

The Teratogenic Effects of Glyphosate Based Herbicide (GBH) on the Development of Chick Embryos

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ABSTRACT

Objective: This study was conducted to investigate the teratogenic effects of GBH by using chick embryo as an animal model.

Methods: The equal volume of 0.1 ml of 0.01%, 0.05%, 0.3%, and 0.5% w/v glyphosate solution were injected into yolk sacs of fertilized White Leghorn eggs at 21 h of incubation and repeated at the volume of 0.05 ml on the 3rd day of incubation. The embryos were observed for abnormalities on day 3, 6 and 10 of incubation.

Results: The results showed that the mortality percentages increased as the concentration of glyphosate increased. Day 3 chick embryos showed retardation of development and several abnormalities, for instance, the irregular shape of the brain vesicles with an opening of anterior neuropore, small eye primordia with the optic cup and lens vesicle retardation, looser of the heart looping with dilated lumen, lesser number of branchial arches, absent of limb bud or tail fold. Day 6 chick embryos showed severe retardation of several organs. Microphthalmia, anophthalmia, ectopia cordis and ectopic viscerae were observed in day 6 chick embryos. On day 10, most embryos died earlier and living embryos showed normal external features but delayed ossifications which were significantly different from the control ($P < 0.05$).

Conclusion: Glyphosate was toxic to chick embryos which directly caused mortality and was also a powerful teratogen which caused growth retardation and malformations on day 3 and day 6 and skeletal alteration on day 10. The effects were predicted to occur in other embryos including humans. Pregnant women should avoid contamination with GBH especially in the first trimester of pregnancy.

Keywords: Glyphosate; ROUNDUP; teratogen; chick embryo (Siriraj Med J 2018;70: 419-428)

INTRODUCTION

The broad spectrum herbicide glyphosate is widely used in agriculture worldwide particularly in association with genetically modified organisms (GMO), but has possible adverse effects on the environment and on human health. Reports of neural defects and craniofacial malformations from regions where glyphosate based herbicides (GBH) are used led us to undertake an embryological approach to explore the effects of low doses of glyphosate on development. Glyphosate has contaminated land, water, air and our food chain since its use with the genetically modified (GM) glyphosate

tolerant crops. The generation of plants tolerant to glyphosate allows farmers to apply glyphosate while crops are growing, theoretically killing every plant, but the crop. The consequence is that crops now contain residual levels, directly exposing consumers. Not only that, glyphosate tolerant crops accumulate the herbicide and transport it to the roots, excreting it into the root zone of the soil, harming the next crop to be planted in the same field.

Glyphosate (N-(phosphonomethyl) glycine) is an organophosphorated, broad-spectrum, non-selective and post-emergence herbicide. It kills unwanted weeds by

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inhibiting the synthesis of aromatic amino acids necessary for protein formation in plants.¹⁻⁴ Glyphosate in form of isopropylamine salt is an active ingredient of Roundup®, herbicide's trade name under Monsanto's company since the 1970s.⁷ Glyphosate is believed to be less toxic than other pesticides and no potential to pose a health risk to humans,⁸ with low toxicity and environmental friendliness. However, several studies showed that glyphosate may not be as safe as previously thought. It has potential adverse health effects to humans as it may induce apoptosis and necrosis in human umbilical cord, embryonic, and placental cells.⁹ Glyphosate acts as an endocrine disruptor by inhibiting activity of enzyme CYP19, an essential component of cytochrome P450 aromatase. CYP19 is responsible for the irreversible conversion of androgens into estrogens.¹⁰ After studying the epididymal region of drakes, the animal model, showed the glyphosate-roundup affected the expression of the steroidogenic acute regulatory protein (StAR) protein, which mediated the initial step of steroid hormone synthesis.¹¹ Furthermore, several studies stated glyphosate acted as a teratogen.¹² The malformations were microcephaly, microphthalmia, cyclopia and craniofacial malformations,¹³ of the fetal skeleton in Wistar rats.¹³ Residual glyphosate was found in many organs of malformed offspring pigs. The piglets showed many abnormalities such as ear atrophy, leg atrophy, spinal and cranial malformations, elephant tongue, female piglet with testes, and piglets born alive having short legs and one eye. Residual glyphosate in GMO foods may effect to pig embryos via pregnant pigs which were fed with GMO foods.¹⁴

This experiment was conducted to study the teratogenic effects of glyphosate based herbicide (Roundup®) on development *in ovo* chick embryos. The serial sections of chick embryos at different stages and the development of bone and cartilage of chick embryos were studied by comparing to normal development. The results of this work may be consistent with the well-known evidences which have occurred to the babies born in the area using this herbicide and come to the conclusion that GBH is a teratogen, so its use should be limited.

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.

