Research Article

Morphological and Skeletal Abnormalities Induced by Commercially Available Insecticides Colonel-s® and Decis® in the Developing Embryo of G*allus domesticus*

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ABSTRACT

In the present work, certain congenital effects induced by commercial formulations of two insecticides *i.e.* dicofol (Colonel-S®) and deltamethrin (Decis®) were illustrated in the developing chick embryo. Fertilized eggs of *Gallus domesticus* were immersed in three aqueous emulsions of each insecticide (250, 500 and 1000 mg L^{-1} of Colonel-S® and 12.5, 25 and 50 mg L^{-1} of Decis®) on their embryonic day (ED) 4 and 7 for 60 min at 37°C and kept for re-incubation till ED 16. All the embryos recovered for sampling on ED 16 were evaluated for their survivability success, wet body weight and gross morphological and skeletal malformations. The result revealed that there was a significant decrease in survivability rate of embryos treated with 50 mg L^{-1} of Decis® on ED 4 and 1000 mg L^{-1} of Colonel-S® on ED 7. Also, the wet body weight of embryos was depleted significantly at 25 and 50 mg L^{-1} of Decis® and 1000 mg L^{-1} of Colonel-S® exposed on ED 4 and ED 7, respectively. A significant increase in the percentage of abnormal survivors exhibiting a spectrum of morphological as well as skeletal malformations was observed in each of the insecticide treated group. These findings suggest that Colonel-S® and Decis® can be considered as teratogenic agents, at the dose levels used at our laboratory conditions.

Keywords: Chick embryo, Deltamethrin, Dicofol, Insecticides, Teratogenicity.

INTRODUCTION

Pesticides are widely used in food production systems and in agriculture sectors of some of the countries because of their increased food demands. Also, a large number of benefits have been derived from the use of pesticides in public health, forestry and domestic sphere. Many other kinds of benefits which are often going unnoticed by general public may be attributed to the use of pesticides. From this point of view, these chemicals can be considered as an efficient tool of pest management being as economic and labor-saving with their great popularity in agricultural sectors.[1] Today, more than 800 products of pesticides are in regular use. The markets of industrialized countries for pesticides are no longer growing as their governments are putting restrictions or limiting the use of pesticides due to their serious health implications to man and his environment.[2,3] Therefore, these companies are looking to developing countries for their increased sales.[2]

Colonel-S® is manufactured and sold in local markets of India as a contact acaricide. It is used against mitigate insect red spider mite that cause harm to agricultural crops such as apples, cotton and citrus cultivates, tomatoes, walnuts, mint, cucurbits, beans and peppers etc. Dicofol [4-chloro-*alpha*-(4-chlorophenyl)-α-(trichloromethyl) benzene-methanol], an organochlorine insecticide, is the active ingredient of Colonel-S®. Dicofol is a nerve poison first introduced in 1957 by US-based multinational company named as Rohm and Haas. It is synthesized from technical DDT which is first chlorinated to an intermediate, Cl-DDT, and then hydrolyzed to

dicofol. Therefore, DDT and Cl-DDT may remain in dicofol products as impurities.[4] A lot of literature is available regarding certain toxic effects of dicofol which include cytokinetic and cytogenetic effects on human lymphoid cell *in vitro*, liver histological changes, neuropsychological and psychological problems, and anti spermatogenic and anti androgenic activity of insecticide which is associated with adverse effects on reproduction.[5-11] It is also reported to be harmful to aquatic animals such as fish, invertebrate and algae. In various species of birds such as in eastern screech-owls *(Otus asio)* and American kestrels *(Falco spar-veruis)*, it is known to be responsible for causing eggshell thinning, reduced hatching success and reduced fertility.[12,13]

Decis® is used to apply on a variety of agricultural crops such as cotton, coffee, corn, hops, maize, artichokes cereals and fruits for controlling their insect pests like mealy bugs, apple and pear suckers, various caterpillars, plum fruit moths, aphids and whiteflies. Decis® is a trade name of insecticide deltamethrin [(S)-a-cyano-3-phenoxybenzyl-(1R)-cis-3-(2, 2-dibromovinyl)-2, 2-dimethylcyclopropane carbo-xylate] which was synthesized in 1974 and belongs to the most recent group (fourth generation) of synthetic pyrethroids.[14] The rate of deltamethrin detoxification in mammal is very high than in insects[15], therefore it is considered quite safe to mammals. But earlier studies with deltamethrin, provided evidences to suggest that so prominently proclaimed 'safe to man' pyrethroid insecticides have various degree of toxicological impacts in developing rat brain at concentrations much lower than those recommended for

its safer use.[16] Extensive literature is available on the toxic effects of deltamethrin on animals such as fish, Japanese quail, freshwater mussel, *Daphnia magna*, South American toad, rats and mice.[14, 17-31]

The present study has been planned to investigate the possible morphological and skeletal defects induced by above mentioned commercially available insecticides (Colonel-S® and Decis®) in the developing chick embryo for examining the mechanisms of teratogenicity as similar patterns of human teratogenesis can also be suspected from these toxic chemicals.

MATERIALS AND METHODS

For estimating the congenital effects of insecticides Colonel-S® and Decis® respectively in developing chick embryo, two experimental plans were proposed which were based on exposure of fertilized eggs to different dose concentrations of each insecticide formulation on two different "critical periods" of chick embryogenesis. All the experimental procedures were carried out according to the guidelines of Animal Ethical Committee of Institute and use of the chick embryos was in conformity with the policies of Institutional Animal Care and Use Committee.

Test chemicals

The commercially available insecticides Colonel–S® (18.5% EC, Emulsifiable Concentrate) manufactured by Indofil Chemicals Company, Mumbai, India and Decis® (2.8% EC) manufactured by Bayer CropScience Limited, Gujarat, India were used for present study.

Test animals

Fertilized eggs of BV 300 breed of *Gallus domesticus* were collected from a commercial hatchery (Kewalramani Hatcheries, Ajmer, India). All the eggs were cleaned and kept in an incubator with capabilities of maintaining and monitoring temperature, humidity and turning the eggs periodically. The temperature in the incubator was maintained at 37°C and relative humidity was kept between 70-80%.

