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RESEARCH ARTICLE

Adverse Effects of Cypermethrin on the Chick (*Galus domesticus*) Development are Reversed by Co-Treatment with Vitamin E and Olive Oil

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ABSTRACT

Cypermethrin (CN) is a type II pyrethroid insecticide that has recently been implicated in reproductive and developmental disorders. The aim of present study was to discover the potential role of vitamin E (vitE) and Olive oil (O) in alleviation of prenatal developmental abnormalities of CN exposure in the domestic chick. Fertilized eggs (60 each) were assigned to vehicle (C); CN, CNO, CNE and CNOE treated groups. Embryos from 20 eggs in each group were recovered on 7, 14 and 21days of incubation. Mean body weight of the hatchlings and the rate of hatching were significantly higher ($P \leq 0.05$) in all groups than that of the CN group. Abnormalities of *in ovo* CN exposure in 7 days old embryos included: growth retardation, reduced beak, microphthalmia, microcephaly, open eyes, abdominal edema and limb deformities. While the 14 and 21 days old embryos exhibited patchy plumage, congenital glaucoma, spina bifida occulta, and limb deformities. Rate of occurrence of these abnormalities were decreased with co-treatment of CN with olive oil or/and vitE. It was thus concluded that CN contained potent avian developmental disruption potentials while the co-treatment of vitamin E and/or olive oil have been found to curtail the chances of CN induced embryonic disruptions in developing chick.

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INTRODUCTION

Because of its rapid insecticidal properties and lesser toxicity in adult land vertebrates cypermethrin (CN) has been frequently used in agricultural and domestic settings in countries like India and Pakistan (Abhilash and Singh, 2009; Khan *et al*., 2012). However, the developing embryos of the non-target animals are much more sensitive to toxic insults as compared to the adults' due to their partial metabolic incapability for detoxification and the easily distortable intricate developmental processes (tissues and organs differentiation) they are rapidly passing through. It is worth mentioning here that the type II pyrethroid insecticides are highly toxic to fish adults (Prusty *et al*., 2015); whereas CN has already been reported to be toxic for the developing zebrafish embryos (Shi *et al*., 2011). Moreover, the combined embryo toxic effects of CN and permethrin in zebrafish have also been reported (Yang *et al*., 2014). Additionally, Svartz *et al*. (2016) has reported the toxic effects of combined exposure of CN and endosulfan on the developing toad (*Rhinella arenarum*) embryos. Furthermore, it has been found that the *in utero* exposure of CN- in mice at 2.5-10 mg/kg/day (pregnant dam's body weight) and in rats at 40-80mg/kg/day- has led to various developmental disruptions in the developing embryos (Farag *et al*., 2007; Madu, 2015). Because of their development in closed eggshell environment the avian embryos (if exposed *in-ovo*) may show severe toxic developmental impacts of insecticides like CN. In this connection, Uggini and Suresh (2013) have indicated the genotoxic potentials of CN (0.1µg/egg) in developing chick embryos.

Cypermethrin exposure has been found to induce oxidative stress mainly through disrupting enzymatic and non-enzymatic antioxidant systems as indicated by the elevated tissue levels of malondialdehyde and the decreased activity of superoxide dismutase, catalase and glutathione peroxidase in rats (Abdul-Hamid *et al*., 2017).

Oxidative stress in turn has been considered to cause various cellular and physiological disorders like cancer, neuronal degenerative and cardiovascular diseases (Rahman *et al.*, 2012). Whereas the natural antioxidants harbor protective capacity against the detrimental effects of free oxygen radicals. Olive oil (a rich source of oleic acid: the monounsaturated fatty acid) has been shown to contain natural anti-oxidative and anti-ageing potentials (Fitó *et al.*, 2007). Good quality extra virgin olive oil (EVO) contains high content of antioxidants, like vitamin K, E, chlorophyll, carotenoids and polyphenols. Additionally, it contains oleocanthal that inhibits the cyclooxygenase activity in prostaglandin biosynthesis and thus contains anti-inflammatory and analgesic effects (Beauchamp *et al.*, 2005). Moreover, oleuropein and its derivatives particularly hydroxyl tyrosol found in EVO have shown superb antioxidant capacity (Oliveras-López *et al.*, 2008). Owing to these unique antioxidant components the routine consumption of EVO has been found to improve expression of antioxidant genes and the overall antioxidant status of human adults without altering the routine metabolic processes (Oliveras-López *et al.*, 2014). The *in ovo* treatment of olive oil has been reported to cause significant decline in embryonic mortality with a simultaneous improvement in hatchability against adverse effects of dietary conjugated linoleic acid (Aydin *et al.*, 2011). Similarly, α-Tocopherol or vitE - an antioxidant nutrient, interferes with lipid peroxidation and protect biological membranes from oxidative damage and thus helps to maintain membrane integrity and cellular functions (Mathur *et al.*, 2015). The treatment of vitE has been found to alleviate toxic hematological effects of CN induced oxidative stress in broiler chicks (Sharaf *et al.*, 2010; Aslam *et al.*, 2010). Moreover, the decreased skeletal ossification in fetal rats on methyl mercury exposure has been found to be reversed with vitE treatment (Abd *et al.*, 2012).