ACUC Guideline

Use and Euthanasia Procedures of Chicken/Avian Embryo

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.

Experiment protocol

Glyphosate based herbicide preparation

Roundup® herbicide, a commercial product of Monsanto Company, Malaysia, which consisted of 48% W/V SL Isopropylamine salt with unknown surfactants, was used to prepare the stock solution. 1 ml of Roundup® herbicide was diluted in 47 ml of normal saline. The working solutions of 0.5%, 0.3%, 0.05% and 0.01% w/v Roundup® were further diluted with normal saline solution.

Animal treatment

The 140 white leghorn hen (*Gallus domesticus*) eggs were obtained from Luang Suwan Vajokkasikij farm, Department of Animal Science, Faculty of Agriculture, Kasetsart University. The eggs were divided into 5 groups, one control and four experimental groups. At 21 hours after incubation, eggs were drilled with dental driller to get a hole at the blunt end and injected with normal saline solution (control group), 0.01%, 0.05%, 0.3% and 0.5% w/v glyphosate (experimental groups), at the equal volume of 0.1 ml into the yolk sac by sterile needle. The holes were sealed with paraffin and further incubated until day 3. Embryos from each group were collected. The remaining were repeated with corresponding dosages at the volume of 0.05 ml to yolk sac and further incubated until days 6 and 10. Day 3 embryos were fixed in dietrich's FAA fixative for about 4 hours, recorded viability and processed for total mount and serial sections. Day 6 embryos were fixed in dietrich's FAA fixative for one day and recorded viability, weight, morphology, and measured external features for instance, crown-rump length (CRL), limb length, and width of head, then they were processed for serial sections. Day 10 embryos were recorded viability, morphology, weight, and measured external features such as crown-rump length (CRL), beak, limb length and width of head then they were deskinning and eviscerated for alcian blue and alizarin red staining transparency to study the cartilage and bone abnormalities.

RESULTS

The survival and mortality rates of day 3 chick embryo

The living embryos were indicated by heart beating and blood circulation. The survival rate of day 3 chick embryos of the control group was 100% while the highest dose of glyphosate injected to group, 0.5% w/v, was 0%. The 0.01%, 0.05% and 0.3% w/v groups showed that the survival rates were 92.86%, 85.71% and 33.33%, respectively, which indicated that the survival rate decreased as the concentration increased.

The mortality and survival rates day 6 chick embryo

The survival rates of the day 6 chick embryos were 100%, 87.50%, 85.71%, 41.67% and 14.29% in the control, 0.01% w/v, 0.05% w/v, 0.3% w/v and 0.5% w/v glyphosate, respectively. The living embryos were observed by the heart beating and blood circulation. The data indicated that the increasing glyphosate concentration affected to the viability as the survival percentage was decreased.

The survival and mortality rates of day 10 chick embryo

Table 3 showed the viability was 100% in the control group injected with NSS, and 42.86% and 50% in the experimental groups injected with 0.01% and 0.05% GBH, respectively. Both 0.3% and 0.5% glyphosate injected groups showed no survival. The surviving embryos were indicated by the blood circulation and heart beating.

The total mount of day 3 chick embryo

The normal day 3 chick embryo by total mount preparation

On day 3, the normal chick embryo developed to the stage 18 of Hamburger and Hamilton.¹⁵ The control group showed normal embryonic development which comprised 36 pairs of somite. The brain developed to the secondary brain vesicles with 5 components, telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. The total mount showed head fold rotation about 2/3 to the right side and illustrated the

TABLE 1. The survival and mortality rates of day 3 chick embryos after injected with 4 different concentrations of GBH and normal saline solution.

Group	% Survival	% Mortality
Control (n=8)	100	0
0.01% (n=14)	92.86	7.14
0.05% (n=14)	85.71	14.29
0.3% (n=12)	33.33	66.67
0.5% (n=8)	0	100
Total (n=56)		

TABLE 2. Percentages of survival and mortality of day 6 chick embryos after injected twice with 4 different concentrations of GBH and normal saline solution

Groups	% Survival	% Mortality
Control (n=5)	100	0
0.01% (n=8)	87.50	12.50
0.05% (n=14)	85.71	14.29
0.3% (n=12)	41.67	58.33
0.5% (n=7)	14.29	85.71
Total (n=46)		

TABLE 3. Percentages of survival and mortality of day 10 chick embryo after injected with 4 different concentrations of GBH and normal saline solution

Group	% Survival	% Mortality
Control (n=7)	100.0	0
0.01% (n=7)	42.86	57.14
0.05% (n=12)	50	50
0.3% (n=8)	0	100
0.5% (n=4)	0	100
Total (n=38)		

dextrorotational view. There were 2 head flexures, one at the midbrain called “cephalic flexure” and at the point between myelencephalon and spinal cord called “cervical flexure”. The otocyst situated at the level of myelencephalon. The eye primordia, optic cups and lens vesicle situated at the level of diencephalon. The optic cup appeared as horseshoe-shaped and lens vesicle located in the middle. About 3-4 branchial arches were visible. Heart tube was S-shaped loop. The neural tube appeared as two parallel dense lines which were parallel to each other to the caudal end along sides with the continuous blocks of somite which also exceeded the caudal end. The limb buds enlarged symmetrically, and the posterior pair were slightly larger than the anterior pair (Fig 1A).