Experimental design

Prior to dosage, all the fertilized eggs were placed in an incubator to initiate embryonic development. Aqueous emulsions of Colonel- S® and Decis® were prepared in distilled water in 250, 500, 1000 mg L^{-1} and 12.5, 25 and 50 mg L^{-1} of concentrations respectively, which were based on their recommended doses (500 mg L^{-1} of Colonel-S® and 25 mg L^{-1} of Decis®) used for plant protection technique in the agricultural field. The predefined numbers of fertilized eggs were immersed in these three dose concentrations of each insecticide on 4th day (stage 24, Hamburger and Hamilton[32]) and 7th day (stage 31, Hamburger and Hamilton[32]) of their incubation for 60 min at 37°C. Vehicle control and untreated control eggs were immersed in distilled water and with no treatment respectively. Thirty eggs were assigned for each treatment groups. All the treated and control eggs

were kept for reincubation and candled daily to determine the survivability of embryos. The infertile eggs as well as dead embryos were discarded. Chick embryos were sacrificed on embryonic day (ED) 16 (stage 42, Hamburger and Hamilton[32]) for analysis of effects of Colonel-S® and Decis® on their survivability rate, wet body weight, gross morphological and skeletal development.

Histological procedure for skeletal staining

For visualizing skeletal deformities, 16 day old chick embryos from each group were randomly selected and processed through cartilage and bone double staining method described by McLeod[33] with some modifications. The embryos were washed with water, eviscerated and then fixed in absolute ethyl alcohol for 7 days. Embryos were stained for 4 days at 37°C in a solution prepared by mixing (a) 1 volume 0.3% (300 mg) filtered Alcian Blue in 70% ethyl alcohol, (b) 1 volume 0.1% (100mg) filtered Alizarin Red-S in 95% ethyl alcohol,(c) 1 volume glacial acetic acid and (d) 1 volume 70% ethyl alcohol. At least 100ml of the resulting staining solution was used per embryo. After staining, all the embryos were washed for 2 hours in tap water and placed in 1% aqueous potassium hydroxide (KOH) solution for 12- 48 hours. Macerated, stained specimens were cleared by aqueous solution of ascending gradual concentration of glycerol (20, 50 and 80 %) diluted with 1% KOH, for 3 days for each step, then transferred into 100% glycerol to which a few crystals of thymol crystals were added to avoid mold proliferation. The stained skeletal elements of embryos were kept and stored in 100 % glycerol until they were examined and photographed.

Incidence of external malformations

The morphological abnormalities observed were comprised of (a) *Head region*: absence of head (acephaly), small size of brain (microcephaly), exposure of brain through the skull (exencephaly), absence of large part of brain (anencephaly), blood patches (hematomas), (b) *Eye*: small eye (microphthalmia), eyes entirely missing (anophthalmia), absence of eyelids (ablepharia), swelling and edema of eye, bulging eyes (exophthalmia), (c) *Neck:* narrow neck, twisted neck, *Beak:* defects in development of beak and cleft or parrot beak, (d) *Lower body*: general growth retardation, internal organ abnormally exposed (ectopia viscera/gastroschisis) subcutaneous hemorrhage, hematomas, (e) *Limbs*: short, undeveloped and/or twisted upper limb or lower limb and their digits.

Incidence of skeletal malformations

The incidence of various abnormalities observed in skeleton of treated animals were of (a) *Skull:* retarded ossification, short maxilla, short mandible, congenital absence of cranium either partial or complete (acrania), (b) *Vertebrae:* not ossified, absent, displaced or fused, lateral curvature of spine (scoliosis), anterior curvature of spine (lordiosis), posterior curvature of spine (kyphosis), reduction in size of caudal part of skeleton, complete

absence of tail or kinky tail (Caudal Regression Syndrome), (c) *Ribs:* incomplete ossification or poorly ossified, absent, displaced, wavy or flying, union of separate bone by osseous tissue (synostosis), (d) *Sternum:* not ossified or poorly ossified, displaced, dumb bell shaped, synostosis, congenital absence of sternum (asternia), (e) *Upper limb:* not ossified or poorly ossified, shortened, bent or displaced (humerus, radius, ulna, matacarpals and phalanges), (f) *Lower limb:* not ossified or poorly ossified, shortened, bent or displaced (pelvis, femur, tibia, fibula, metatarsals and phalanges). Any other changes in the axial and appendicular skeleton (such as absence of bones and lack or reduction of cartilage) were also examined.

Analytical methods

Student's "t" test was used to analyze the wet body weights of all the chick embryos and their values were expressed as mean± standard error (SE). The quantified data for survivability and number of abnormal survivors with external and skeletal malformations were represented in percentages and statistically analyzed using Mann-Whitney "U" test. The values of p were considered to be significant at 0.05, 0.01 and 0.001 against that of control II.

RESULTS AND DISCUSION

From Table 1 it can be estimated that when the eggs were treated on ED 4 with different doses of each insecticide, no significant ($p{\geq}0.05$) decrease in number of surviving embryos was observed after Colonel-S® exposure, but in case of Decis® a significant ($p{\leq}0.05$) decrease was exhibited by the embryos treated with its 50 mg L^{-1} of dose concentration. Whereas, exposure of eggs with each of the insecticide on ED 7 resulted in a significant ($p{\leq}0.05$) decrease of survivability rate of their developing embryos only at 1000 mg L^{-1} of Colonel-S® application (Table 2).

The mean body weight of embryos did not change with respect to Colonel-S® treatment on ED 4, but Decis® showed significant decrease at 25 mg L^{-1} (11.50±1.37 g; $p{\leq}0.05$) and 50 mg L^{-1} (11.44±1.07 g; $p{\leq}0.01$) of its dose concentrations when compared with that of control II (Table 1). The exposure of eggs towards Colonel-S® and Decis® on ED 7 resulted in significant ($p{\leq}0.05$) decrease of embryonic mean body weight (10.26±0.68 g) only at 1000 mg L^{-1} of Colonel-S® (Table 2). Whereas, embryonic body weights of other insecticide treated groups and control groups remained unaffected.

