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Endosulfan Impacts on the Developing Chick Embryos: Morphological, Morphometric and Skeletal Changes

[1,2]Y.M. Mobarak and [1]M.A. Al-Asmari
[1]Department of Biology, Faculty of Science, Taif University, Taif, Kingdom Saudi Arabia
[2]Department of Zoology, Faculty of Science, Suez Canal University, Ismailia, Egypt

Corresponding Author: Yomn Mohamed Shahat Mobarak, Department of Biology, Faculty of Science, Taif University, Taif, Kingdom Saudi Arabia

ABSTRACT

This study aims to explore the effects of the organochlorine pesticide Endosulfan (35% EC) on the developing chick embryos. After 24 h of eggs incubation, a single dose of 7 or 14 or 21 mg Endosulfan/egg was administered through the egg air space at once. The eggs were opened on embryonic days 6 and 12 and the embryos were evaluated for viability, wet body weights and various morphological, morphometric and skeletal changes. Skeletons of 12-day-old embryos were stained by alizarin red S and Alcian blue using a whole mount double cartilage and bone staining technique. Comparing the three doses with control and with each others, the high dose treatment resulted in statistically significant more embryonic deaths, while the mid-dose caused statistically more malformed embryos. On both embryonic days, the treated embryos exhibited dose-related growth retardation, as reflected by significant reductions of embryonic wet body weight, anterior-posterior head and crown-rump lengths as well as generalized edema and hematomas formations. Also, on embryonic day 12 significant reductions of beak length, eye diameters and measurements of wing and hind-limb parts were recorded. Abnormal survivors showed high percentages of limb deformities (as limb paralysis, clinodactyly, flexion and shortness of limbs or digits), microphthalmia, microtia and omphalocele. The skeleton of treated embryos showed anomalies and incomplete chondrification and/or ossification of some skull parts (interorbital septum, frontals, parietals, palatines and external auditory apertures), cervicals, scapulae, ribs, sacrals and caudals. These findings suggest that Endosulfan exhibits embryotoxic and teratogenic effects on the developing chick embryos in terms of growth retardation, external and skeletal malformations.

Key words: Growth retardation, hematomas, beak shortness, microphthalmia, ribs

INTRODUCTION

Because of their persistence in the environment, pesticides are common contaminants in soil, water and wildlife and are present in tissues of mothers and children, eggs and chicken meat samples especially in regions devoted to intensive agriculture (Botella *et al*., 2004; Ahmad *et al*., 2010). Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1,5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepin-3-oxide) is a broad-spectrum organochlorine pesticide (insecticide and acaricide) first registered for use in the United States in 1954 to control agricultural insect and mite pests on a variety of fruits, vegetables, rice, grains, tea, coffee, cotton and also in animal farms and

houses (US EPA, 2002). Results from a global monitoring network for persistent organic pollutants revealed that Endosulfan is abundant in the environment and its use is increasing (Pozo *et al.*, 2006; Harner *et al.*, 2006). It reaches aquatic systems through direct application, as well as spray drift and runoff from agricultural areas (Leonard *et al.*, 2000, 2001; Broomhall, 2002; Jergentz *et al.*, 2004; Rand *et al.*, 2010). It is a xenoestrogen and it can act as an endocrine disruptor, causing reproductive toxicity and congenital malformations in both animals and human's offspring exposed to it during pregnancy and/or lactation (Dalsenter *et al.*, 1999, 2003; Nurminen, 2001; Edwards *et al.*, 2006; Varayoud *et al.*, 2008). Maternal exposure to pesticides is associated with urogenital malformations, semen quality impairment and testicular, prostate, ovarian and breast cancer (Koifman *et al.*, 2002). Pre-and post-natal exposures to Endosulfan are confirmed by measures of residues in human breast milk, placenta, cord blood and adipose tissue (Campoy *et al.*, 2001; Shen *et al.*, 2008). Twenty five insecticides were tested for their toxicity to avian embryos at various concentrations using an egg injection technique. Of the two major groups studied, the organophosphorus compounds are much toxic than the organochlorine (Gilbertson *et al.*, 1991; Dunachie and Fletcher, 2008). Teratogenic effects of organochlorine insecticides are experimentally induced to the embryos of the developing chick (Sandhu and Waters, 1980; Lenselink *et al.*, 1992; Kumar and Devi, 1992). The embryotoxcity, neurotoxicity and teratogenicity of Endosulfan on mammals (Singh *et al.*, 2007, 2008; Cabaleiro *et al.*, 2008; Silva and Gammon, 2009; Silva and Beauvais, 2010), amphibians (Kang *et al.*, 2008; Brunelli *et al.*, 2009) and fish (Stanley *et al.*, 2009) as well as its genotoxic effects *in vitro* (Lu *et al.*, 2000; Nandi *et al.*, 2009; Sandal and Yilmaz, 2010) are studied. However, the data concerning the effects of Endosulfan on the developing chick or avian embryos are rare and represented by some physiological, histochemical and histopathological studies (Vila and de Viale, 1982; Pourmirza, 2000; Bargar *et al.*, 2001; Pushpanjali *et al.*, 2005; Jalili *et al.*, 2007; Prakash *et al.*, 2009). Therefore, the present study was suggested to find out the possible embryotoxic and teratogenic effects of Endosulfan on the morphological, skeletal and morphometric characteristics of the developing chick embryos on embryonic days (EDs) 6 and 12.

MATERIALS AND METHODS

Toxicant: The pesticide used in the present study was a technical-grade Endosulfan [Endocel (Batch 3, EPA Reg. No. 11678-25)], in the form of a light yellow coloured liquid (35% EC) is composed of two stereochemical isomers [alpha]-Endosulfan and [beta]-Endosulfan, in concentrations of approximately 70 and 30%, respectively, stored at ambient temperature. It was kindly provided by Al-Wadi Agricultural Development Company, Al-Taif, K.S.A. The Endosulfan was administered into the egg's air space through a minute hole without any dilutions at three dosage levels (7, 14 and 21 mg egg^{-1}) to 24 h-incubated eggs in volumes of 0.02, 0.04, 0.06 mL egg^{-1}, respectively. After Endosulfan administration, the hole was sealed with melted paraffin wax and the eggs were re-incubated.

Egg incubation: The present study was carried out in the laboratory of Zoological Research, Biology Department, Faculty of Science, Taif University, KSA. This research was conducted from January-2009 to February-2010. Fertile dark brown leghorn chicken eggs (each weighing about 55 g) were generously provided by El-Fakeeh Poultry Company, Taif, KSA. Eggs were stored in a refrigerator at 4°C for 24 h to allow egg contents to return to steady state after transport. The eggs were then transferred and maintained at 37.5°C in a full automatic egg incubator with full

Table 1: Percentages of mortality rate in control and endosulfan-treated chick embryos (N = 16)

Endosulfan mg egg^{-1}	Mortality (%)
Control	0.00
2	0.00
4	0.00
6	0.00
8	0.00
1	0.00
12	6.25
14	6.25
16	12.50
18	12.50
20	18.75
22	25.00
24	31.25
26	43.75
27	43.75
28	50.00
30	62.50

Mortality rate was recorded 24 hours following endosulfan treatment

automatic control of humidity (relative 55%), egg turning, fan speed, ventilation and alarm until the desired stages of development (24 hrs embryos, EDs 6 and 12) were reached. Eggs were candled before treatment and the unfertilized eggs were excluded from the experiments.

Experimental protocol: To estimate the survival rate in Endosulfan intoxicated chick embryos and Endosulfan-induced embryotoxicity and teratogenecity in the chick, fertilized eggs were marked, numbered and divided into three groups as follows:

Group I. LD_{50} determination: To evaluate the viability and LD_{50} of Endosulfan a total of 256 (24 h-incubated) eggs were divided into 16 groups (16 eggs each). Dilutions of Endosulfan were made in corn oil then eggs were administered different doses (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 27, 28, 30 mg egg^{-1}) of Endosulfan and the eggs were re-incubated. 24 h following Endosulfan administration, the eggs were opened and the survival rate of the embryos was recorded. The estimated LD_{50} of Endosulfan was 28 mg egg^{-1}. The doses selected for the present study were 1/4, 1/2, 3/4 of LD_{50} (7, 14, 21 mg egg^{-1}) (Table 1).