The total amount of glyphosate injected groups

All treated groups showed more or less malformations and growth retardation. The 0.01% treated group (Fig 1B) showed severe loosening of heart looping with minor growth retardation of brain, eye, branchial apparatus and limb buds. Retardation of formation of brain caused no cervical flexure. Moreover Fig1C showed abnormal body bending. Fig 1D showed severe abnormality of brain and spinal cord with opened anterior and posterior neuropores, abnormal heart looping, eye primordia was rudimentary and neural tube showed unparallel dense lines. There were retardations in somite formation and absence of branchial arch and limb bud. The 0.3% glyphosate group in Fig 1E showed retardation of head bending which

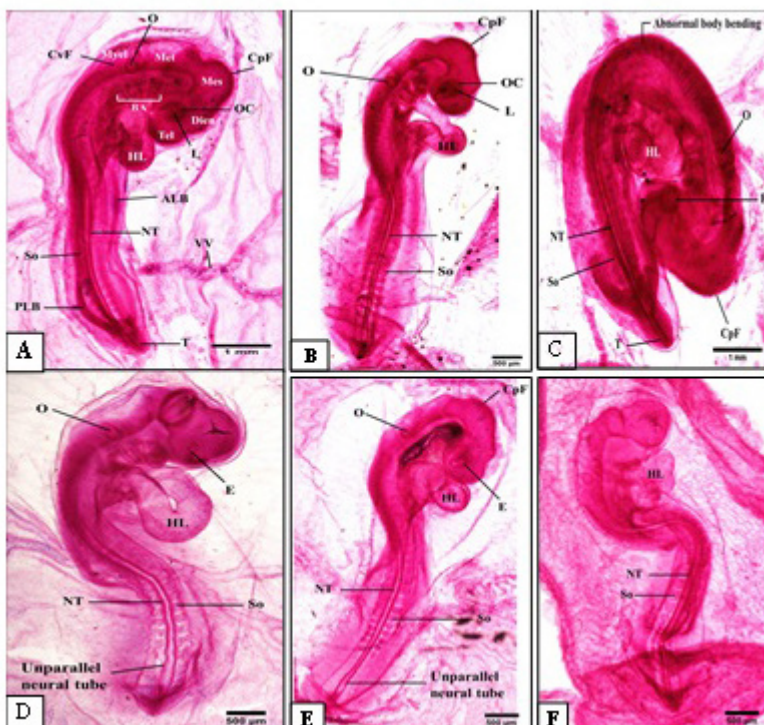


Fig 1. A Micrograph of the total amount of the 3rd day chick embryo of the control group showed normal development of Hamburger and Hamilton stage 18. Tel, Telencephalon; Dien, Diencephalon; Mes, Mesencephalon; Met, Metencephalon; Myel, Myelencephalon; CpF, cephalic flexure; CvF, cervical flexure; BA, branchial arch; O, otocyst; OC, optic cup; L, lens; HL, heart loop; So, somite; NT, neural tube; ALB, anterior limb bud; PLB, posterior limb bud; VV, vitelline vessel; T, tail fold.

B was the total amount of chick embryo of the 0.01%w/v treated group, C and D were the 0.05%w/v treated group. B showed severe loosening of heart looping with minor growth retardation of brain, eye and limb buds. C showed abnormal body bending. D showed severe abnormality of brain and spinal cord with opened anterior and posterior neuropores, abnormal heart looping, eye primordia was rudimentary and neural tube showed unparallel dense lines. O, otocyst, OC, optic cup, L, lens E, eye, NT, neural tube, So, somite. E and F were total amount of chick embryos of the 0.3% and 0.5% w/v group, D showed retardation of head bending which appeared only the cephalic flexure (CpF), abnormal heart looping (Ht), delay of somite formation (So) with unparallel dense lines of neural tube and absent of limb bud. E showed abnormal body bending, abnormal heart looping, anophthalmos and opened anterior neuropore, (HL, heart looping), NT, neural tube.

showed only the cephalic flexure (CpF), abnormal heart looping (Ht), and delay of somite formation (So) with unparallel dense lines of neural tube and absence of limb bud. The 0.5% glyphosate in Fig 1F showed abnormal body bending, abnormal heart looping, anophthalmia and opened anterior neuropore, absence of branchial apparatus and limb bud.