Table 1: Toxicity of Colonel-S® and Decis® on embryonic day 16 of chick embryo recovered from eggs treated on 4th day of incubation

Treatment	Dose	Number of eggs taken	Number of surviving embryos	Wet body weight of surviving embryo# (g)	Abnormal survivors						
					No of surviving embryos with malformations	Incidence of external malformations					
						Head	Eye	Beak	Neck	Lower body	Limb
Control I	0	30	25 (83%)	16.53±0.94	3 (12%)	1	1	1	1	2	0
Control II	Vehicle	30	27 (90%)	15.57±0.64	3 (11%)	2	1	0	0	2	2
Colonel-S®	250 mg L^{-1}	30	23 (77%)	15.61±1.38	8 (35%)	3	1	2	2	3	1
	500 mg L^{-1}	30	21 (70%)	15.92±0.76	10 (48%) †	1	0	3	3	4	3
	1000 mg L^{-1}	30	19 (63%)	15.83±0.82	8 (42%)	1	1	3	3	4	3
Decis®	12.5 mg L^{-1}	30	20 (67%)	12.72±1.34	8 (40%)	2	1	2	2	3	2
	25 mg L^{-1}	30	19 (63%)	11.50±1.37*	12 (63%) ††	3	3	4	4	5	5
	50 mg L^{-1}	30	17 (57%) †	11.44±1.07**	13 (76%) †††	3	3	3	4	6	5

each value represents Mean±Standard error of 5 animals per treatment group; Statistical difference from the control II: † significant at p≤0.05; †† significant at p≤0.01; ††† significant at p≤0.001 using Mann- Whitney "U" test; * Significant at p≤0.05; ** significant at p≤0.01 using student "t" test.

Table 2: Toxicity of Colonel-S® and Decis® on embryonic day 16 of chick embryo recovered from eggs treated on 7th day of incubation

Treatment	Dose	Number of eggs taken	Number of surviving embryos	Wet body weight of surviving embryo# (g)	Abnormal survivors						
					No of surviving embryos with malformations	Incidence of external malformations					
						Head	Eye	Beak	Neck	Lower body	Limb
Control I	0	30	24 (80%)	13.05±0.49	3 (11%)	1	1	2	1	2	1
Control II	Vehicle	30	25 (83%)	12.05±0.22	2 (7%)	0	0	0	0	1	0
Colonel-S®	250 mg L^{-1}	30	21 (70%)	12.19±0.47	9 (43%) †	1	1	2	3	4	3
	500 mg L^{-1}	30	18 (60%)	12.70±0.62	8 (44%) †	1	1	1	2	3	2
	1000 mg L^{-1}	30	16 (53%) †	10.26±0.68*	9 (56%) ††	1	2	3	2	5	4
Decis®	12.5 mg L^{-1}	30	23 (77%)	12.76±0.48	8 (35%)	1	0	2	2	3	4
	25 mg L^{-1}	30	21 (70%)	12.65±1.06	10 (48%) †	2	2	3	3	5	4
	50 mg L^{-1}	30	17 (57%)	12.15±0.59	9 (53%) †	1	1	3	3	4	4

each value represents Mean±Standard error of 5 animals per treatment group; Statistical difference from the control II: † significant at p≤0.05, †† significant at p≤0.01 using Mann- Whitney "U" test; * Significant at p≤0.05 using student "t" test.
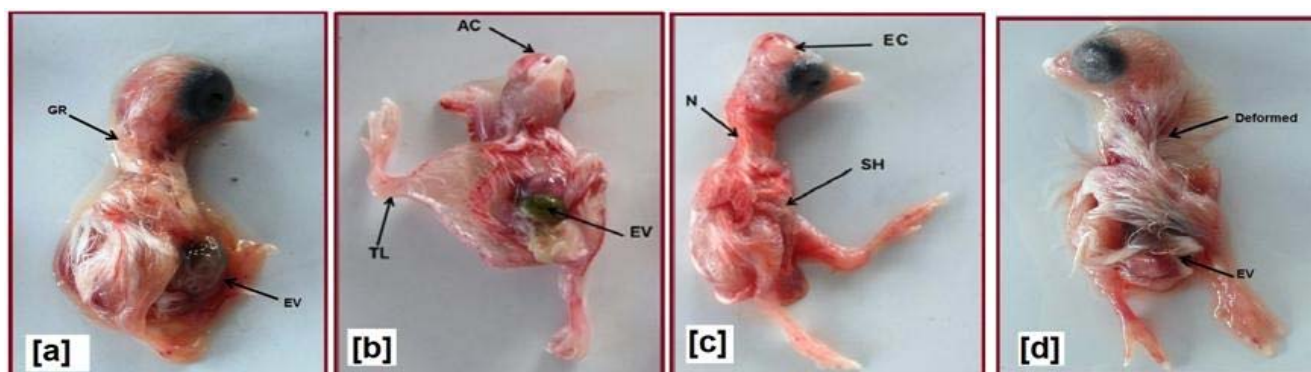
Figure 1: Photographs of 16 day old chick embryo [a] treated with 1000 mg L^{-1} of Colonel-S[®] on ED 4 showing ectopia viscera (EV) and overall growth retardation (GR). [b] treated with 50 mg L^{-1} of Decis[®] on ED 4 showing anencephaly (AC), twisted leg (TL) and ectopia viscera (EV). [c] treated 25 mg L^{-1} of Decis[®] on ED 7 showing exencephaly (EC), narrow neck (N) and subcutaneous hemorrhage (SH).[d] deformed embryo treated with 250 mg L^{-1} of Colonel-S[®] on ED 7 showing ectopia viscera (EV).