Group II. Endosulfan administration: To evaluate the developmental toxicity and teratogenicity of Endosulfan, 24 h-incubated 480 eggs were divided into three replicates each formed of eight subgroups as follows:

Subgroup I : 20 eggs served as controls and opened on ED 6
Subgroup II : 20 eggs were administered 7 mg Endosulfan egg^{-1} at once and opened on ED 6
Subgroup III : 20 eggs were administered 14 mg Endosulfan egg^{-1} at once and opened on ED 6
Subgroup IV : 20 eggs were administered 21 mg Endosulfan egg^{-1} at once and opened on ED 6

Subgroup V : 20 eggs served as controls and opened on ED12.
Subgroup VI : 20 eggs were administered 7 mg Endosulfan egg^{-1} at once and opened on ED 12
Subgroup VII : 20 eggs were administered 14 mg Endosulfan egg^{-1} at once and opened on ED 12
Subgroup VIII : 20 eggs were administered 21 mg Endosulfan egg^{-1} at once and opened on ED 12

Eggs of the above subgroups were re-incubated and then the ratios of living and dead, living and malformed embryos were recorded on both experimental days (6th and 12th) of chick development. The living embryos were wet weighed and examined for the presence of any external malformations (of the head, limbs, body and tail) under a dissecting microscope. On ED 6, the Crown-Rump (C-R) length and Anterior-Posterior (A-P) dorsal head length were measured. For the 12th day of chick development, measurements of C-R length, eye diameter, A-P dorsal head and beak lengths, fore limb (humerus, radius and ulna, metacarpus) and hind limb (femur, tibia and fibula, metatarsus) lengths were recorded. The measurements of the C-R and A-P dorsal head lengths were made by a caliper, while the fore and hind-limb parts were measured by silk threads fixed from the beginning to the end of the limb part and then the actual thread length was estimated on a ruler (Mobarak, 1995).

Histological procedures for skeleton staining: On ED 12, control and endosulfan-treated embryos were randomly selected and processed for staining with a whole mount double cartilage and bone staining technique previously described by Lamb *et al.* (2003) with some modifications. The embryos were removed from their extraembryonic membranes, washed in saline, injected under skin and within viscera with 95% ethanol, immersed for 1 h in ethanol then they were skinned, eviscerated and fixed in pure ethanol for 24 h. The embryos were then stained overnight at room temperature in Alcian blue [0.01 g Alcian blue 8GX (SIGMA) was added to equal volumes of 75% ethanol and 25% glacial acetic acid] for one day. The skeletons were then stained with 0.015% alizarin red S in 0.5% KOH for 48 h, cleaned in the following ratios of 0.5% KOH in distilled water to glycerol: 80:20, 60:40, 40:60, 20:80 and finally were stored in pure glycerol.

Incidence of external and skeletal malformations: The abnormalities of the central nervous system were represented by neural tube defects in the form of spina bifida (a defect that involves the incomplete development of the spinal cord or its coverings), microcephaly (disproportionately small head) and delay of brain development. Microphthalmia was defined as a reduced size of the eye. Microtia or the external ear malformations (external auditory aperture in birds) was represented by narrowing or complete absence of the aperture. Oedema is an abnormal accumulation of fluid beneath the skin. Omphalocele is characterized by the absence of abdominal muscles and skin and the abdominal wall covering is replaced by a membrane. This body wall closure defect exists in the abdomen or in both thoracic and abdominal regions. Limb deformities involved: limb paralysis, clinodactyly (deflection of digits from the central axis), flexed limbs (ranging from simple flexion to excessive flexion or bending of a complete limb or a joint), short limbs or digits and complete missing of a limb or digit. Caudal Regression Syndrome (CRS) was diagnosed when the caudal part of the embryo or the tail was reduced in size or the whole tail was lacking. The stained axial and appendicular skeletons were examined for any changes (absence, irregular shapes, axis deviations and lack or reduction of cartilage and bone formation).

Statistical analysis: The fresh body weight and the measurements of C-R length, eye diameter, A-P head and beak lengths, fore limb (humerus, radius and ulna, metacarpus) and hind limb (femur, tibia and fibula, metatarsus) lengths of the control and treated groups were expressed as group Mean±SD and were compared with the control by one-tailed student's t-test. Then a one-way parametric ANOVA was used to compare replicate results with each others. Both tests were carried out using Microsoft Office Excel (Frye, 2003). A Fisher's Exact test (Preacher and Briggs, 2001) was performed to compare the number of alive normal and dead embryos and the number of alive normal and alive abnormal embryos in the Endosulfan-treated groups with each others and with the control.

RESULTS

Mortality rate: Within a period of 24 h following Endosulfan treatment, a dose-dependent mortality rate was observed in embryos treated once with a single dose (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 27, 28, 30 mg egg^{-1}) of Endosulfan. However, no mortality was observed up to 24 h after treatment with 02 or 04 or 06 or 08 or 01 mg egg^{-1} doses. The embryos that received the doses of 12 or 14 or 16 or 18 or 20 or 22 or 24 or 26 or 27 or 28 mg egg^{-1} of Endosulfan showed 6.25, 6.25, 12.5, 12.5, 18.75, 25, 31.25, 43.75 43.75 and 50% mortality after 24 h of Endosulfan treatment, respectively. At the dose of 30 mg egg^{-1}, 62.5% mortality was recorded within the same period (Table 1).

The embryotoxic effect (the proportion of living malformed and dead embryos) of Endosulfan-treated chick embryos was dependent on the dose given; time elapsed after the treatment and day of embryonic investigation. The study did not exclude spontaneously dead or spontaneously malformed embryos from the evaluation, to obtain the total frequency of all affected embryos. When the results obtained from the three replicates were compared with each others no statistically different changes were recorded. Therefore, during the statistical evaluation of the results each three replicates were considered as one group.

On ED 6, there were 1.16% spontaneously dead and 1.69% spontaneously malformed embryos in the control groups. When comparing the living normal and dead embryos in the three Endosulfan-treated groups, statistically more deaths were identified in the high (21 mg egg^{-1}) Endosulfan dose treated group than in the low (7 mg egg^{-1}) and in the mid (14 mg egg^{-1}) doses treated groups ($p<0.001$, $p>0.05$ and $p<0.002$, respectively, vs. control) (Table 2). When comparing the living normal and malformed embryos in the three Endosulfan-treated groups, statistically more malformations were identified in the mid-dose treated group than in the low and high doses treated groups ($p<0.0001$, $p>0.002$ and $p<0.001$, respectively, vs. control) (Table 2). Compared to controls, the percentage of the sum of living malformed + dead embryos was higher in the mid and high dose treated groups than in the low dose treated one.