External appearance of the day 6 chick embryo

The normal day 6 chick embryo developed to the Hamburger and Hamilton stage 29,¹⁵ and the external appearance of the day 6 chick embryo showed the prominent mesencephalon and cephalic flexure. The eyes were black prominent protruding organs of the head. The upper and lower limbs distinctly appeared. The wing bent at the elbow with the second digit distinctly longer than the other. The ends of the upper and lower limbs were paddle-shaped with groove between the 1st, 2nd and 3rd digits. The 2nd to the 4th toes were prominent as ridges separated by distinct grooves with the webs between them. Beak was prominent, but the egg-tooth was not visible. Mandibular processes lengthened and were broadly fused. The visceral organs were enclosed by the body wall except the area of umbilicus. The control showed normal development while the experimental groups showed smaller body, eye and head. The upper and lower limbs were shorter than the control by observation. An

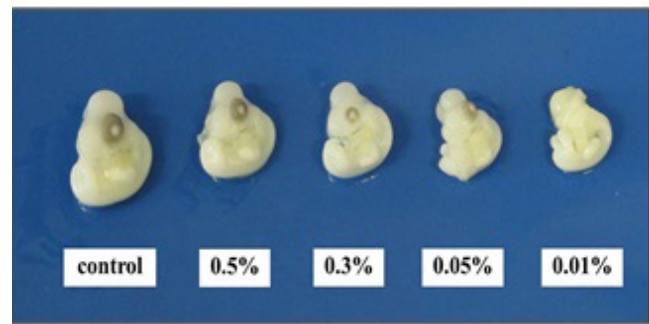


Fig 2. The photographs showed the comparison of external features between the experimental groups and the control group. All showed smaller than the control. The 0.01% w/v group was the smallest and showed anophthalmia and anencephaly.

embryo treated with 0.3% w/v glyphosate indicated the unilateral microphthalmia and an embryo treated with 0.01% w/v glyphosate indicated bilateral anophthalmia with opening of anterior neuropore.

Measurement and analysis of body parameters

The comparison of body parameters of day 6 chick embryos between the control group and the experimental groups of 0.01% w/v, 0.05% w/v, 0.3% w/v and 0.5% w/v GBH were shown in Table 4. The data analysis of body weight, crown rump length (CRL), width of head, width of eye, length of beak, length of upper limb and length of lower limb were tested statistically using Statistical

TABLE 4. The effect of GBH on body parameters of the 6th day chick embryo development showed the data by the mean \pm standard error which compared with the control group.

Body parameter	Control (n=5)	0.01% (n=7)	0.05% (n=12)	0.3% (n=5)	0.5% (n=1)
Body weight (mg \pm SE)	0.34 \pm 0.02	0.25 \pm 0.02*	0.27 \pm 0.01*	0.19 \pm 0.04*	0.19 \pm 0.01*
CRL (mm \pm SE)	14.08 \pm 0.15	12.27 \pm 0.52*	12.20 \pm 0.33*	12.06 \pm 0.60*	11.28 \pm 0.0*
Width of eye (mm \pm SE)	3.43 \pm 0.04	2.90 \pm 0.48	2.98 \pm 0.12	2.34 \pm 0.20*	2.52 \pm 0.0*
Width of head (mm \pm SE)	7.53 \pm 0.22	6.35 \pm 0.56*	6.67 \pm 0.15	6.40 \pm 0.69	6.07 \pm 0.0*
Upper limb (mm \pm SE)	3.66 \pm 0.09	3.27 \pm 0.16*	3.37 \pm 0.09	3.48 \pm 0.11	3.16 \pm 0.0*
Lower limb (mm \pm SE)	4.18 \pm 0.06	3.72 \pm 0.18*	3.97 \pm 0.13	4.10 \pm 0.16	3.65 \pm 0.0*

Significant difference from control: * $P < 0.05$. One-way ANOVAs was used for statistical analysis.

Table 4 indicated that the body weight and the CRL of the day 6 chick embryo of all experiment groups were significantly lesser than the control. Moreover all parameters of the 0.5% w/v glyphosate treated group were also significantly lesser than the control. The width of head, upper limb and the lower limb of the 0.01% w/v glyphosate treated group were significantly lesser than the control.