The toxicological outcome of CN in developing embryos may include diverse etiologies like membranous, DNA and chromosomal damages leading to apoptotic changes in various embryonic tissues and organs including neurodevelopmental toxicity. However oxidative stress seems to play a mediatory role in all such toxicological processes. Thus, it seemed imperative to study the role of natural antioxidants (vitE and olive oil) against the teratological potentials of a widely used insecticide (cypermethrin) exposure in chick embryos for future possible human benefits.

MATERIALS AND METHODS

Ethical deceleration: This randomized study was conducted on domestic chick eggs under permission on the ethical and technical issues by Higher Studies and Research Board, University of Sargodha.

Materials used

Cypermethrin: A commercially available 10%EC (10% emulsifiable Concentrate i.e. 10g CN /100ml) formulation of CN: marketed by Arrow International, manufactured by: M/s. Gharda Chemicals Ltd., Mumbai, India; Imported, Formulated & Packed by: M/s. Pak China Chemicals 1 Km, Bhoptian Chowk Defence Road, Off Raiwind Road, Lahore was used in this study.

Olive oil: Extra Virgin Olive oil, a product of *ACEITES BORGES PONT. S.A.U,* Avda J Trepat s/n; TARREGA, SPAIN, was used in this study.

Vitamin E: (dl alpha tocopheryl acetate, USP**)** available as capsules of 200mg under the brand name EVION; manufactured by Merck Pharmaceuticals (private) Limited F126, S.I.T.E. Karachi Pakistan; Mfg. Lic. No: 000043 and Reg. No: 008753 were purchased from the local market for use.

Corn oil: Edible corn oil, a product of Rafhan food products Ltd. Pakistan, was used as vehicle.

Eggs and the experimental groups: Freshly laid fertilized eggs of common chick (*Gallus domesticus*) golden black variety were brought in the lab, within 24hrs, from the villages around Sargodha city. These were washed in (boiled and cooled) lukewarm tap water, dried with paper towels, cleaned with alcohol (70%) moistened cotton tissues (to remove any surface bacteria), air dried and randomly distributed in 5 groups (60 each). Eggs were marked (accordingly for each group) as C (control), CN (cypermethrin), CNO (cypermethrin + olive oil), CNE (cypermethrin + vitamin E) and CNOE (cypermethrin + olive oil + vitamin E). The eggs were placed in the incubator for development after application of their respective treatments.

Dosage and method of intoxication: Dilutions for CN and CNE groups were made in corn oil while for CNO and CNOE groups olive oil was used as vehicle instead of corn oil. The injectable volume for each egg was maintained at 0.1ml. Thus, the required amounts of CN, vitE and olive oil (0.1µg each) were present in 0.1ml dose volume. Eggs in C group received 0.1ml corn oil injections only.

Sterilized disposable insulin syringes were used to deliver the relevant material directly into the center of the yolk. Before injection each egg was placed horizontally (Fig. 1) in cardboard egg tray for 10 minutes so that the embryo would migrate to the top of the yolk and would not be damaged by the syringe needle at the time of injection. The treatment was applied in the egg yolk through a tiny hole produced on the lateral side of the eggshell by syringe needle. This tiny hole in each eggshell was closed with molten wax immediately after application of the respective treatment.

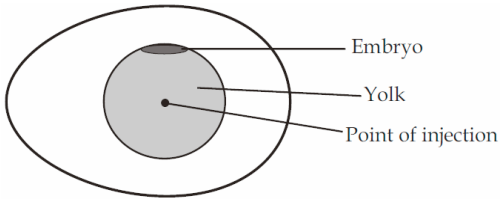

Fig 1: Diagram showing position of the embryo and location of injection.

Incubation: Eggs were incubated at 37±0.5°C with 60-65% humidity in an automatic incubator. During the entire period of incubation, eggs were manually rotated every 8hourly. On fifth day of incubation, each egg was examined by candling to locate the developing embryo.

The eggs showing no embryo formation were considered unfertilized and thus replaced with fresh ones to maintain the number of eggs (60) in each group.