Table 2: Percentages of living normal, dead and malformed embryos in 6-day-old chick embryos, control and endosulfan-treated groups

	Egg groups			
Embryos condition	Control	Treated (7 mg egg^{-1})	Treated (14 mg egg^{-1})	Treated (21 mg egg^{-1})
Living N + Dead E	96 68 + 1.66	75 + 6.66°	43.33 + 11.66*	36.66 + 36.66**
Living N + Malformed E	96.66 + 1.66	75 + 18.33*	43.33 + 45***	36.66 + 26.66**

Malformed embryos exhibited one type or 2-4 types of malformations. ° $p>0.05$, *$p<0.002$, **$p<0.001$ ***$p<0.0001$, vs. control (One-sided fisher's exact test). N: Normal; E: Embryos

Table 3: Percentages of living normal, dead and malformed embryos in 12-day-old chick embryos, control and endosulfan-treated groups

	Egg groups			
Embryos condition	Control	Treated (7 mg egg^{-1})	Treated (14 mg egg^{-1})	Treated (21 mg egg^{-1})
Living N + Dead E	98.33 + 0.0	78.33 + 08.33*	46.66 +18.33***	35 + 41.66***
Living N + Malformed E	98.33 + 1.66	78.33 + 13.33**	46.66 + 40.00***	35 + 23.33***

Malformed embryos exhibited one type or 2-4 types of malformations. *p<0.05, **p<0.01, ***p<0.0001, vs. control (One-sided Fisher's Exact test). N: Normal; E: Embryos

Table 4: Mean wet body weight (g), anterior-posterior head and crown-rump lengths (in centimeter) of living 6-day-old chick embryos, control and endosulfan-treated groups

	Egg groups			
Measurements	Control	Treated (21 mg egg^{-1})	Treated (14 mg egg^{-1})	Treated (7 mg egg^{-1})
Wet body weight	0.54±5.60	0.53±0.10°	0.50±0.10*	0.44±0.11***
C-R length	1.93±0.13	1.86±0.20°	1.85±0.18*	1.72±0.27***
A-P head length	1.21±5.13	1.18±0.14°	1.10±0.13***	1.13±0.18**

The data are Mean±SD. °p>0.05, *p<0.05, **p<0.01, ***p<0.001 (Student t-test) compared to control. A-P: Anterior-Posterior; C-R: Crown-Rump

On ED 12, the percentage of the sum of living normal + dead embryos was significantly higher ($p<0.02$, $p<0.0001$ and $p<0.0001$, one-sided fisher exact test) in the low, mid and high dose-treated groups, respectively, compared to controls. Also, the sum of living normal + living malformed embryos was significantly higher ($p<0.01$, $p<0.0001$ and $p<0.0001$, one-sided Fisher exact test) in the low, mid and high dosage levels, respectively (Table 3). When comparing the number of the living malformed embryos in the three Endosulfan-treated groups, high percentage of malformations was identified in the 14 mg Endosulfan egg^{-1}-treated group than in the 7 and 21 mg treated groups. Compared to controls, the sum of living malformed + dead embryos was higher in the mid and high dose-treated groups (Table 3).

External morphology

I-Six-day-old chick embryos (control and endosulfan-treated)

Control embryos: The external morphology of the normal chick embryo on ED 6 was shown in Fig. 1a. At this stage of development, the size of head was great as compared to its body size and it gave the embryo a bird appearance. The eyes were large, spherical in shape and occupied a central position of the head. They were surrounded-from all sides except the ventral one-by the three brain regions {forebrain (Prosencephalon) anteriorly, midbrain (Mesoncephalon) dorsally and the hindbrain (Rhombencephalon) posteriorly}. The fore and mid-brains appeared transparent with the blood vessels easily detected. The beak was slightly developed since only the upper one was appeared, while no sign of the lower beak was detected. The mouth was represented by a narrow slit under the upper beak. The cervical region was slightly curved, while the cranial region made an angle of about 90° on the body axis. The heart was still-but partially-exposed from the thoracic cavity; its ventricular apex was exposed through a small hole of the thoracic wall. The tail was short and curved, whereas the fore and hind limb parts were still in the original paddle-shaped appendage buds with no detectable digits. The means of wet body weights, C-R and A-P head lengths recorded for these 6-day-old control chick embryos were 0.54±5.6, 1.93±0.13 and 1.21±5.13 cm, respectively (Table 4).

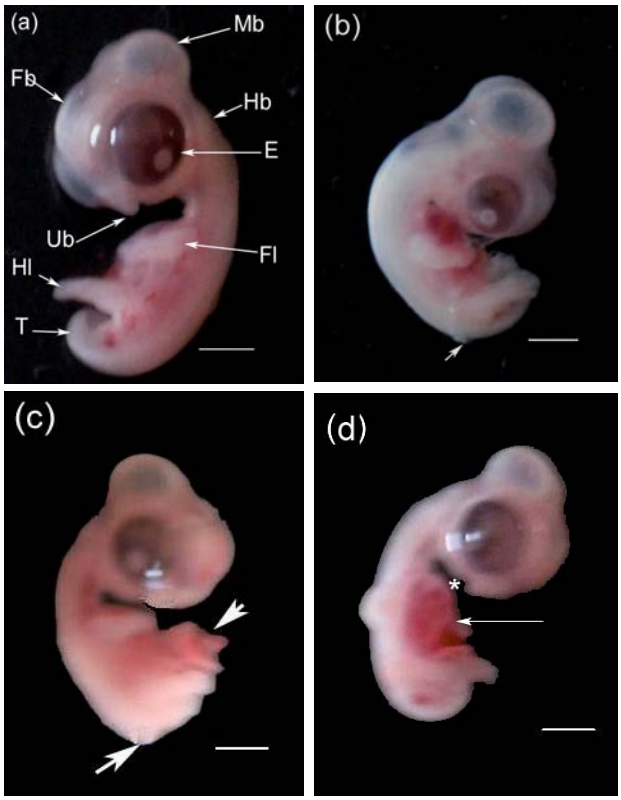

Fig. 1: Photographs of 6-day-old chick embryos, control and endosulfan-treated. (a) A control embryo, (b) a 7 mg endosulfan-treated embryo exhibits microphthalmia, haemorrhage in the thoracic and abdominal regions, reduced body size and spina bifida (arrow), (c) an embryo treated with 14 mg endosulfan displays absence of tail (arrow), abdominal omphalocele (arrow head) and reduced size of limbs and (d) a 21 mg endosulfan-treated embryo reveals both abdominal and thoracic omphalocele (arrow), reduced size of fore-limbs and exposed heart (*). Fb: Fore-brain; Mb: Mid-brain; Hb: Hind-brain; E: Eye; Fl: Fore-limb; T: Tail; Hl: Hind-limb; Ub: Upper beak. Scale bar = 5 mm

Endosulfan-treated: The mean of wet body weights, C-R and A-P head lengths were slightly-but insignificantly-decreased than in the control ($p>0.05$) in embryos treated with the low Endosulfan dose. However, in embryos received the mid-dose there was alike significant ($p<0.05$, vs. controls) decreases in mean of both wet body weights and C-R lengths, while the decrease in the A-P head lengths was highly significant ($p<0.001$, vs. controls). The embryos treated with the high Endosulfan dose exhibited highly significant ($p<0.001$, $p<0.001$, $p<0.01$, *versus* controls) decreases in the mean of wet body weights, C-R and A-P head lengths, respectively (Table 4).

The abnormalities encountered (18.33%) in embryos-treated with the low Endosulfan dose were found in 3 embryos, each one exhibited one type of malformation (CRS or paralyzed left wing or microphthalmia) and 8 embryos displayed 2-4 type of malformations as microphthalmia, haemorrhage and haematomas formation, ventral body wall defect in the thoracic and/or abdominal regions and spina bifida (Fig. 1b). However, in groups treated with the mid dose, a relatively higher percentage (40%) of the embryos was suffering from different types of malformations. Five of these embryos exhibited one type of abnormality and 19 embryos each of

Table 5: Frequency percentages of particular developmental defects in living 6-day-old chick embryos, control and endosulfan-treated groups

	Egg groups			
Type of malformation	Control	Treated (7 mg egg^{-1})	Treated (14 mg egg^{-1})	Treated (21 mg egg^{-1})
Haematomas F	0.00	5.00	15.00	8.33
CNS Malformations	0.00	1.66	3.33	3.33
Delay of beak D	1.69	0.00	3.33	0.00
Microphthalmia	0.00	08.33	8.33	0.00
Limb deformities	0.00	1.66	6.66	8.33
Caudal regression S	0.00	1.66	3.33	0.00
Ventral body WD	0.00	5.00	8.33	11.66

Note: Malformed embryos exhibited 1-4 types of malformations. F: Formations; CNS: Central Nervous System; D: Development; S: Syndrom; WD: Wall Defect

which had 2 or 3 or 4 types. The most frequent types of abnormalities recorded were the microphthalmia, all forms of limb deformities and the abdominal omphalocele. The last type of malformation was always associated with 1-3 types like haematomas formation or CRS and/or limb deformities as shown in Fig. 1c. The high dose treatment resulted in 26.66% of embryos with external malformations of which the ventral body wall defect was the most frequent one (11.66%) followed by equal percentages (8.33%) of haematomas formation and limb deformities (Table 5). Figure 1d showed one these embryos exhibited both abdominal and thoracic omphalocele, exposed heart and reduced size of fore-limbs.