Package for the Social Science (SPSS) by one way analysis of Variance (ANOVA), and the data represented by mean ± SD with significant difference at $p < 0.05$

The glyphosate affects to the eye development of day 6

The normal day 6 eyes comprised oval-shaped optic cup and lens. The optic cup comprised two layers, the outer pigment layer and inner nervous layer which were closely 4/5 attached and obliterated the intraretinal space. The posterior 4/5 of the optic cup which is called pars optica retinae comprised of single layer of outer pigment layer and thick inner nervous layer with numerous cell layers developed into retina. Lens comprised of anterior lens epithelium and posterior lens fiber. The anterior lens epithelium with the mitosis cells migrated to the equatorial zone and elongated to form the posterior lens fiber that developed to obliterate the lens cavity. Anterior to it were anterior chamber and cornea. The eye in the control group showed normal eye development following HH stage 29.

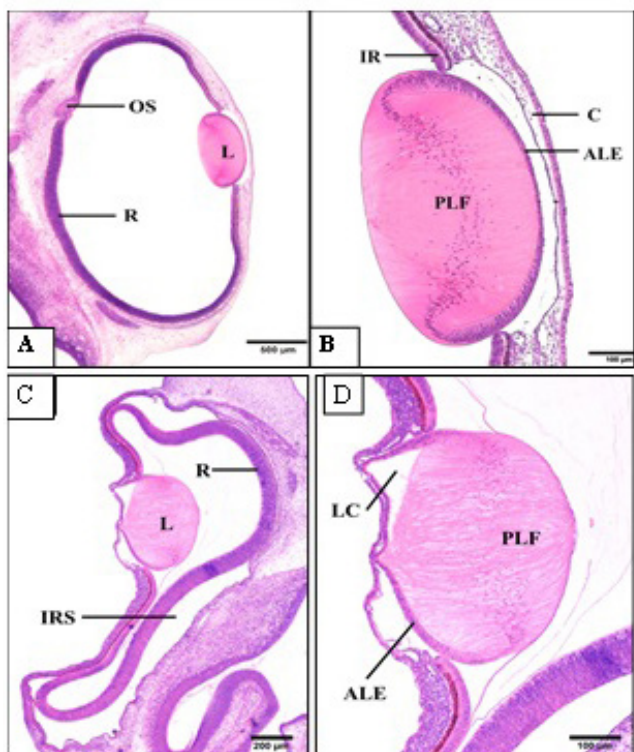


Fig 3. The micrographs showed eye development of the control A, B and in 0.05% w/v of glyphosate injected group C, D. Fig A exhibited normal optic cup which was oval-shaped without intraretinal space B showed lens without lens cavity, cornea (C) and smooth anterior chamber. Fig C and D showed irregular-shaped of optic cup and lens. R, retina; L, lens; IRS, intraretinal space; LC, lens cavity; ALE, anterior lens epithelium; PLF, posterior lens fiber. Other experiment groups showed the same appearances as in C and D.

The severe malformation of the eye was shown in an embryo that was injected with 0.01% w/v glyphosate. Both eyes were absent, called “anophthalmia”. In addition, the other embryos in 0.01% glyphosate injected group and other experimental groups showed under development of the eye compared with the control. The embryo showed irregular-shape of the optic cup with wide intraretinal space. The lens protruded into the anterior chamber and contacted with the cornea which obstructed the anterior chamber. The lens cavity still persisted between anterior lens epithelium and posterior lens fiber. The embryos in 0.05% injected group (Fig 3) showed collapsed optic cup with persistence of the intraretinal space which as well as irregularity of the cornea and displacement of lens to obstruct the anterior chamber. Non homogenous lens could be observed at area of posterior lens fiber which might cause congenital cataract.

One of the day 6 chick embryos treated with 0.3% w/v of glyphosate exhibited unilateral microphthalmia (Fig 4), the right eye showed normal size with slightly retarded optic cup while the left eye exhibited much smaller optic cup with relatively large lens with persistence of hyaloids canal called the microphthalmic eye.

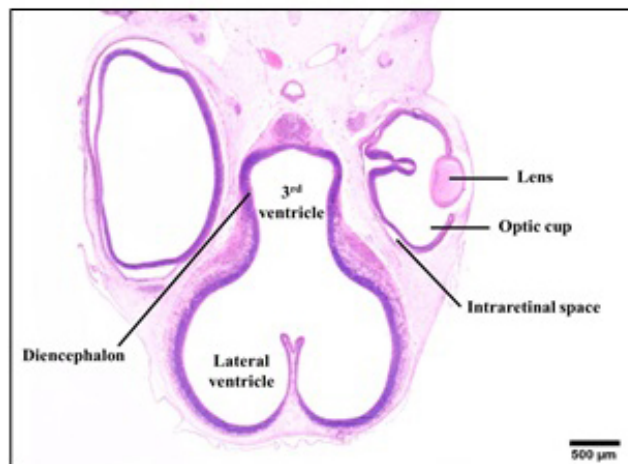


Fig 4. The micrograph showed unilateral microphthalmia in 6-day-old chick embryo after injected with 0.3% w/v of glyphosate.