Embryo collection: Embryos were recovered on day 7 and 14 post incubation from 20 eggs in each group separately for both stages. The remaining 20 eggs of each group were allowed to complete *in ovo* development and hatch normally. Chicks unable to hatch naturally by day 21 were gently removed from their shells and hatching rate in each group was recorded. Each hatchling was weighed on a digital balance to calculate mean group weight±SEM. All hatchlings were observed for morphological abnormalities. Embryos recovered on days 7 and 14 of incubation were fixed in Bouin's fluid (48hours) and finally stored in 70% ethanol for study of the developmental anomalies. Photographs of selected hatchlings and the 7th and 14th day embryos were obtained on a 7.2 MP digital camera (Sony DSC W35) in super-macro-mode.

Statistics analysis: The data collected was processed using χ^2, analysis of variance (ANOVA) and Duncan's multiple range tests.

RESULTS

Highest mean weight of hatchlings was recorded in C followed by CNOE, CNE and CNO groups; while CN group showed the lowest mean neonatal weight. Analysis of variance showed highly significant variation ($P<0.001$) in the overall data while the post hoc analyses indicated significant variations ($P\leq0.05$) of CN with rest of the five groups. On the other hand, C and CNOE groups did not differ significantly with each other. Furthermore, there was no significant variation between CNO and CNE groups; however, both of these groups showed significant difference ($P\leq0.05$) with C and CNOE groups (Fig 2).

Maximum growth retardation and abnormalities were observed in CN, CNO and CNE groups; while the embryos in CNOE groups were mostly normal. Additionally, chick embryos in CNOE groups showed improved growth at 7 and 14 days of incubation. Statistical (χ^2) analysis of the data pertaining to the rate of natural hatching, the number of normal hatchlings and the number of embryos in the CN, CNO and CNE groups recovered at 7 and 14 days of incubation showed significant difference compared to the C and CNOE groups (Table 1).

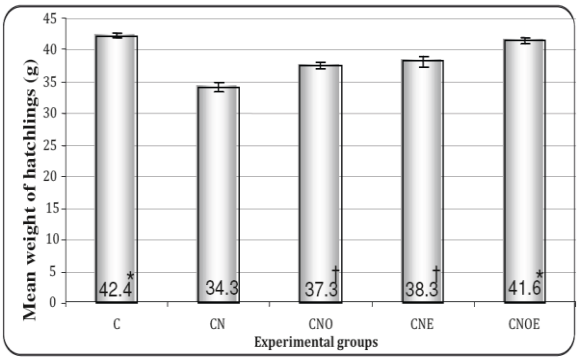

Fig. 2: Histogram showing mean neonatal body weights±SEM of the hatchlings in various experimental groups; C: control; CN: cypermethrin; CNO: cypermethrin+ olive oil; CNE: cypermethrin+ vitamin E; CNOE-cypermethrin+ olive+ vitamin E treated groups; *†any two groups values not sharing a common symbol differs significantly from each other.