11-Twelve-day-old chick embryos (control and endosulfan-treated)

Control embryos: By this stage of chick development, the embryonic body parts became covered by short and sparse down feathers, the beaks enlarged and became hard with white scales covered the tip of the upper one (Fig. 2a). The nostrils were narrow slits and the external auditory apertures were in the form of narrow spherical openings with elevated edges and were located at right angle of a distance about 2 mm behind the eyes (Fig. 3a). The eyes were of relatively larger size comparable to head size and had well developed eyelids, where they met each others when the eye was closed. The wing of the embryo showed the normal wing parts of the hen (humerus, radius and ulna, first digit, metacarpus, second and third digits) and the leg had no scales, while their digits were made of distinct phalanges ended by small claws. Each leg consisted of femur, tibia and fibula, metatarsus and four digits (Fig. 2a). The mean of fresh body weights, C-R and A-P head lengths recorded for such 12-day-old control chick embryos were 6.97±0.78 g, 6.24±0.71, 1.54±0.19 cm, respectively (Table 6). The measurements of different body parts (beak length; eye diameter; humerus, radius and ulna, metacarpus; femur, tibia and fibula and metatarsus) recorded for these embryos were listed in Table 6.

Endosulfan-treated: In embryos received the low dose the mean of wet body weights and C-R, humerus, radius and ulna and femur lengths recorded were significantly ($p<0.01$ and $p<0.001$, $p<0.05$, $p<0.001$ and $p<0.01$, respectively) decreased compared to controls (Table 6). However, the reduction in the measurements of the A-P head length, beak length; eye diameter, metacarpus and metatarsus lengths was insignificant (Table 6). The treatment with the mid-Endosulfan dose had resulted in significant ($p<0.01$, $p<0.05$, $p<0.01$, $p<0.001$, $p<0.01$, $p<0.001$, $p<0.001$, vs. controls) decrease in the mean of wet body weights, C-R, A-P head lengths, eye diameter and lengths of

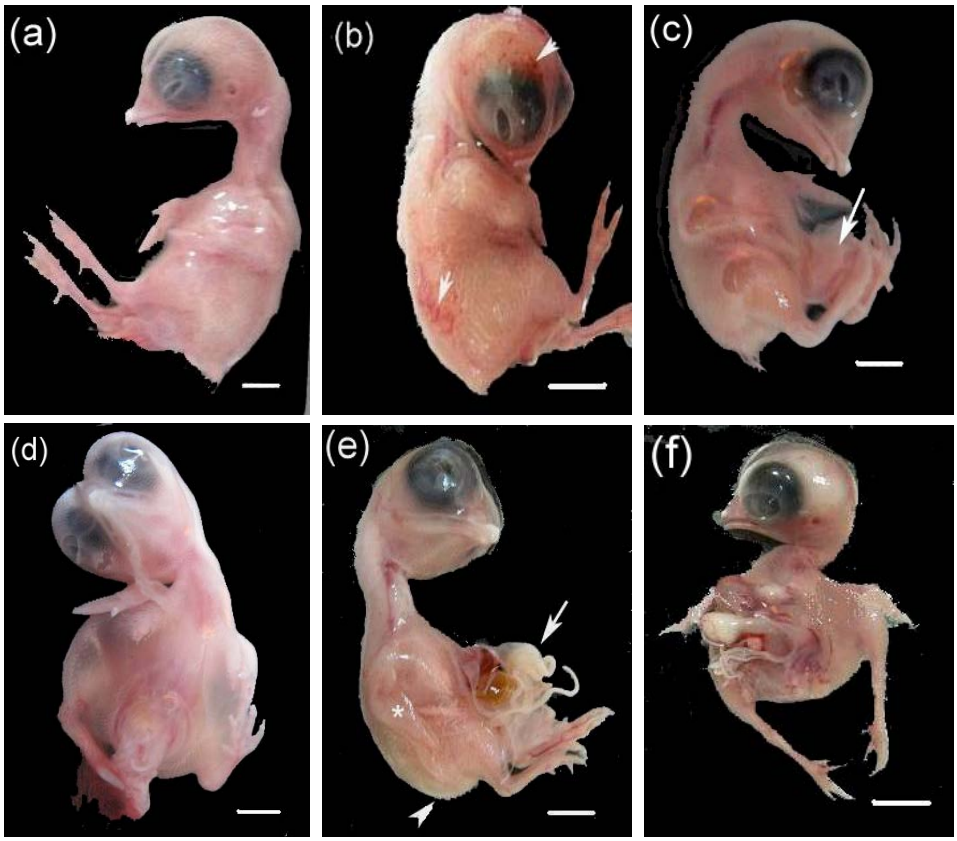

Fig. 2: Photographs of 12-day-old chick embryos, control and endosulfan-treated (a) A control embryo, (b) an embryos treated with 0.7 μg endosulfan shows hematomas formations on head and dorsal body wall (arrow heads), (c) another 7 mg endosulfan-treated embryo exhibits omphalocele with some viscera externally located (arrow), flexed limbs and clinodactyly, (d, e) embryos received 14 mg of endosulfan exhibiting poorly developed abdominal wall, shortness and flexing of limbs and joints as well as edematous body (d), (e) the abdominal contents, including liver, stomach and gut, extrude ventrally within enlarged membranous sac (arrow), paralyzed wing (*) and caudal regression syndrome (arrow head) and (f) an embryo received 21 mg of endosulfan suffering from growth retardation, microtia, microphthalmia with poorly developed eye lids, omphalocele and CRS. Scale bar = 3 mm

beak, humerus, radius and ulna and femur; respectively. Nonetheless, the embryos treated with the high Endosulfan dose exhibited similar highly significant ($p<0.001$, vs. controls) reductions in mean of wet body weights, C-R and A-P head lengths, eye diameter and lengths of beak, humerus, radius and ulna, metacarpus, femur and metatarsus (Table 6).

External malformations were recorded in 13.33% of the embryos treated with the low dose. Two of them exhibited one type of abnormality (one with delay of beak development and the other embryo (Fig. 2b) had haematomas on the head and dorsal body wall), whereas 6 embryos each of which had 2 or 3 or 4 type of malformations. Figure 3b demonstrated a head of an embryo treated with 7 mg endosulfan egg^{-1} exhibited microcephalia, poorly developed external auditory aperture and edematous swelling under the eye. The most frequent abnormalities recorded were of the limb

Table 6: Mean wet body weight (in grams), eye diameter, anterior-posterior head, crown-rump and limb lengths (in centimeter) of control and endosulfan treated 12-day-old chick embryos

	Egg groups			
Measurements	Control	Treated (7 mg egg^{-1})	Treated (14 mg egg^{-1})	Treated (21 mg egg^{-1})
Wet body weight	6.97±0.78	6.18±0.98**	6.15±0.57**	5.18±0.12***
C-R length	6.24±0.71	4.77±0.49***	5.52±0.95*	4.81±0.15***
A-P head length	1.54±0.19	1.45±0.13°	1.32±0.17**	1.34±0.15***
Eye diameter	1.35±0.12	1.33±0.12°	1.29±0.08°	1.24±0.13***
Beak length	1.55±0.14	1.45±0.3°	1.44±0.18***	1.43±0.13***
Humerus length	0.86±0.13	0.81±0.13*	0.80±0.03**	0.78±0.11***
Radius and ulna length	0.83±0.14	0.76±0.12***	0.75±0.12***	0.72±0.12***
Metacarpus length	0.78±0.12	0.77±0.11°	0.76±0.13°	0.69±0.12***
Femur length	1.34±0.14	1.24±0.14**	1.22±0.13***	1.21±0.12***
Fibula length	1.16±0.23°	1.15±0.13°	1.14±0.13°	1.08±0.12**
Metatarsus length	1.08±0.13°	1.06±0.13°	1.05±0.13°	0.97±0.21***