Effect to the heart of day 6 chick embryo

The control group showed thin atrium lied dorsal to the thick ventricle. They were divided internally by fused endocardial cushion with two opening of left and right atrioventricular canals. The atrium was divided to two chambers, left and right atrium by septum primum that extended from the roof of atrium to the endocardial cushion. The left and right ventricles were divided by

muscular part of the interventricular septum. On this stage, the chamber of the ventricle was not completely divided and they were connected by the interventricular foramen. The results showed both abnormal and delayed developments of the heart. The ectopiacordis, the congenital displacement of the heart were observed in the embryos which were injected with 0.01% and 0.3% w/v of glyphosate. In addition in the 0.3% group, the embryo showed severe heart retardation when compared with the control. Some embryos in 0.05% and 0.3% injected group showed thin heart wall and the ventricle wall was thinner than the control. The embryo of the highest dose, 0.5% w/v of glyphosate showed that the heart size was smaller than the control without any malformation.

The day 6 embryo treated with 0.01 % w/v glyphosate (Fig 5A) showed the heart and liver (Li) situated outside the body cavity (ectopiaviscerae). The heart was severely retarded with the unfused endocardial cushion with the thinned-wall and underdeveloped atrium and ventricle. Figs 5B and C showed ectopiacordis with the concentration

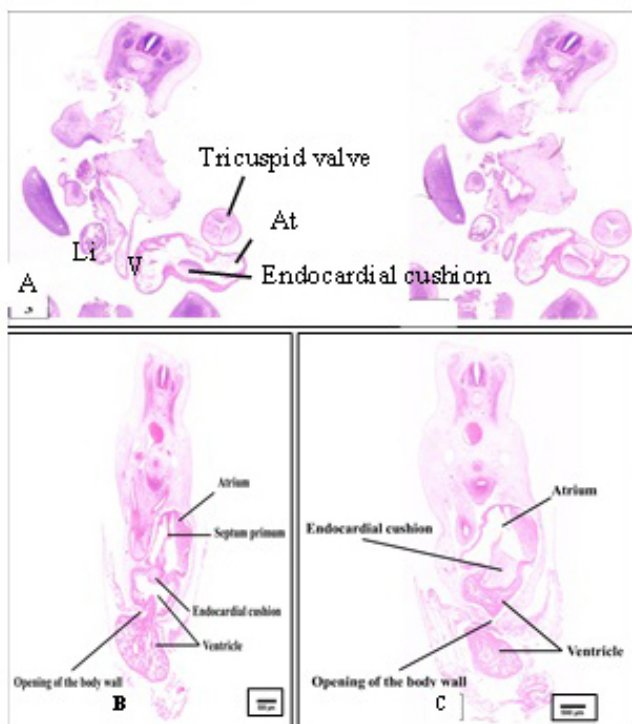


Fig 5. A The day 6 embryo treated with 0.01% w/v glyphosate showed the heart and liver(Li) situated outside the body cavity (ectopia viscerae). The heart showed severe retardation, the unfused endocardial cushion with the thinned-wall and underdeveloped atrium and ventricle. Fig B and C Micrographs showed the serial sections of 0.3% w/v of glyphosate injected group at the heart developing level. This embryo showed a part of the ventricle situated outside the body cavity. This occurred as a result of abnormal lateral body folds fused at the abnormal dorsal level. This was accompanied with small and retarded heart.

of 0.3% w/v of glyphosate-based herbicid. This heart composed of atrium and ventricle. The atrium situated inside the body cavity and showed more or less retardation of development. Ventricle was separated into two parts the proximal lied inside the body cavity while the distal part lied outside (Fig 5B and C) and the ventral body wall separated them as indicated in Fig 5C.

The day 10 embryo developed to stage 40 of Hamburger and Hamilton,¹⁵ and the embryo showed an increasing length of beak and toe. The egg tooth which is used to break the eggshell for hatching developed. The longer feather-germs covered the body and wing. The four to five rows of feather-germs were at the edges of lower eyelids. The eyes showed the opening between lids. Both the control group and the experiment groups showed normal development of the living embryos by external feature observation. Only the 0.01% and 0.05% survived, and the other two groups totally died. The dead embryo of the 0.3% treated group showed anophthalmia, microphthalmia and claw digits (Fig 6).