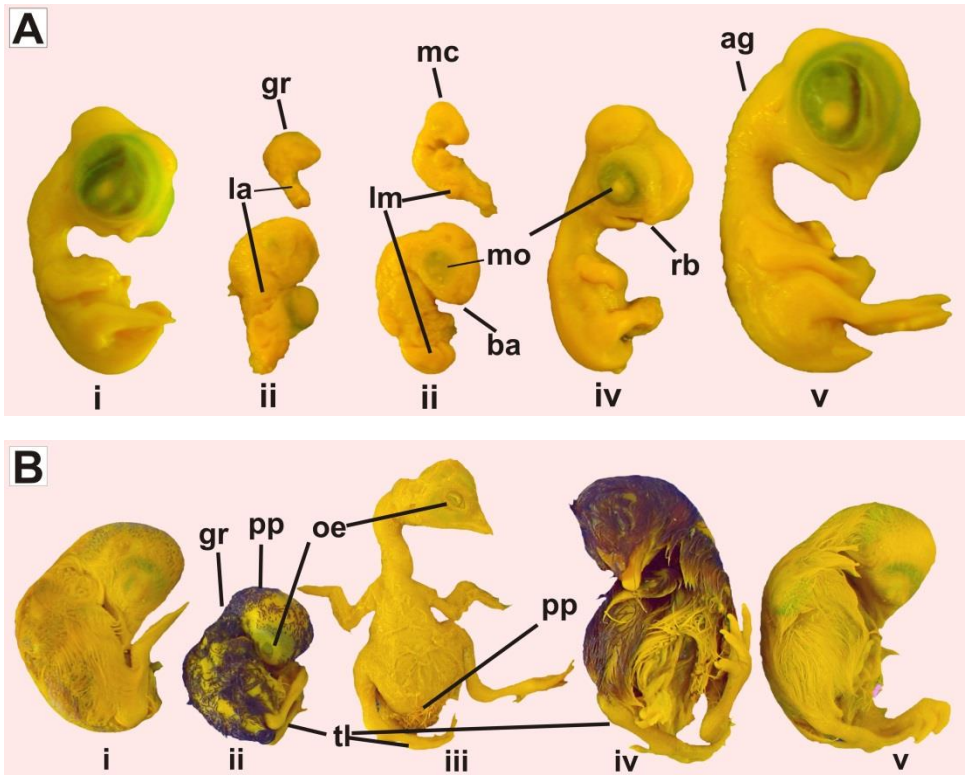

Fig 3: Developing chicklings exteriorized after 7 (A) and 14 days of incubation (B) - **i**: control; **ii**: cypermethrin; **iii**: cypermethrin and olive oil; **iv**: cypermethrin and vitamin E; **v**: cypermethrin, olive and vitamin E); **ag:** above normal growth, **rb:** rudimentary beak, **mo:** micropthalamia, **mc:** Microcephaly, **ba:** beak agenesis, **lm:** limb micromelia, **la:** limb Amelia, **gr:** growth retardation, **pp:** patchy plumage, **oe:** open eyes, **tl:** torted limbs.

Table 1: Chi square analysis of the rate of hatching and number of normal embryos and hatchlings recovered after 7, 14 and 21 days of incubation

Parameters	Groups (Number of normal embryos)				
	C	CN	CNO	CNE	CNOE
Normal embryos recovered on 7days incubation	20	6	10	11	13
Normal embryos recovered on 14days incubation	20	8	9	12	15
Normal embryos recovered on 21days incubation	20	7	11	13	14
Hatching rate	19	7	13	16	18
χ^2		33.03***	16.99**	10.17*	5.55

C: control; CN: Cypermethrin; CNO: Cypermethrin + olive oil CNE: Cypermethrin + Vitamin E; CNOE: Cypermethrin + olive oil + Vitamin E; ***P≤0.001; **P≤0.01; *P≤0.05.

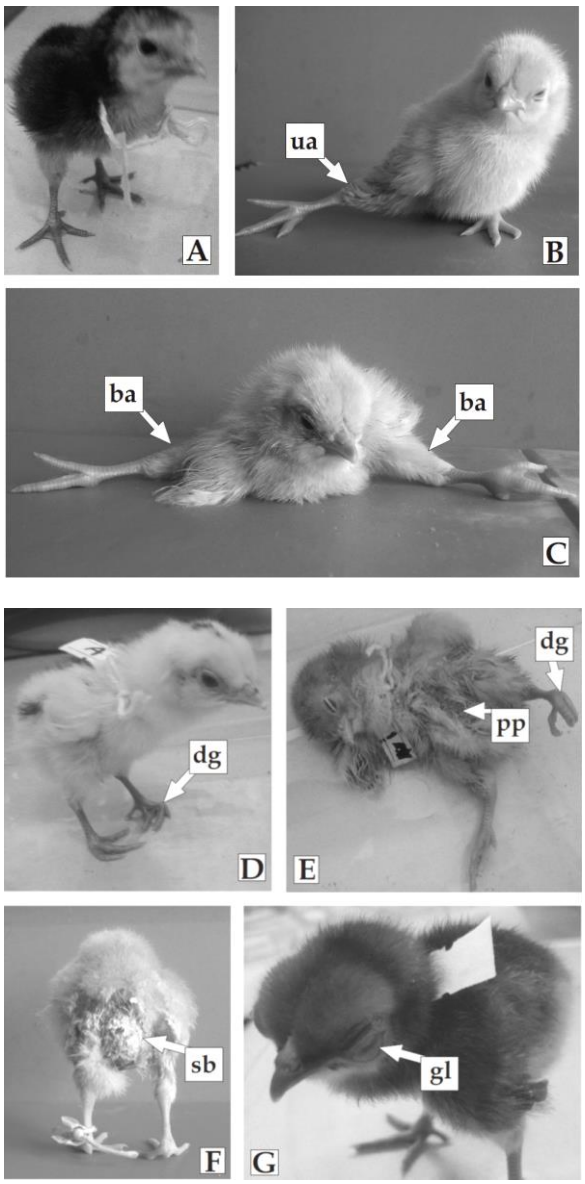

Fig 4: Selective abnormalities seen in hatchlings (21 days) :(A-control; B-G: cypermethrin treated hatchlings; **ua:** Lower limb unilateral axial deformity; **ba:** bilateral axial deformity; **dg:** digitory deformity; **pp:** Patchy plumage; **sb:** Spina bifida occulta; **gl:** congenital glaucoma