The data are Mean±SD. °p>0.05, *p<0.05, **p<0.01, ***p<0.001 (Student t-test) compared to control. C-R: Crown-Rump; A-P: Anterior-Posterior

Table 7: Frequency percentages of particular developmental defects in 12-day-old chick embryos, control and endosulfan-treated groups

	Egg groups			
Type of malformation	Control	Treated (7 mg egg^{-1})	Treated (14 mg egg^{-1})	Treated (21 mg egg^{-1})
Haematomas F	0.0	6.66	16.66	23.33
Delay of Feather D	0.0	0.00	3.33	3.33
CNS malformatious	0.0	1.66	3.33	3.33
Delay of beak D	0.0	3.33	10.00	5.00
Microtia	0.0	0.00	05.00	6.66
Microphthalmia	0.0	0.00	1.66	3.33
Limb deformities	0.0	8.33	30.00	6.66
Caudal R S	0.0	0.00	3.30	3.33
Ventral BWD	0.0	3.33	13.33	8.33
Edema	0.0	6.66	13.33	0.00

Malformed embryos exhibited 1-4 types of malformatious. F: Formatious; CNS: Central nervous system; D: Development; R S: Regression syndrom; BWD: Body wall defect

defects and abdominal omphalocele (Table 7). The later malformation was associated with limb deformities (Fig. 2c). The mid dose treatment resulted in 40% of embryos with external malformations of which the limb deformities, haematomas formation, ventral body wall defect and edema were the most frequent ones (Table 7). Three of these embryos were shown (Fig. 2d, e and 3c). The first one exhibited poorly developed abdominal wall, shortness and flexion of limbs and joints as well as edematous body (Fig. 2d); the second embryo had abdominal omphalocele, paralyzed wing and CRS (Fig. 2e) and the third one with microcephaly, hematomas formations on head, narrow neck and reduced external auditory aperture (Fig. 3c). The malformations encountered (23.33%) in embryos treated with the high dose were found in 11 embryos of which 5 exhibited one type of malformation (paralysis of a wing or a hind limb or CRS) and 6 embryos displayed 2-4 type of malformations such as delay of feather and beak development, microphthalmia, haemorrhage and haematomas formation, ventral body wall defect, microtia and CRS. Figure 2f and 3d show embryos with growth retardation, microtia, microphthalmia with

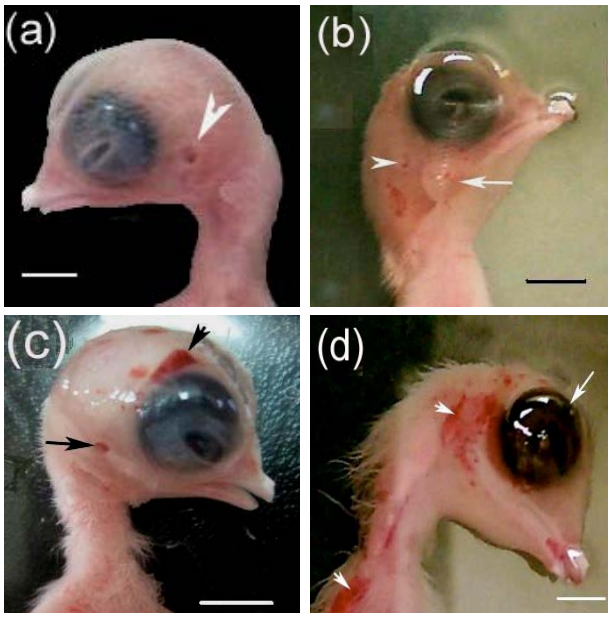

Fig. 3: Head and neck region Photographs of 12-day-old chick embryos, control and endosulfan-treated (a) A control embryo exhibits normally developed eye and external auditory meatus (arrow head), (b) an embryo treated with 7 mg endosulfan shows microcephalia, poorly developed external auditory aperture (arrow head) and edematous swelling under the eye (arrow), (c) a 14 mg endosulfan-treated embryo with microcephaly, hematomas formations on head (arrow head), narrow neck and reduced external auditory aperture (arrow) and (d) embryo received 21 mg of endosulfan showing swelling and edema of the eye (arrow), hematomas formations on the head and shoulders (arrow heads) and complete absence of the external auditory aperture. Scale bar = 5 mm

poorly developed eye lids, omphalocele and CRS (Fig. 2f); swelling and edema of the eyes, hematomas formations on the head and shoulders and complete absence of the external auditory aperture (Fig. 3d).

Skeletal elements in 12-day-old chick embryos (control and endosulfan-treated)

Control embryos: On ED 12, the skull and lower jaw parts that showed complete ossification were represented by the premaxilla, maxilla, nasal, lachrymal, frontal, palatine, supraorbital arch, inferior temporal arch, parietal, pterygoid, exoccipital, basioccipital, outer surfaces of dentary and angular. The quadrate and supraocciptal were more or less ossified, while the interorbital septum, orbitosphenoid, otic, squamosal, inner parts of dentary and articular were fully cartilaginous and exhibited an intense blue staining with Alcian blue (Fig. 4a and 5a). The vertebral column made of 42 vertebrae (14 cervicals, 7 thoracic, 7 lumbars, 7 sacrals and 7 free caudals followed by pygostyle), respectively. The cervical vertebrae 3-12 showed partial ossification of their centra and tips of their transverse processes, while the remained parts of vertebrae were fully cartilaginous. In the thorax, the first two and the seventh thoracic vertebrae were free, while the vertebrae 3-6 were fused (Fig. 6a). The centra of the thoracic vertebrae were partially ossified together with middle parts of the vertebral portions of ribs and the central portions of the ilium and ishium of the pelvic girdle, lumbar and sacral regions. The lumber region consisted of 7 fused vertebrae, while