Table 3: Effect of Colonel-S® and Decis® on skeleton development of 16 day old chick embryo recovered from eggs treated on 4th day of incubation

Treatment	Dose	Number of embryo skeleton examined	Number of embryo with skeleton abnormalities	Incidence of skeleton abnormalities					
				Axial				Appendicular	
				Skull	Vertebrae	Ribs	Sternum	Upper Limb	Lower Limb
Control I	0	15	2 (13%)	0	1	1	0	0	0
Control II	Vehicle	13	1 (8%)	0	1	0	0	0	0
Colonel-S®	250 mg L^{-1}	12	5 (42%)	2	3	2	0	1	1
	500 mg L^{-1}	11	5 (45%)	1	3	2	1	1	0
	1000 mg L^{-1}	12	6 (50%)	2	4	2	0	1	1
Decis®	12.5 mg L^{-1}	10	4 (40%)	1	2	2	0	1	1
	25 mg L^{-1}	9	5 (56%)	2	3	3	1	1	0
	50 mg L^{-1}	11	6 (55%)	2	4	3	1	2	1

Table 4: Effect of Colonel-S® and Decis® on skeleton development of 16 day old chick embryo recovered from eggs treated on 7th day of incubation

Treatment	Dose	Number of embryo skeleton examined	Number of embryo with skeleton abnormalities	Incidence of skeleton abnormalities					
				Axial				Appendicular	
				Skull	Vertebrae	Ribs	Sternum	Upper Limb	Lower Limb
Control I	0	14	2 (14%)	0	1	1	0	0	1
Control II	Vehicle	12	2 (17%)	1	1	1	0	1	1
Colonel-S®	250 mg L^{-1}	10	5 (50%)	1	3	2	1	2	2
	500 mg L^{-1}	7	3 (43%)	1	2	2	0	1	1
	1000 mg L^{-1}	8	3 (38%)	1	2	2	0	1	1
Decis®	12.5 mg L^{-1}	12	4 (33%)	2	4	3	1	2	1
	25 mg L^{-1}	10	4 (40%)	2	3	2	0	1	1
	50 mg L^{-1}	8	5 (63%)	2	4	3	1	2	2

The 16 day old untreated and vehicle treated control embryos treated on ED 4 and 7 showed normal and identical development according to the stage 42 described by Hamburger and Hamilton.[32] Malformations observed in some of these animals were within normal standard range, while a higher percentage of abnormal survivors were recorded in all the insecticide treated groups (Figure 1).

The Colonel-S[®] treatment on ED 4 resulted 35 (p≥0.05), 48 (p≤ 0.05) and 42% (p≥0.05) of abnormal survivors respectively at 250, 500 and 1000 mg L^{-1} of its dose applications, while Decis® revealed 40 (p≥0.05), 63 (p≤ 0.01) and 76% (p≤ 0.001) of abnormal living embryos at 12.5, 25 and 50 mg L^{-1} of its dose concentrations, respectively. The lower body malformations such as general growth retardation and ectopia viscera were quite common in surviving embryos followed by limb defects. For example, 35% (6 out of 17) and 29% (5 out of 17) of embryos exhibited lower body and limb malformations, respectively when they were exposed to 50 mg L^{-1} of Decis®(Figure 1b) on ED 4.

The eggs which were treated on ED 7 with 250, 500 and 1000 mg L^{-1} of Colonel-S® showed 43% (p≤ 0.05), 44 % (p≤ 0.05) and 56 % (p≤ 0.01) of abnormal survivors respectively, whereas Decis® exposure at dose levels of 25 mg L^{-1} and 50 mg L^{-1} resulted in 48 and 53% of abnormal survivors respectively, which was significantly (p≤ 0.05) higher than that of control II (Table 2). Most of the abnormal survivors from insecticide treated group exhibited lower body malformations such as ectopia viscera and hematoma, followed by limb and eye defects. 31 % (5 out of 16) of embryos treated with 1000 mg L^{-1} of Colonel-S® on ED 7 showed lower body defects, whereas 25% (4 out of 16) of embryos exhibited limb defects.

Table 3 demonstrates that the higher percentages (insignificant, p≥0.05) of embryos with skeletal anomalies were recorded in the groups treated with insecticide on ED 4 when compared with that of vehicle treated control group. The groups of egg treated with 250, 500 and 1000 mg L^{-1} of Colonel-S® had 42, 45 and 50% of embryos with skeletal defects, while Decis® resulted in 40, 56 and 55% of embryos with skeletal defects respectively, at 12.5, 25 and 50 mg L^{-1} of its dose applications (Table 3). The spectrum of skeletal defects observed in these animals was same as described earlier in incidence of skeletal malformations (Figure 2). Double stained skeleton element of embryos showed that most of these animals exhibited vertebrae defects followed by ribs and skull defects. 36% (4 out of 11) of embryos treated with 50 mg L^{-1} of Decis® on ED 4 revealed vertebrae defects, while skull and ribs malformations were observed only in 18% (2 out of 11) and 27% (3 out of 11) of embryos.

Similarly, embryos treated with each of the insecticide on ED 7 showed certain abnormalities in their axial and appendicular skeleton which were found as insignificant (p≥0.05). Maximum number of embryos (63%; 5 out of 8) with skeletal malformations was observed in the group treated with 50 mg L^{-1} of Decis®. Among these, 50 % (4 out of 8) of embryos exhibited vertebral defects (such as poor ossification, synostosis, lordiosis and CRS) of their axial skeleton element (Table 4). The skeleton elements of embryos from both untreated and vehicle treated control groups had normal development with complete ossification of their bones.
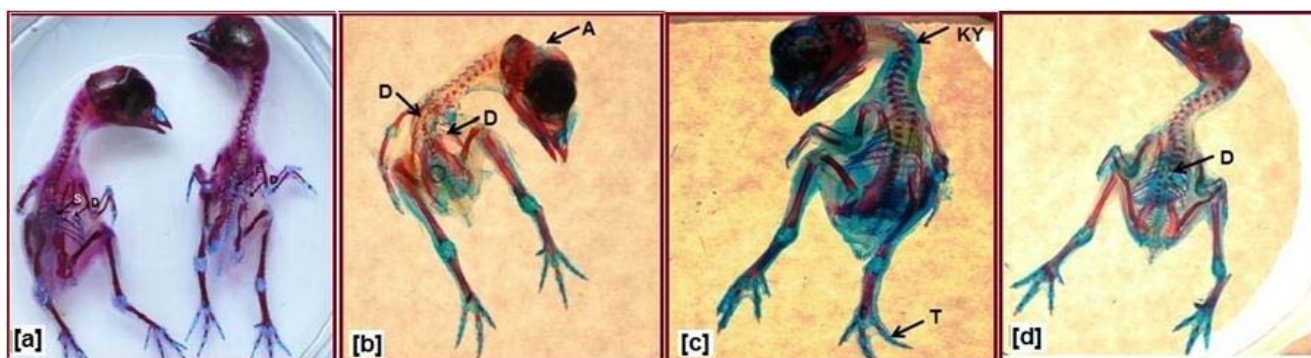

Figure 2: Photographs of double stained skeleton element of 16 day old chick embryo [a] treated with 250 mg L^{-1} of Colonel-S[®] on ED 4 showing scoliosis (S) of spine, displaced (D) ribs and fused (F) vertebrae. [b] treated with 250 mg L^{-1} of Colonel-S[®] on ED 7 showing poor ossification with acrania (A), displaced (D) vertebrae and displaced (D) ribs. [c] treated with 1000 mg L^{-1} of Colonel-S[®] on ED 7 showing kyphosis (KY) and twisted (T) phalanges of leg. [d] treated with 12.5 mg L^{-1} of Decis[®] on ED 7 showing displaced (D) vertebrae.