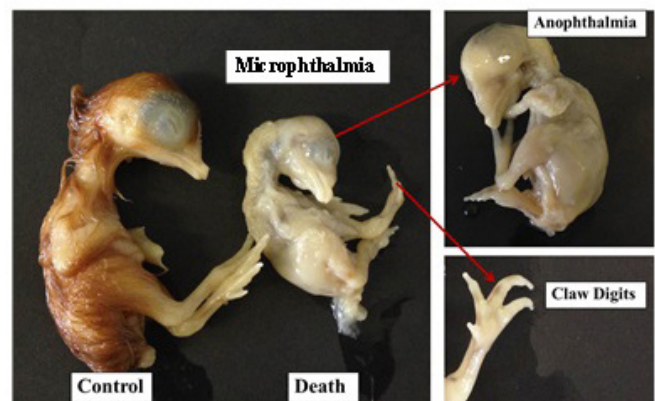


Fig 6. The photographs showed abnormal external appearance of dead chick embryo. The body size was smaller than the control. The anophthalmia, microphthalmia and claw digits were observed in the dead embryo.

Most embryos died before day 10 and the surviving embryos showed normal external appearance with no significantly difference of body parameters. The alcian blue and alizarin red staining showed lesser ossification of several bones. The ossification area of the skull, beak, mandible and the cervical vertebrae were stained red by the alizarin red while the cartilaginous parts stained blue by the alcian blue. Fig 7A was the control group which showed more ossification area of the skull, beak, mandible and the cervical vertebrae while Fig 7B of the 0.01% and Fig 7C of the 0.05% showed lesser ossification.

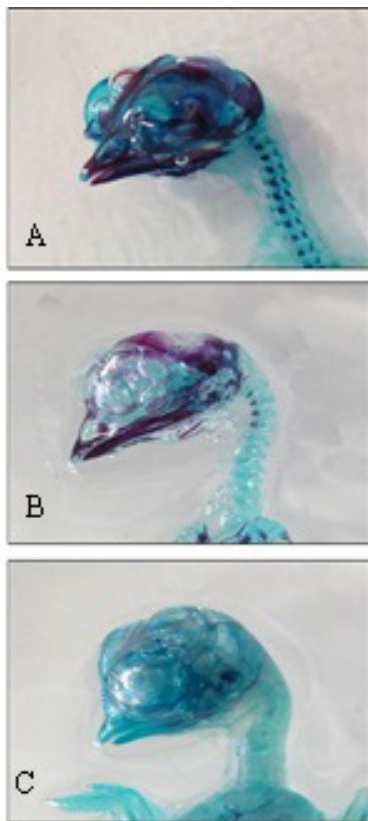


Fig 7. The photographs showed the head of day 11 chick fetus which were stained with alcian blue and alizarin red. The normal formation showed in the control group (Fig A) and the 0.01% glyphosate injected group (Fig B). The retardation of skull ossification showed in the 0.05% glyphosate injected group (Fig C).

DISCUSSION

Humans may expose to Roundup herbicide residues by agricultural practices¹⁶ or when the residues enter the food chain.¹⁷ Glyphosate residues in animal food, which is from GMO's products may be the cause of malformed piglets.¹⁵

The mortality rates were equal to the enhancing rate of glyphosate concentration. This result corresponded to Yusof *et al.*, 2014 who studied the effects of GBH on Java medaka (*Oryzias javanicus*).¹⁸ The survival and hatching percentage of fish embryos decreased as glyphosate concentration and time increased. In addition, the rats which were treated with high dose, 1000 mg/kg glyphosate-Roundup showed 50% mortality between days 7-14 of pregnancy.¹⁴ This study also indicated that glyphosate-Roundup was highly toxic and lead to embryonic death.

In 2003, Lajmanovich *et al.*, found the external malformations in tadpole stage of frog embryos (*Scinax nasicus*) such as craniofacial and mouth deformities, eye abnormalities and bent curved tails.¹⁹ These abnormalities occurred in all tests and increased with time and glyphosate-Roundup concentrations. In

2014, Yusof *et al.*, found the effects of GBH in pre-hatching stage of the fish embryo that showed several developmental abnormalities such as shrunken yolk, abnormal body curvature, growth retardation and disproportion of head and body size. In post-hatching stage, birth defect was found as absence of cornea and pectoral fins, permanently bent tail, abdominal enlargement and cell disruption in fin head and abdomen.¹⁸ In this study, day 3 embryos showed retardation of brain vesicles, delayed flexing of head, looser of heart looping with thin heart's wall, fewer branchial arches, retardation of optic cup and lens and absence of limb buds. The day 6 chick embryos showed several significantly smaller parameters ($P < 0.05$, one way ANOVA) such as body weight, CRL, width of eye, width of head, length of upper limbs and length of lower limbs. The serial sections revealed growth retardation of optic cup, lens and heart. The delayed development corresponded to the study of toxicity of GBH to four North American frog species.²⁰ Glyphosate-Roundup inhibited transcription of hatching enzyme and correlated with a delay in hatching process of sea urchin embryos.²¹