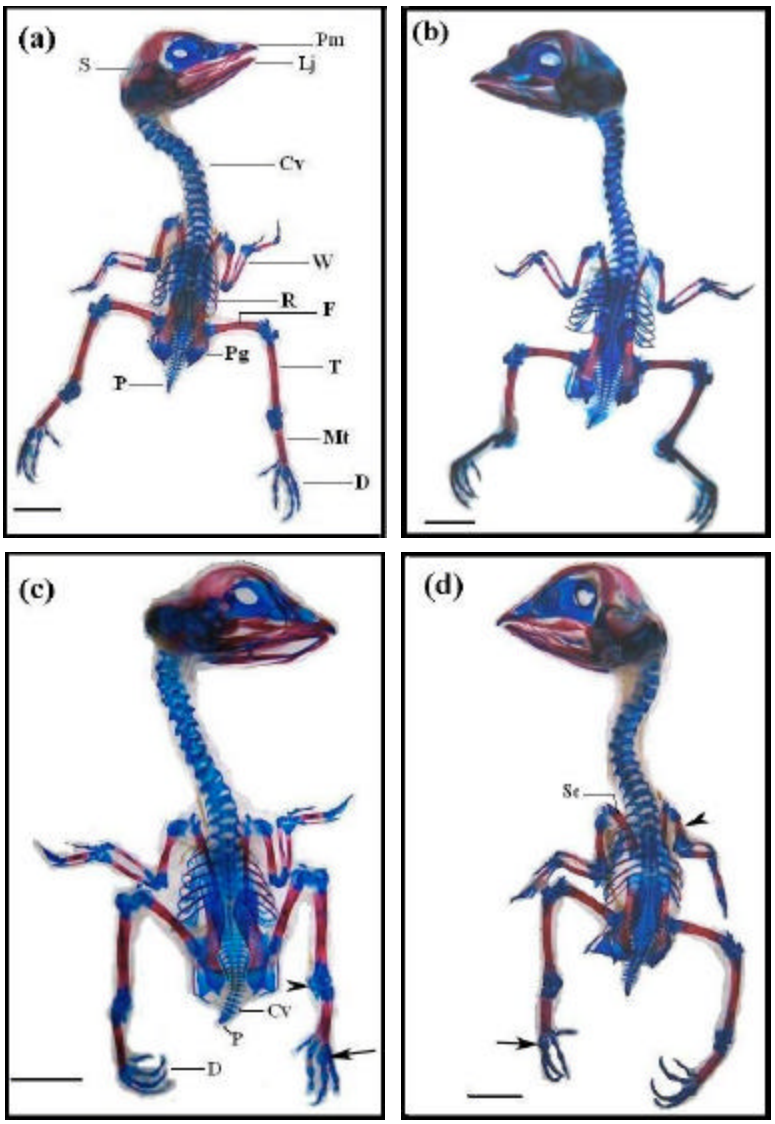

Fig. 4: Photographs showing endoskeleton dorsal views of 12-day-old chick embryos, control and endosulfan-treated. (a) A control embryo, (b) an embryo treated with 7 mg endosulfan shows reduced ossification of ribs, cervical vertebrae, metacarpus and digits, (c) an embryo treated with 14 mg endosulfan exhibits short kinked caudal vertebrae and reduced pygostyle, abnormal limb joint (arrow head) and swollen common origin of the third and fourth digits (arrow) and flexed digits, (d) an embryo treated with 21 mg endosulfan exhibits incomplete skeletal formation of ribs and pelvic girdle, shortness of humerus (arrow head) and scapula, swollen common origin of the third and fourth digits (arrow) with thin flexed digits. Pm: Premaxilla; Lj: Lower jaw; S: Skull; Cv: Cervical vertebrae; W: Wing; R: Ribs; F: Femur; T: Tibia; Mt: Metatarsus; Pg: Pelvic girdle; Cv: Caudal vertebrae; P: Pygostyle; D: Digits. Alizarin red S and Alcian blue staining. Alizarin red S and Alcian blue staining. Scale bar = 3 mm

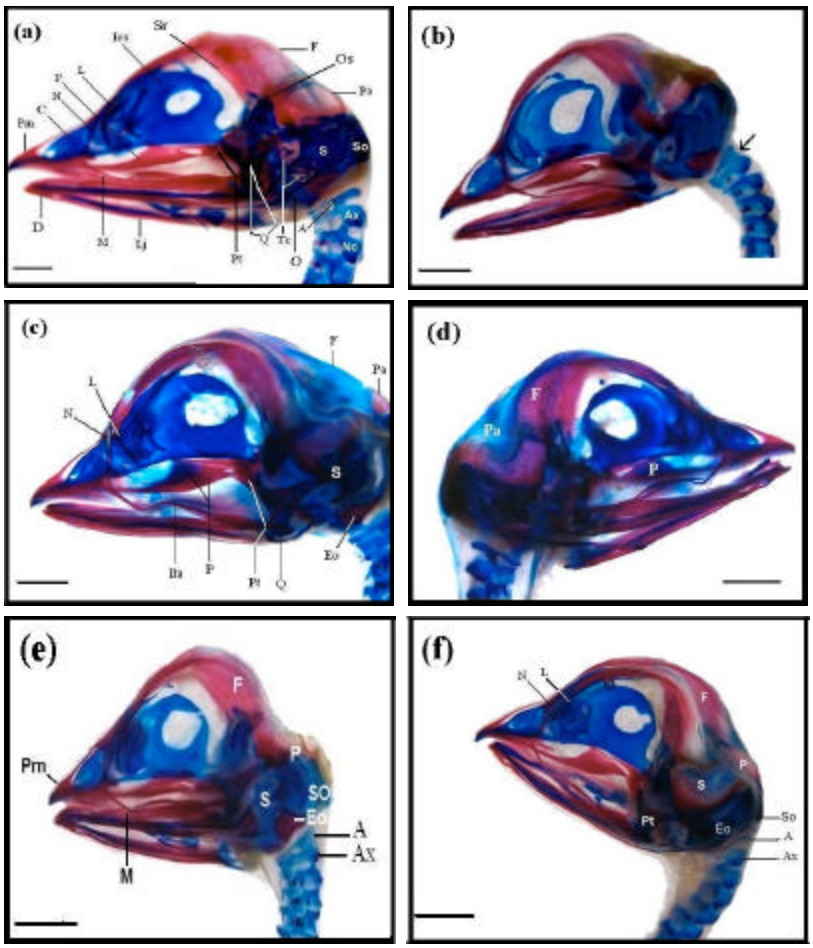

Fig. 5: Photographs showing lateral views of skull with a few cervical vertebrae of 12-day-old chick embryos, control and endosulfan-treated. (a) The skull of Fig. 4a magnified to show the normal ossified and cartilaginous parts of a control, (b) small-sized skull of an embryo treated with 7 mg endosulfan shows reduced cartilage formation of interorbital septum, delay of frontal and parietal ossification, shortness and fusion of atlas and axis vertebrae (arrow) and delay of development of normal cervical vertebrae, (c-e) skulls of embryos treated with 14 mg endosulfan, (c) shows unossified hind part of frontals and pterygoid, abnormally curved inferior temporal arch, (d) incomplete ossification of the frontals, parietals and palatine, complete absence of tympanic cavity, (e) short beak, abnormally formed frontals and parietals, reduced cartilage formation of interorbital septum and fused atlas and axis vertebrae and (f) small-sized skull of an embryo treated with 21 mg endosulfan shows shortness of the beak, reduced nasals and lachrymals, lower position of pterygoid, partially ossified frontals and parietals, reduced supraoccitals and the atlas and the axis are highly reduced. Pm: Premaxilla; M: Maxilla; C: Core; N: Nasal; P: Pterygoid; L: Lachrymal; Ios: Iterorbital septum; F: Frontal; Os: Orbitosphenoid, Pa: Parietal; So: Supraocciptal; S: Squamosal; Tc: Tympanic cavity; Q: Quadrate; Pt: Pterygoid; D: Dentary; Lj: Lower jaw; O: Otic; A: Atlas; Ax: Axis; Nc: Normal cervical. Alizarin red S and Alcian blue staining. Scale bar = 7 mm