A large number of environmental as well as manmade chemicals such as drug, toxin, solvents and pesticides exert their toxic effect by interfering with fundamental developmental mechanisms of an organism and evert them from reaching their proper end points.[34] These environmental stressors are also known as teratogens, responsible for causing teratogenicity (which includes permanent structural and functional birth abnormalities) in developing embryo or fetus even though these agents have either negligible or no maternal effects. These changes can include lethal events resulting before or shortly after birth.[35] Susceptibility of teratogenicity in an organism towards any teratogen depends on many factors such as genotype of an organism including species as well as strain differences, critical developmental stage at which the organisms are exposed, dose and route of teratogen and also on the types of initiating mechanism of teratogenesis.[36]

Oxidative stress is reported to be a major mechanism followed by other mechanisms such as mutation, chromosomal abnormalities, mitotic interference, interference with nucleic acid function, nutrient deficiencies, deficient or altered energy supply, change in osmolarity, ultrastructure changes in cell membrane and

disruption of retinoid acid signaling pathway by which different types of teratogen exert their developmental effects.[36,37] The utilization of oxygen in metabolism is critical to early developmental stages of organisms.[37] It is associated with the generation of reactive oxygen species (ROS) such as hydrogen oxides, alkyl peroxides and hydroxyl radicals.[38] These ROS serve as second messenger to play important role in signal transduction and affect several physiological and pathological functions in an organism which include ion transport, immunological host defense, transcription and apoptosis of unwanted cells.[37,39] However, ROS levels must be continuously checked by antioxidants or anti oxidative enzymes to prevent them from binding covalently or irreversibly to cellular macromolecules (e.g., proteins, lipids, DNA and RNA) which can otherwise lead to inactivation of many enzyme and cell death.[39] A number of non toxic chemicals or xenobiotics are responsible in increasing oxidative stress (an imbalance between ROS generation and its control) as these can be enzymatically bioactivated to highly toxic electrophilic or free radical reactive intermediates which react directly or indirectly with molecular oxygen to initiate the formation of ROS.[35] van Gelder et al.[39] stated that susceptibility of developing embryo to high level of ROS get increased by its weak antioxidant defense mechanism, particularly during early stages of organogenesis causing various teratogenic effects.

The present study is an attempt to evaluate the teratogenic effects of insecticides Colonel-S® and Decis®. The data presented in this study revealed that exposure of fertilized eggs to both of these insecticides resulted in decreased numbers of surviving embryos at their high doses. Similar to our results, Petrovova et al.[40] also reported increased mortality of chick embryo exposed to bendiocarb on embryonic day 2, 3, 4, 5 and 10. Further, the present findings are in agreement with work reported by several investigators who reported decreased rate of survivability in chick embryo exposed to various pesticides such as carbaryl, methyl parathion, malathion and endosulfan, dimecron, dimethoate containing formulation, dimethoate, benfluralin and S-metolachlor, flufenoxuron and lufenuron.[41-48] The toxicity of deltamethrin containing insecticide as described presently with Decis® were also reported by earlier investigators using different experimental models. Koprucu and Aydin[17] in their study with *Cyprinus carpio* reported that the number of dead embryo/larvae increased significantly in response to deltamethrin concentrations (0.005, 0.05, 5, 25 and 50 µg/L) when exposed during their embryonal stage. Similarly, Datta and Kaviraj[14] and Koprucu et al.[19] also reported mortality in early stages of freshwater catfish *Clarias gariepinus* and European catfish *Silurus glanis* due to exposure of deltamethrin containing insecticide formulations; K-Obiol and Decis, respectively.

Presently, the prominent decrease in body weight was observed in those surviving embryos which were recovered from eggs exposed with 25 and 50 mg L^{-1} of Decis® on 4th day of incubation and 1000 mg L^{-1} of Colonel-S® on 7th day of incubation which ultimately resulted in overall growth retardation of developing animals. Petrovova et al.[40] have also noticed decrease in body weight of chick embryo with clear correlation with dose concentrations of bendiocarb when administrated on embryonic day 5 and 10. Further, overall growth retardation, because of decreased body weight was also observed in the methyl-parathion treated chick embryo, which was evident only when this insecticide was exposed on 4th day of incubation at dose concentrations of 10 or 50 µg and on the 6th day of incubation only at 50 µg of dose level.[42] Similarly, dose dependent decrease in the body weight was also reported in the chick embryo exposed to malathion at 5mg/egg of dose level.[43]

In the present study, exposure of developing chick embryo on two different critical period of embryogenesis (embryonic period; ED 4 and fetal period; ED 7) with the three dosage levels of each insecticide; Colonel-S® and Decis® resulted in higher percentage of abnormal survivors which indicate teratogenic susceptibility of these insecticides. Wells et al.[35] and Levi[36] stated that the embryonic period and fetal period of embryogenesis are hallmarks of teratological risks as organs are usually more susceptible to abnormal development only if exposed either during early events of their formation *i.e.* during organogenesis phase (embryonic period) or during their differentiation and functional development (fetal period).

