJ, et al. Non-neuronal acetylcholine, a signaling molecule synthesized by surface cells of rat and man. Naunun-Schmiedeberg's Arch Pharmacol 1997; 355: 515-23.
9. Kirkpatrick CJ, Bittinger F, Nozadze K, Wessler I. Expression and function of the non-neuronal cholinergic system in endothelial cells. Life Sci. 2003; 72: 2111-16.
10. Danielson P, Andersson G, Alfredson H, Forsgren S. Extensive expression of markers for acetylcholine systhesis and of M2 receptors in tenocytes in therapy-resistant chronic painful patellar tendon tendinosis - a pilot study. Life Sci. 2007; 80: 2235-8.
11. Resende RR, Alves AS, Britto LRG, Ulrich H. Role of acetylcholine receptors in proliferation and differentiation of P19 embryonal carcinoma cells. Exp Cell Res. 2008;314(7):1429-43.
12. Caterina M, Agati PD, Cangialosi MV, Mansueto V, Puccia E. Further data on expression of acetylcholinesterase during oogenesis and embryonic development of Cionaintestinalis and Ascidia malaca (Ascidiacea, Urochordata): a biochemical and histochemical study. Caryologia 2006; 59(4): 388-94.
13. Sandra FM, Gunther SS. Appearance and localization of acetylcholinesterase in embryos of the leech Helobdella Triserialis. J Neurosci. 1981;1(8):901-7.
14. Laasberg T, Neuman T. Changes in the acetylcholinesterase and choline acetyltransferase activities in the early development of the chick embryo. Roux's Arch Developmental Biology 1985; 194: 306-10.
15. Vidal CJ. Expression of cholinesterases in brain and non-brain tumours. Chem Biol Interact. 2005;157-158:227-32.
16. Ural MS, Calta M. Acute toxicity of dichlorvos (DDVP) to Fingerling Mirror Carp, Cyprinuscarpio L. Bull Environ Contam Toxicol. 2005;75(2):368-73.
17. Ural MS, Koprucu SS. Acute toxicity of dichlorvos on fingerling European catfish, Silurus glanis. Bull Environ Contam Toxicol. 2006;76(5):871-6.
18. Bellairs R, Osmond M. The atlas of chick development, second edition. London, UK: Elsevier, 2005.