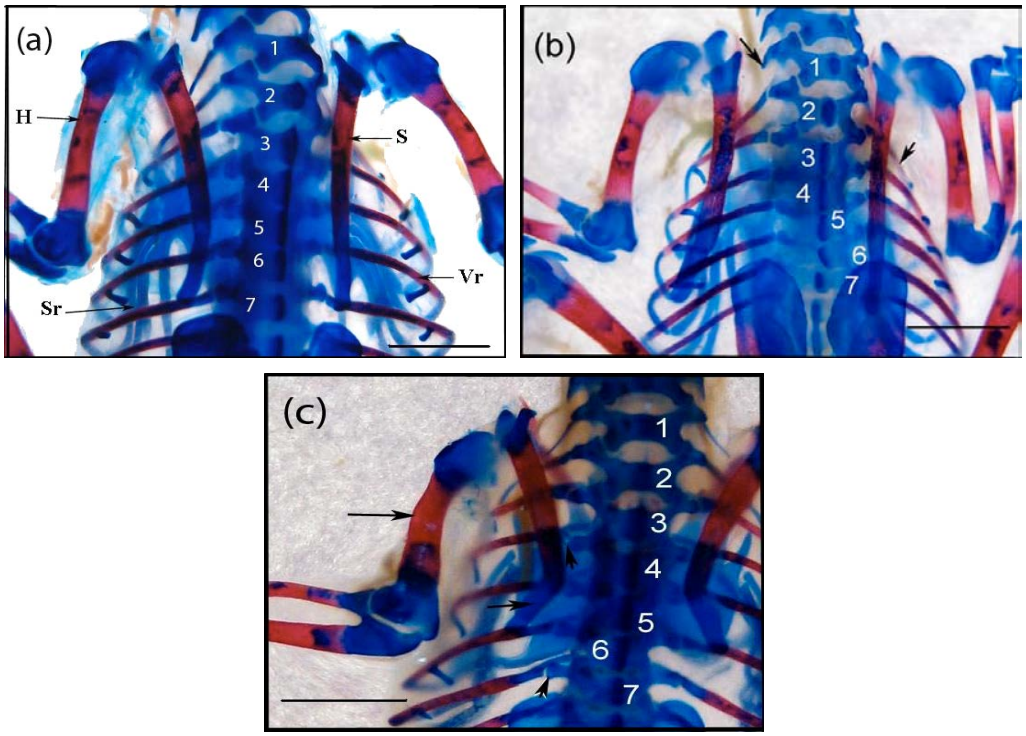

Fig. 6: Photographs showing dorsal view of thoracic skeleton of 12-day-old chick embryos, control and endosulfan-treated. (a) A control showing normal structures of the thoracic vertebrae (1,2,7 are free and 3-6 are fused), partially ossified scapulae and vertebral portions of ribs, fully cartilaginous sternal portions of ribs and humerii each with three strips of cartilage, (b) an embryo treated with 14 mg endosulfan shows absence of the transverse process of the first thoracic (arrow, 1), poorly developed (5-7) and deviation of the 6th vertebra and (c) an embryo treated with 21 mg endosulfan shows deviation of ribs from their vertebral origin (arrow heads), curved ends of scapulae (short arrow) and short curved humerus (long arrow). S: Scapula; H: Humerus; Vr: Vertebral portion of ribs; Sr: Sternal portion of ribs. Alizarin red S and Alcian blue staining. Scale bar = 9 mm

the external margins of the sacral transverse processes were fused forming a complete oval ring (Fig. 7a). The joints, ends of the long bones, radiale, ulnale, interclavicle, sternal portions of ribs, sternum, lumbars, sacrals, free caudal vertebrae and pygostyle were fully cartilaginous. The long bones (humerii, radii and ulnae, metacarpals of the fore limb; ilium, femur, tibia and fibula, metatarsals of the hind-limb) were fully ossified except the presence of cartilage remains as 2-3 narrow stripes along the length of these bones (Fig. 6a and 7a). Ossification was also observed in the first 1/3 part of scapulae, middle of coracoids and the first half of clavicles and a few phalanges of the hind limb digits.

Endosulfan-treated: The embryos treated with 7 mg Endosulfan showed small-sized skulls with reduction of cartilage formation of interorbital septum, delay of frontal and parietal ossification, shortness and fusion of atlas and axis vertebrae and delay of development of normal cervical vertebrae, incomplete ossification of ribs, metacarpus and digits (Fig. 4b and 5b). The embryo that

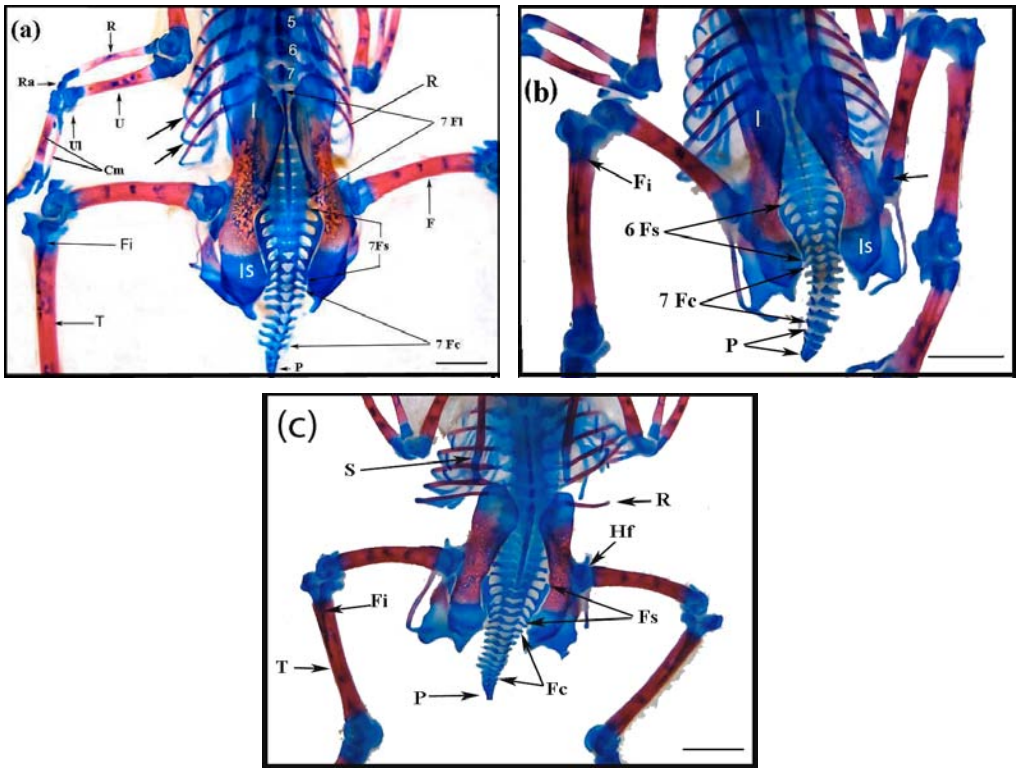

Fig. 7: Photographs illustrating dorsal views of lower body skeleton of 12-day-old chick embryos, control and endosulfan-treated. (a) A control showing the cartilaginous sternal portions of ribs (arrows), long bones, joints of long bones, the last three thoracic vertebrae (5,6,7), sacrals, lumbars, caudals and pelvic girdle (b) an embryo treated with 14 mg endosulfan shows shortness of the right clavicle, reduced size of the pelvic girdle; head of femur (arrow) and fibula, incomplete fusion of sacrals and (c) an embryo treated with 21 mg endosulfan shows incomplete formation of the 7th rib, reduced cartilage formation of head of femur, curved end of the right scapulae and delay of sacrals fusion. S: Scapula; R: Radius; U: Ulna; Ra: Radiale; Ua: Ulnale; Cm: Carpometacarpus; F: Femur; Fi: Fibula; Hf: Head of femur; I: Ilium; Is: Ischium; R: Rib; Fl: Fused lumbars; Fs: Fused sacrals; Fc: Free caudals; P: Pygostyle; Vertebral portion of ribs; Sr: Sternal portion of ribs Alizarin red S and Alcian blue staining. Scale bar = 6 mm

received 14 mg Endosulfan exhibited short kinked caudal vertebrae and reduced pygostyle, abnormal cartilage of limb joint, abnormally swollen common origin of the third and fourth digits and flexed digits (Fig. 4c). Skulls of these embryos showed unossified hind part of the frontals and pterygoid, abnormally curved inferior temporal arch, incomplete ossification of the parietals and palatine, complete absence of tympanic cavity, shortness of beaks, abnormally formed frontals and parietals, reduced cartilage formation of interorbital septum and fused atlas and axis vertebrae (Fig. 5c-e). Furthermore, absence of the transverse process of the first thoracic vertebrae, poorly developed and deviation of the 6th thoracic vertebra, shortness of the right clavicle, reduced size of the pelvic girdle; delay of cartilage formation of femur head and fibula and incomplete formation of sacrals were also observed (Fig. 6b and 7b).

Embryo treated with 21 mg Endosulfan exhibited incomplete skeletal formation of ribs and pelvic girdle, shortness of humerus and scapula, swollen common origin of the third and fourth digits with thin flexed digits (Fig. 4d). Skulls of these embryos showed shortness of the beak, reduced nasals and lachrymals, lower position of pterygoid, partially ossified frontals and parietals, reduced supraoccitals and the atlas and the axis were highly reduced in size (Fig. 5f). Also, deviation of ribs from their vertebral origin, curved ends of scapulae and short curved humerus, incomplete formation of the 7th rib, reduced cartilage formation of the head of femur and delay of sacrals transverse process fusion were also found (Fig. 6c and 7c).