Each malformed embryo observed in the present study exhibited one type or 2-4 type of external and skeletal malformations. Some of presently observed morphological malformations are in accordance with the work of Swartz[41] who have observed flexion in phalanges of legs in carbaryl treated chick embryo and attributed cause of such deformities in legs to specialized effect of insecticide at neuromuscular junction of the peripheral nerves. Similarly, our present findings are also in accordance with reports of Kumar and Devi[42] who found predominant teratological changes such as short neck, abdominal hernias and hemorrhagic spots on the brain and upper body of 20 day old methyl parathion treated chick embryos and with reports of Friedberg and Gartner[49] who noted cranial hematomas and various type of eye and beak deformities in the chick embryo treated with formocresol. Sahu and Ghatak[44] noticed few malformations such as abnormal development of brain filled with blood clot, exencephaly, unilateral anophthalmia, parrot beak and ectopia viscera in chick embryo treated with insecticide dimecron and suggested that formation and development of eye could be affected by injury of roof plate of neural tube and also the suppression of nicotinamide adenine dinucleotide level in the embryo might have contributed toward various other malformations. Further, Seifert and Casida[50] found that certain organophosphorus insecticides and carbaryl treated chick embryos responded by developing structural anomalies such as micromelia and parrot beak

which could have resulted from inhibition of kynurenine formamidase which impairs conversion of tryptophan to essential pyridine nucleotide cofactors in the yolk sac membrane possibly in the embryonic liver at later stage of development. Indyk[51] reported that exposure of fertilized eggs with propotox prior to, on 3rd and 19th day of incubation revealed several type of anomalies such as beak deformities (parrot beak), twisted neck and bent toes in the surviving chick embryos. External malformations such as reduction in size of head and size of eye, incomplete development and in some cases totally absence of beak were also observed by Anwar[52,53] in 7 and 11 day old chick embryo following treatments with 100, 200 and 400 ppm of cypermethrin.

The present results confirm the finding of several workers who reported that the treatment of insecticide was clearly associated with severe alterations in the development of bones and cartilage of chick embryo which ultimately resulted in various axial and appendicular skeletal anomalies. According to, Uggini et al.[34], the insecticides are known to affect the neurotransmission and such hindrance in neural and acetylcholinesterase activity can attribute to various vertebral malformations. Our findings are in synchronization with the findings of Friedberg and Gartner[49] who reported alteration in the formation of cartilage and bone and various types of limb deformities in the chick embryo exposed with formocresol. Similarly, Misawa et al.[54] reported the inhibition in the growth of skeletal elements and fused cervical rings in 9 day old chick embryo following exposure to two organophosphate insecticides- diazinon and dicrotophos on 3rd day of incubation. Furthermore, some malformations particularly of distal portions of lower limbs such as tibial and metatarsal angulations with their curtailment were observed by L'Abbate et al.[55] in chick embryo exposed to insecticide carbaryl on 5th and 6th day of incubation. These authors suggested that interference of insecticide in the synthesis of nicotinamide adenine dinucleotide might have been responsible for causing such skeletal defects of lower limbs. The incomplete or poor ossification of bone as observed in the present study can result from interference of insecticide with cellular and molecular mechanism of two principal pathways of embryonic skeletal development; endochondral pathway and intramembranous pathway which involve programmed differentiation and morphogenesis of mesoderm. The process of endochondral bone formation occurs in the long bones and vertebrae during which mesenchymal cell condense and expand to form a structure similar to that of long bone, undergo chondrogenesis and form cartilage. Subsequently, chondrocytes of formed cartilage undergo hypertrophy and finally replaced by bones.[56, 57] On the other hand, intramembranous ossification pathway of skeletal development involves direct differentiation of mesenchymal cells and osteoblasts, with no cartilage intermediate. This process is observed in various

craniofacial bones.[58] Khalid et al.[59] suggested that the activation of estrogen receptors α and β by the insecticides may result in various skeletal malformations as development and growth of bones are affected by estrogens. Our results are in accordance with Pinakin et al.[48] who had observed skeletal anomalies such as deformed ribs and vertebrae and CRS in the chick embryos treated with lufenuron, while Heinz et al.[60] recorded malformations in spine (lordiosis and scoliosis) of methymercury treated chick embryos. The skeletal defects such as scoliosis, fused cervical vertebrae, lordiosis, wavy ribs and bill defects were also reported by Hoffman and Gay[61] in avian embryos exposed to insecticide parathion on embryonic day 3. Furthermore, our results are also in conformity with the findings of Verrette et al.[62], Sullivan[63], Maci and Arias[64], Garg et al.[65] and Natekar[66] who reported several types of skeletal deformities in developing chick embryos induced with various other xenobiotics.

CONCLUSION

The results of present study emphasized that a single dipping treatment of fertilized eggs by immersion technique in aqueous solutions of commercial available insecticide formulations *i.e.* Colonel-S**®** and Decis® were teratogenic for chick embryo. And therefore, it should be recommended that usage of these or similar types of insecticide formulations should be prevented or limited in the environment where pregnant animal or woman live.

REFERENCES

1. Damalas CA, Eleftherohorinos IG, Pesticide exposure, safety issues, and risk assessment indicators, Int. J. Environ. Res. Public Health, 8, 2011, 1402-1419.

2. Pretty J, Hine R, Pesticide use and the environment, In the Pesticide Detox: Towards a More Sustainable Agriculture (Edited by Pretty J), EARTHSCAN, London, Sterling, UK, 2005, 1-22.

3. Savithri Y, Sekhar PR, Rani KU, Doss PJ, Toxicity of chlorpyrifos on total proteins, free amino acids and aminotransferase activity levels in liver and kidney tissues of albino rats, Adv. Pharmacol. Toxicol, 11, 2010, 13-19.

4. Extension Toxicology Network (EXTOXNET), Pesticide Information Profile - Dicofol, 1996. Available at- Extoxnet.orst.edu/pips/dicofol.htm.

5. Sobti RC, Krishna A, Davies J, Cytokinetic and cytogenetic effects of agricultural chemicals on human lymphoid cells *in vitro.* II. Organochlorine pesticides, Arch. Toxicol, 52, 1983, 221-231.

6. Sato H, Toyoda K, Furukawa F, Hasegawa R, Takahashi M, Hayashi Y, Subchronic oral toxicity test of dicofol (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethanol) as the basis for the design of a long-term carcinogenicity study in $B_6C_3F_1$ mice, Bulletin of National Institute of Hygienic Sciences, 105, 1987, 42-45.

7. Grasso P, Sharratt M, Davies DM, Irvine D, Neurophysiological and psychological disorders and occupational exposure to organic solvents, Food Chem. Toxicol, 22, 1984, 819-52.

8. Jadarmkunti UC, Kaliwal BB, Possible mechanisms for anti-implantation action of dicofol in albino rats, J. Basic. Clin. Physiol. Pharmacol, 12, 2001, 217-226.