Abnormalities seen in embryos incubated for 7 days included: rudimentary beak, micro-ophthalmia, microcephaly, forelimb micromelia, hind limb amelia and micromelia (Fig. 3A). Abnormalities observed in embryos incubated for 14 day included: open eyes, abdominal edema, patchy plumage and hind limb deformities (Fig. 3B). The abnormalities observed in newly hatched chicks included: unilateral and bilateral axial limbic deformities, bilateral distal or digit deformities, patchy plumage, congenital glaucoma and spina bifida occulta (Fig. 4).

DISCUSSION

Embryonic development is a syntax of highly ordered biological processes starting with extremely specific metabolic activities in result of fertilization leading to the most precisely controlled cell divisions and cellular differentiations to produce body tissues, organs, organ systems and finally a complete new individual. The intricate processes of embryonic development are highly delicate and thus can be easily derailed by slight variations in the chemical or physical environment. Unfortunately, insecticides like CN have shown teratogenic potentials in experimental animal studies (Ullah *et al.,* 2006; Farag *et al*., 2007; Uggini *et al.,* 2012; Ahmad *et al.,* 2012). From the intracellular toxicological prospective the oxidative stress induced by CN exposure has been found to cause DNA damage and cellular apoptosis (Jin *et al.,* 2011). In developing zebra fish embryos CN exposure has led to increased production of malondialdehyde while the activities of antioxidant enzymes (catalase and superoxide dismutase) were decreased (Shi *et al*., 2011). A similar developmental study conducted on the common carp (*Cyprinus carpio* L.) embryos also indicated that CN exposure has led to growth retardations and embryonic death. In these embryos the estimated antioxidant enzymes (glutathione-S-transferase, glutathione reductase, and glutathione peroxidase) activities were significantly lowered than the control group embryos (Richterova *et al.,* 2015). Thus, it seems customary to believe that the oxidative stress induced by CN exposure must play a pivotal role in embryonic developmental disruptions (Huang *et al.,* 2016; Wu *et al.,* 2017). Dietary antioxidants such as vitE and the precious ingredients of EVO (oleocanthal, oleuropein and hydroxyl tyrosol) may play an important role in alleviation of the oxidative stress and stress related biochemical, cellular and developmental changes (Sharaf *et al.,* 2010; Oliveras-López *et al.,* 2008; Oliveras-López *et al.,* 2014).

Although the observed developmental abnormalities (rudimentary beak, micro-ophthalmia, microcephaly, forelimb micromelia, hind limb amelia and micromelia general growth retardations in 7days embryos) (open eyes, abdominal edema, patchy plumage and hind limb deformities in 14 days embryos) (unilateral and bilateral axial limbic deformities, bilateral distal or digit deformities, patchy plumage, congenital glaucoma and spina bifida occulta in 21days embryos) were not completely bottled-up by vitE or olive oil treatment; the results indicate suppressive effects of each of them (vitE and EVO) against these etiologies of CN exposure. In this context the combined treatment of vitE and EVO was found to provide batter rescuing effects on these teratological outcomes of cypermethrin. Additionally, along with the

general suppression of the embryo-toxic and teratological effects the combined EVO and vitE treatment was also found to significantly ameliorate the CN exposure effects on embryonic growth retardation and hatchability. Our results indicate that CN potentially hamper the intricate developmental processes at least partially by means of induced oxidative stress. This is because that the co-treatment of vitE and/or EVO have clearly shown rescuing effects from these detrimental developmental outcomes mainly by means of their established role for the maintenance of membrane integrity and enhanced intracellular antioxidant capacity protecting the cytoplasmic and nuclear components from oxidative damages (Rahmani *et al*., 2014; Mathur *et al*., 2015). Present findings indicate the benefits of vitamin vitE and EVO consumption during pregnancy to safeguard against any such probable/accidental oxidative threats to the developing human embryos.

Conclusions: It is clearly indicated that CN treatment disrupts avian developmental processes causing increased in ovo mortality and various malformations together with general embryonic growth retardations and hatchability; whereas vitE and EVO co-treatments have shown protective effects against the harmful effects of CN exposure.

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Authors contribution: KRA and AU conceived and chalked this study; KS did the experimental work; KR and KRA interpreted the data and wrote the principal draft; MAK and TA generated the photographic and numerical data. All authors approved the final version.

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