The irregularly-shaped brain with opening of anterior neuropore presented on day 3 and day 6 chick embryos. The width of head of the embryos in 0.01% and 0.5% experimental groups were significantly smaller than the control ($P < 0.05$, one way ANOVA) which revealed microcephaly. This agreed with the study of Pagamelli *et al.*, in 2010, in which brain microcephaly occurred in vertebrate embryos; amphibians and chickens at different developmental stages.¹³

Abnormalities were presented in eye development on day 3 and day 6 chick embryos. The range of abnormal eye development included irregular-shaped optic cup, lens displacement, congenital cataract, microphthalmia, and anophthalmia. GBH caused bilateral anophthalmia and unilateral microphthalmia that were distinctly presented in 0.01% and 0.3%, respectively on the day 6 chick embryos. This result was in agreement with a previous study reported in 2010 by Paganelli *et al.*, who found GBH and pure glyphosate produced microphthalmia in amphibians (*Xenopus laevis*) at neurula stage, tail bud stage and tadpole stage. Injecting of glyphosate into bilateral side on the frog embryo at 2-cell stage produced cyclopia. Similarity of glyphosate and glyphosate-based herbicide induced bilateral microphthalmia in chick embryos on Hamburger and Hamilton stage 8. During embryonic development, the *pax6* protein was thought to activate genes involved in the formation of the eyes, the brain and spinal cord. According to Paganelli *et al.*, the potential effects of GBH were by increasing retinoic acid signaling which inhibited or decreased sonic hedgehog

(*Shh*), *otx2*, *sox9* and *pax6* genes which caused cyclopia, microcephaly, affected cranial neural crest as well as craniofacial malformations.¹³

Cardiac abnormalities have been reported in rabbit fetuses that mothers were exposed to glyphosate during pregnancy such as defects of aorticopulmonary (spiral) septum, ventricular septal defects, dilated heart, dilated ventricle, and cardiomegaly.²² In this study, there was dilated heart tube with thin wall and looser of heart looping on day 3, and small heart size with thin wall of atrium and ventricle on day 6. The ectopiacordis showed severe abnormality which was found on day 6 chick embryos of 0.3% and 0.01% w/v of glyphosate concentration. There was no report that glyphosate herbicide exposure in chick embryos caused ectopic viscerae. This abnormality was found in chick embryos which were exposed to various concentrations of the insecticides.²³

Most embryos died before day 10 and the surviving embryos showed normal external appearance with no significantly difference of body parameters. The alcian blue and alizarin red staining showed lesser ossification of several bones. This agreed with the previous study that revealed 21-day-old fetus skeletal development retardations consisted of incomplete skull ossification and enlarged fontanel in all test groups of tadpole stage of amphibians that were exposed to low concentrations of glyphosate and GBH (Roundup).¹⁹

The results of this study indicated that mortality rate increased with the concentration while degrees of the severity of abnormalities were not dependent on the increasing concentrations of the GBH. The malformations randomly occurred in all experimental groups which did not correspond with the previous studies that stated the malformation increased with dose and time of exposure.^{14,18} The abnormalities were unpredictable and also the repeated exposures or single exposure of this herbicide on various periods of development also produced uneven results. This issue needs to be emphasized in further study.

The result of this study indicates that glyphosate based herbicide is highly toxic to chick embryo which leads to several developmental abnormalities and embryonic death. The mortality rate increased with the concentration of glyphosate while degree of abnormalities did not depend on the increasing concentration. Glyphosate is therefore predicted to be harmful to humans.

CONCLUSION

Glyphosate-based herbicide (Roundup) caused lethality in every concentration on day 3, 6 and day 10 chick embryo which indicated that it was an embryocidal agent which may cause the same effect, miscarriage, to humans.

Moreover glyphosate also caused several malformations and growth retardation. The living embryos of day 10 showed normal external features but revealed delayed ossification. The dead fetus had unilateral anophthalmia and microphthalmia, and small body size with claw digits. This indicated that most of the abnormal embryos could not survive and died before day 10. The pregnant should be highly cautious when living in the area using this herbicide during pregnancy to avoid abortion or congenital malformations. The results of this work showed that the malformations produced by GBH in chick embryos were embryonic death, the abnormality of nervous system (anencephaly), eye (anophthalmia, microphthalmia), heart, ectopiacordis, ectopia viscerae, delayed ossification, which were consistent with the well-known whichevidences occurred to the babies born in the areas using such herbicides. Why is GBH still promoted as "SAFE" despite damning evidence of serious harm to health and environment? Thus, future studies should investigate the teratogenic effects of GBH in different routes such as oral route, inhalation to mimic contamination to this herbicide from the residue in soil, water, air, vegetable or fish and meat fed with the GMO products.

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