9. Jadaramkunti UC, Kaliwal BB, Dicofol formulation induced toxicity on testes and accessory reproductive organs in albino rats, Bull. Environ. Contam. Toxicol, 69, 2002, 741-748.

10. El-Kashoury AA, Salama A F, Selim AI, Mohamed RA, Animal model study of reproductive toxicity of the chronic exposure of dicofol, Life Science Journal, 6, 2009, 1-18.

11. El-Kashoury AA, Salama A F, Selim AI, Mohamed RA, Chronic exposure of dicofol promotes reproductive toxicity in male rats, Life Science Journal, 7, 2010, 5-19.

12. Clark DR, Flickinger EL, White DH, Hothern RL, Belisle AA, Dicofol and DDT residues in lizard carcasses and bird eggs from Texas, Florida and California, Bull. Environ. Contam. Toxicol, 54, 1995, 817-824.

13. MacLellan KN, Bird DM, Cowles JL, Reproductive and morphological effects of o, p'-dicofol on two generations of captive American kestrels, Arch. Environ. Contam. Toxicol, 30, 1996, 364-372.

14. Datta M, Kaviraj A, Acute toxicity to the synthetic pyrethroid deltamethrin to freshwater catfish *Clarias gariepinus,* Bull. Environ. Contam. Toxicol, 70, 2003, 296-299.

15. Patro N, Mishra SK, Chattopadhyay M, Patro IK, Neurotoxicological effects of deltamethrin on the postanatal development of cerebellum of rats, J. Biosci, 22, 1997, 117-130.

16. Shrivastava B, Shrivastava A, Kumar A, Bhatt JL, Bhajpai SP, Parihar SS, Bhatnagar V, Impact of deltamethrin on environment, use as an Insecticide and its Bacterial degradation- A preliminary study, International Journal of Environmental science, 1, 2011, 77-985.

17. Köprücü K, Aydin R, The toxic effect of pyrethroid deltamethrin on the common carp (*Cyprinus carpio* L.) embryos and larvae, Pest. Biochem. Physiol, 80, 2004, 47-53.

18. Ural MS, Saglam N, A study on the acute toxicity of pyrethroid deltamethrin on the fry rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792), Pest. Biochem. Physiol 83, 2005, 124–131.

19. Koprucu SS, Koprucu K, Ural MS, Acute toxicity of the synthetic pyrethroid deltamethrin to fingerling European Catfish, *Silurus glanis* L, Bull. Environ. Contam. Toxicol, 76, 2006, 59-65.

20. Velíšek J, Dobšíková R, Svobodová Z, Modrá H, Lusková V, Effect of deltamethrin on the biochemical profile of common carp (*Cyprinus carpio* L.), Bull. Environ. Contam. Toxicol, 76, 2006, 992–998.

21. Sharma DK, Ansari BA, Effect of deltamethrin and a neem based pesticide Achook on some biochemical parameters in tissues liver, ovary and muscle of Zebrafish *Danio rerio* (Cyprinidae), Res. J. Chem. Sci, 1, 2011, 125-134.

22. Amin KA, Hashem KS, Deltamethrin-induced oxidative stress and biochemical changes in tissues and blood of catfish (*Clarias gariepinus*): antioxidant defense and role of alpha-tocopherol, BMC Veterinary Research, 8, 2012, 45.

23. Martin PA, Effects of carbofuran, chlorpyrifos and deltamethrin on hatchability, deformity, chick size and incubation time of Japanese quail (*Coturnix japonica*) eggs, Environmental Toxicology and Chemistry, 9, 1990, 529–534.

24. Koprucu K, Seker E, Acute Toxicity of deltamethrin for freshwater Mussel, *Unio elongatulus eucirrus* Bourguignat, Bull. Environ. Contam. Toxicol, 80, 2008, 1–4.

25. Xiu R, Xu Y, Gao S, Toxicity of the new pyrethroid insecticide, deltamethrin, to *Daphnia magna*, Hydrobiologia, 188/189, 1989, 411-413.

26. Salibian A, Effects of deltamethrin on the South American toad, *Bufo arenarum*, Tadpoles, Bull. Environ. Contam. Toxicol, 48, 1992, 616-621.

27. WHO, Environmental Health Criteria 97-Deltamethrin, International Programme on Chemical Safety (IPCS), WHO, Geneva, Switzerland, 1990, 1-133.

28. Abdel-Khalik MM, Hanafy MS, Abdel_Aziz MI, Studies on the teratogenic effects of deltamethrin in rats, Dtsch Tierarzti Wochenschr, 100, 1993, 142-143.

29. Manna S, Bhattacharyya D, Mandal TK, Das S, Repeated dose toxicity of deltamethrin in rats, Indian J. Pharmacol, 37, 2005, 160-164.

30. Moid N, Patel F, Shrimali A, Desai KR, Highland HN, Toxicological implications of type II pyrethroid, deltamethrin on certain tissues of Swiss albino mice, International journal of Pharmaceutical science and health care, 5, 2012, 41-54.

31. Ozkan O, Ustuner O, Investigations about genotoxicity of deltamethrin, Kafkar. Univ. Vet. Fak. Derg, 18, 2012, 69-74.

32. Hamburger V, Hamilton HL, A series of normal stages in the development of the chick embryo, J. Morph, 88, 1951, 49-92.

33. McLeod MJ, Differential staining of cartilage and bone in whole mouse fetuses by Alcian blue and Alizarin red S, Teratology, 22, 1980, 299-301.

34. Uggini GK, Patel PV, Balakrishnan S, Embryotoxic and teratogenic effects of pesticides in chick embryos; a comparative study using two commercial formulations, Environ. Toxicol, 27, 2012, 166-174.

35. Wells PG, Bhuller Y, Chen CS, Jeng W, Kasapinovic S, Kennedy JC, Kim PM, Laposa RR, McCallum GP, Nicol CJ, Parman T, Wiley MJ, Wong AW, Molecular and biochemical mechanisms in teratogenesis involving reactive oxygen species, Toxicol. Appl. Pharmacol, 207, 2005, S354 – S366.

36. Levi PE, Toxic Action, In A Textbook of Modern Toxicology (Edited by Hodgson E, Levi PE), New York: Elsevier, McGraw-Hill Companies, 1987, 133-184.