DISCUSSION

The general morphology of the chick embryos, on EDs 6 and 12, described here is quite similar to the observations described previously by some other investigators (Butler and Juurlink, 1987; Bellairs and Osmond, 1998; Mobarak, 2009). The current results provide the first detailed analysis of Endosulfan toxicity and teratogenicity in the chick embryo for which there are no detailed data in the literature. It is difficult to compare the presently estimated LD_{50} (28 mg egg^{-1}) to other studies of Endosulfan effects on the chick embryos because there is forty-two Endosulfan products which contain Endosulfan as the active ingredient. These products are placed into eight batches in accordance with the active and inert ingredients and type of formulation. For example Pourmirza (2000) investigated LD_{50} of 5 mg egg^{-1} Endosulfan (99.6% active ingradients), while Pushpanjali *et al.* (2005) estimated 5 μg AR grade α-endosulfan/egg as LD_{50} for chick embryos. Also, the LD_{50} of endosulfan varied widely depending on the route of administration, species, vehicle and sex of the animal. In the present study Endosulfan was administered through the egg air space which might reduce the amount of Endosulfan that reaches the embryo within 24 h of the LD_{50} test. This assumption is supported by the currently obtained high rates of mortality with the three administered Endosulfan doses (7 or 14 or 21 mg egg^{-1}) on both EDs comparable to mortalities obtained with the same three doses after the period of 24 h (LD_{50} test) of embryos exposure. Endosulfan LD_{50} is investigated in different adult mammals: rat 10-23 mg kg^{-1} (female) and 48-160 mg kg^{-1} (male) (GFEA-U, 2007); guinea pig 35 mg kg^{-1}; rabbit 35-40 mg kg^{-1} (WHO, 2005) and also in non-mammalian species such as birds: mallard duck 3.1 mg kg^{-1}, bobwhite quail 16 mg kg^{-1}, hen 137 mg kg^{-1} (US EPA, 2007).

The present results revealed statistically more embryonic deaths using the high (21 mg Endosulfan egg^{-1}) dose with highly significant reductions of wet body weight and C-R lengths as well as high rates of edema and hematomas formations. On the other hand, statistically more malformed embryos are identified in the mid-(14 mg Endosulfan egg^{-1}) dose treated group. Therefore, the high dose of Endosulfan might be considered the embryotoxic dosage level, while the mid-dose is the teratogenic dosage level. Similarly, in fish-eating birds, exposure to high doses of organochlorine contaminants induces embryotoxicity, a condition known as Great Lakes embryo mortality, edema and deformity syndrome (GLEMEDS) (Gilbertson *et al.*, 1991).

In the present study treatment of the developing chick embryos with the three dosage levels of Endosulfan also resulted in significant reductions in the mean of wet body weights (on ED 12) in comparison to controls, while on ED 6 the reduction was only significant following the mid and high dosage treatments. This result is in accordance with Pourmirza (2000), who declared that with either compound (malathion or endosulfan), an increased dose generally resulted in a decrease of chick embryonic body weight. Also, our results strengthen findings from other animal models that correspondingly indicated reduced maternal and fetal body weights of Wistar rats exposed to

Endosulfan either alone or in combination with citrinin (Singh *et al.*, 2007); exposure of eggs of a South American caiman (similar to alligators) to Endosulfan caused loss of egg weight and reduced weight in the hatchlings, thought to be as a result of disrupting the metabolism of the embryo and the signals that control development (Beldomenico *et al.*, 2007). Similarly, low birth weight and adverse behavioural effects of Endosulfan are noted on rat offspring exposed *in utero* and during lactation (Cabaleiro *et al.*, 2008).

In the current study, embryos examined on both EDs exhibited dose dependant general significant (vs. control) reductions in the mean of C-R and A-P head lengths, eye diameter, beak length and lengths of wing and leg parts. This result is in agreement with reports from Singh *et al.* (2007) on rat embryos and Sepulveda *et al.* (2006) on the American Alligator embryos exposed to organochlorine pesticides. This growth retardation effect of Endosulfan on the developing chick could be due to its inhibitory effects on metabolism (Garg *et al.*, 2004), or suppression of gluconeogenesis (main energy source in embryonic life) as suggested by Pushpanjali *et al.* (2005) or after disruption of the retinoid signalling pathway in cells, which play an essential role in the proliferation, development and differentiation of cells and disruption that can lead to malformation or abnormal development of the eye, brain, heart and limbs (Lemaire *et al.*, 2005).

In the presently three dosage levels of Endosulfan-treated embryos that examined on EDs 6 or 12, significantly higher percentages (compared to controls) of external malformations (each malformed embryo exhibited one type or 2-4 types of malformations) were obtained that suggest a teratogenic effect of Endosulfan on the chick embryos. This suggestion is supported by previous studies that proved Endosulfan teratogenicity in human (ATSDR, 2000; Saiyed *et al.*, 2003), in rat Singh *et al.* (2007) and teratognicity induced by other organochlorine insecticides when injected into the chick egg during the embryonic development (Sandhu and Waters, 1980; Lenselink *et al.*, 1992; Kumar and Devi, 1992).

The presently obtained generalised haematomas (extravasation of blood) and edema (abnormal retention of body fluids) formation effects of Endosulfan on both EDs might be considered additional modes by which Endosulfan exerts its embryotoxic and teratogenic effects on the developing chick embryos. Similarly, subcutaneous, pericardial and peritoneal edema are recorded in fish-eating birds, exposed to organochlorine contaminants (Gilbertson *et al.*, 1991). Furthermore, Smith and Cole (1973), Monod (1985), Fry (1995), Guiney *et al.* (1997) and Henry *et al.* (1997) declared that exposure of early-life stages of some fish to organochlorine pesticides resulted in high rates of mortality that is associated with yolk sac and pericardial edema, craniofacial alterations and severe and generalized vascular damage. The obtained variable malformations (ranging from 1.66-13.33%) suggest that there is no specific type of abnormality for teratogenicity of Endosulfan except hmb deformities which recorded 30% on ED 12. Other abnormalities included: the delay of CNS development, microphthalmia, omphalocele, CRS, delay of feather and beak development and microtia. Of these malformations the omphalocele and microtia are newly recorded malformations. The majority of these malformations are in agreement with previous studies on Endosulfan teratogenicity in rat (Singh *et al.*, 2007); in anuran *Bombina orientalis* embryos (Kang *et al.*, 2008) and in *Bufo bufo* (Brunelli *et al.*, 2009). The neurotoxic effect of endosulfan is manifested here in the form of the obtained limb paralysis of some embryos. This result is in accordance with Howe *et al.* (2004) who investigated extensive paralysis of Endosulfan-exposed tadpoles of three anurans species (*Rana sylvatica, Bufo americanus* and *R. clamitans*).

In the present study the skeletal malformations of 12-day old chick embryos treated with the three dosage levels of endosulfan are represented by deficient and abnormality of cartilage formation of interorbital septum, joints, caudal vertebrae, sacrals, femur head and reduced pygostyle; size reduction of cervical vertebrae, ribs, pelvic girdle, long bones and digits. Also, incomplete ossification of some skull parts, ribs, scapulae, poorly developed and deviation of the 6th thoracic vertebra. This result confirms the findings of (Singh *et al.*, 2007) that endosulfan was clearly associated with severe alterations in the development of rat embryo cartilage and bones. Furthermore, these results are in accordance with the skeletal abnormalities obtained in gulls embryos following exposure to another organochlorine pesticide (o,p'-DDT, polychlorinated biphenyls) (Fry, 1995). The present study concluded that, Endosulfan directly interferes with the normal development of the chick embryo and causes growth retardation, as reflected by significant reductions of embryonic wet body weight, anterior-posterior head and crown-rump lengths, significant reductions of beak length, eye diameters and measurements of wing and hind-limb parts as well as generalized edema and hematomas formations. Also, treated embryos exhibited high percentages of limb deformities, microphthalmia, microtia and omphalocele, plus anomalies and incomplete chondrification and/or ossification of the skeletal system. Therefore, these results strengthen findings from *animal* models that similarly indicate embryotoxicity and teratogenicity of endosulfan.

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