37. Paskova V, Hilscherova K, Blaha L, Teratogenicity and embryotoxicity in aquatic organisms after pesticide exposure and the role of oxidative stress, Rev. Environ. Contam. Toxicol, 211, 2011, 25-61.

38. Han D, Hanawa N, Saberi B, Kaplowitz N, Mechanisms of liver injury. III. Role of glutathione redox status in liver injury, Am. J. Physiol. Gastrointest. Liver. Physiol, 291, 2006, G1-G7.

39. van Gelder MM, van Rooij IA, Miller RK, Zielhuis GA, de Jong-van den Berg LT, Roeleveld N, Teratogenic mechanisms of medical drugs, Hum. Reprod. Update, 16, 2010, 378-394.

40. Petrovova E, Mazensky D, Vdoviakova K, Massanyi P, Luptákova L, Smrco P, Effect of bendiocarb on development of the chick embryo, J. Appl. Toxicol, 30, 2010, 397-401.

41. Swartz WJ, Long and short term effects of carbaryl exposure in chick embryo, Environ. Res, 32, 1981, 463-472.

42. Kumar S, Devi KS, Teratogenic effects of methyl parathion in developing chick embryo, Vet. Hum. Toxicol, 34, 1992, 408-409.

43. Pourmirza AA, Toxic effects of malathion and endosulfan on chick embryo, J. Agr. Sci. Tech, 2, 2000, 161-166.

44. Sahu CR, Ghatak S, Effect of dimecron on developing chick embryo: malformations and other histopathological changes, Anat. Histol. Embryol, 31, 2002, 15-20.

45. Budai P, Fejes S, Várnagy L, Somlyay IM, Szabó ZK, Teratogenicity test of dimethoate containing insecticide formulation and Cd-Sulphate in chicken embryos after administration as a single compound or in combination, Commun. Agric. Appl. Biol. Sci, 68, 2003, 795-798.

46. Keseru M, Budai P, Varnagy L, Szabo R, Juhasz E, Babinszky G, Pongracz A, (2004) Teratogenicity study of some pesticides in chicken embryos, Cummum. Agric. Biol. Sci, 69, 2004, 803-806.

47. Rachid R, Houria DB, Reda DM, Impact of flufenoxuron, an IGR pesticide on *Gallus domesticus* embryonic development *in ovo*, J. Cell. Anim. Biol, 2, 2008, 087-091.

48. Pinakin W, Deshpande SG, Salokhe SG, Studies on the effect of the insect growth regulator lufenuron on embryogenesis of chick *Gallus domesticus* (White leghorn strain), Int. J. Pharm. Bio, 1, 2011, 82-88.

49. Friedberg BH, Gartner LP, Embryotoxicity and teratogenicity of formocresol on developing chick embryos, J. Endodont, 16, 1990, 434-437.

50. Seifert J, Casida JE, Relation of yolk sac membrane kynurenine formamidase inhibition to certain teratogenic effects of organophosphorus insecticides and of carbaryl and serine in chicken embryos, Biochem. Pharmacol, 27, 1978, 2611-2615.

51. Indyk F, Effects of insecticide propotox M on survival, hatching success, and development of the chick embryo, Zool. Pol, 44, 1999, 47-57.

52. Anwar K, Cypermethrin, a pyrethroid insecticide induces teratological and biochemical changes in young chick embryo, Pakistan. J. Biol. Sci, 16, 2003a, 1698-1705.

53. Anwar K, Toxic effects of cypermethrin on the biochemistry and morphology of 11th day chick embryo (*Gallus domesticus*), Pakistan Journal of Applied Sciences, 3, 2003b, 432-445.

54. Misawa M, Doull J, Uyeki EM, Teratogenic effects of cholinergic insecticides in chick embryos. III. Development of cartilage and bone, J. Toxicol. Environ. Health, 10, 1982, 551-63.

55. L'Abbate N, Virgintino D, Ribatti D, Roncali L, Miccoli MG, Ambrosi L, Effects of carbaryl on the morphogenesis of the extremities in chick embryos, G. Ital. Med. Lav, 8, 1986, 123-126.

56. Caplan AI, Boyan BD, Endochondral bone formation: The lineage cascade. In: Bone, Vol. 8. (Edited by Hall BK,), CRC Press, Boca Raton, FL, 1994, 1-46.

57. Bayatli KA, Fadhil S, Mohammed, Shakir MM, Morpho-histological study of skull bones development in indigenous goose embryo (*Anser anser domesticus*), International Journal of Advance Biological Research, 2, 2012, 469-476.

58. Langille RM, Differentiation of craniofacial mesenchyme. In: Bone, Vol. 9. (Edited by Hall BK,), CRC Press, Boca Raton, FL, 1994, 1-46.

59. Khalid, Tahir M, Shoro AA, Ginseng induced fetal skeletal malformations, Biomedica, 24, 2008, 96-98.

60. Heinz GH, Hoffman DJ, Klimstra JD, Stebbins KR, Kondrad SL, Erwin CA, Teratogenic effects of injected methylmercury on avian embryos, Environ. Toxicol. Chem, 30, 2011, 1593-1598.

61. Hoffmann DJ, Gay ML, Embryotoxic effects of benzo(a)pyrene, chrysene, and 7,12-dimethylbenz(a)anthracene in petroleum hydrocarbon mixtures in mallard ducks, J. Toxicol. Environ. Health, 7, 1981, 775-787.

62. Verrett MJ, Mutchler MK, Scott WF, Reynaldo EF, Mclaughlin J, (1969) Teratogenic effects of captan and related compounds in the developing chicken embryo, Ann. Ny. Acad. Sci, 160, 1969, 334-343.

63. Sullivan GE, Paralysis and skeletal abnormalities in chick embryos treated with physostigmine, Aust. J. Zool, 23, 1975, 1-8.

64. Maci R, Arias E, Teratogenic effects of the fungicide maneb on chick embryos, Ecotox. Environ. Safety, 13, 1987, 169-173.

65. Garg UK, Pal AK, Jha GJ, Jadhao SB, Pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticide on the bone health of boiler chicks, Toxicol. Pathol, 32, 2004, 364-369.

66. Natekar P, Methotrexate induced gross malformations in chick embryo, J. Hum. Ecol, 21, 2007, 223-226.

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