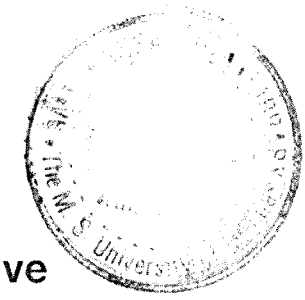

Embryotoxic and Teratogenic Effects of Pesticides in Chick Embryos: A Comparative Study Using Two Commercial Formulations

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ABSTRACT: Developmental toxicity of two different classes of commercial formulations of insecticides was studied by *in ovo* treatment of fertilized Rhode Island Red eggs. The first one was a combination of chlorpyrifos and cypermethrin and the second one was spinosad, a fermentation product of soil bacterium, *Actinomycetes*. In this study, the combination pesticide and spinosad of different concentrations were administered as a single dose *in ovo* in volumes of 50 μL per each egg on day "0" of incubation. Embryonic growth and development, morphological and skeletal malformations, and hatchability were assessed. The combination insecticide induced explicit alterations in the embryonic growth and development and resulted in malformations particularly to the axial and appendicular skeletal structures, whereas the changes were trivial in case of the spinosad exposure. © 2010 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2010.

Keywords: chlorpyrifos; cypermethrin; spinosad; Rhode Island Red; *in ovo*; teratogenicity; skeletal deformities

INTRODUCTION

Agricultural practices as well as the household maintenance today have a serious addiction to the use of pesticides. The world-wide annual consumption of pesticides is about two million tons, of which 24% is consumed in United States alone, 45% in Europe, and 25% in the rest of the world. The usage of pesticides in India accounts for more than 500 pesticide formulations, with an annual consumption of 164,080 tons of active ingredients, which average for 0.5 kg ha^{-1}. Globally, herbicides are the leading category of agrochemical used, followed by insecticides and fungicides. Conversely, in India, insecticides account for 80% of the total pesticides used, and the herbicide usage is insignificant (Abhilash and Singh, 2009; FAO, 2005; Gupta, 2004).

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Till the recent past, organochlorine insecticides were among the most commonly used pesticides in the developing countries in Asia. However, the concerns of environmental persistence and bioaccumulation of the organochlorine resulted in a change in the preference toward more environmentally safer pesticides like organophosphate carbamates and pyrethroids (Abhilash and Singh, 2009).

The new generation pesticides are designed such that they are short lived in the environment and do not accumulate in the human and animal tissues. However, owing to their very nature, that is, to disable and/or kill, they still pose a threat to the nontarget species. Several pesticides used as herbicides, insecticides, and fungicides are known to be endocrine-disrupting chemicals. Mixture of carbamates, organophosphates, phenoxy acids, pyrethroids, and other pesticides in a study showed to induce hormonal imbalances even when exposed within the reference values (Straube et al., 1999). Adult exposure to these chemicals is certainly an important factor; however, the concern is compounded when the exposure gets associated with the

developing organisms, because they are extremely sensitive to perturbations by chemicals with hormonelike activity. The protective mechanisms that are available to the adult such as DNA repair mechanisms, a competent immune system, detoxifying enzymes, liver metabolism, and the blood/brain barrier are not fully functional in the fetus or newborn (Newbold et al., 2007). Even a brief exposure during critical windows of reproductive development can cause permanent adverse effects. Several reports (Landrigan, 2001; Landrigan et al., 1999; Rice and Barone, 2000; Slotkin, 1999, 2004; Weiss et al., 2004) demonstrate that certain insecticides have detrimental effects on the development of the living organisms at far lower exposures than those that elicit signs of systemic intoxication. Hence, unwarranted effects on the fetal body structure could occur in absence of any obvious recognition that exposure has taken place. Several studies in children (Adgate et al., 2001; Barr et al., 2004; Shalat et al., 2003), pregnant women (Berkowitz et al., 2003; Bradman et al., 2005; Whyatt et al., 2002), and fetuses (Bradman, 2003; Whyatt and Barr, 2001) using urine, blood, amniotic fluid, and/or meconium samples demonstrated detectable pesticide levels in the majority of the cases. Adverse outcomes of these exposures during the preconceptional or developmental period may be observed immediately, or they may be expressed as latent effects that are not evident until later in life (Selevan et al., 2000; WHO, 2007).

Nevertheless, pesticide usage is validated by its many important contributions to the society, the fact that the nontarget species become vulnerable to its deleterious effects is a matter of grave concern. Therefore, understanding the consequences of exposure of insecticides and the causative levels of exposure, during the critical periods of embryonic development, is of prime significance. The chick embryos were chosen to conduct the study owing to its ease of availability, accessibility, and experimentation. The method is advantageous over the *in vivo* system by offering an elimination of the maternal influences such as biotransformation of the compound. Moreover, the developing embryo in the egg carries a complete set of developing morphogenetic system and manifests an advantage over *in vitro* systems, which have limited survival (Kotwani, 1998).

The present investigation was undertaken to understand the embryotoxic effects of two commercially available pesticides. Various studies demonstrated that the inert ingredients in the pesticide formulations enhance the toxicities of active ingredients and suggested that pesticide registration and their environmental monitoring should include full assessment of formulations (Cox and Surgan, 2006; Mansour et al., 2008a). Therefore, two different commercial formulations were chosen as the test chemicals for this study.

The first formulation was a combination of chlorpyrifos (50%) and cypermethrin (5%) as emulsifiable concentrate (EC). Chlorpyrifos and cypermethrin belong to organophosphorus and pyrethroid insecticides, respectively. Chlorpyrifos is an acetylcholinesterase inhibitor, and its insecticidal activity is due to the overstimulation of cholinergic receptors by excess acetylcholine. Cypermethrin causes a sustained opening of Na^+ channels in nerve membranes, which lead to the continued impulses in neurons and eventually leads to the death of target organism (Cui et al., 2006). Cypermethrin as an individual compound is quickly metabolized in mammals. The product of hydrolysis thus formed is nonactive and rapidly excreted out of the body (Wielgomas and Krechniak, 2007). With the concurrent exposure of cypermethrin and organophasphate, the later causes a nonreversible inhibition of esterases, which leads to slowing down of enzyme activity responsible for cleavage of ester bonds in pyrethroid molecules (Gaughan et al., 1980; Latuszynska et al., 2001). Thus, when applied together, the organophosphates enhance pyrethroids toxicity (Ray and Forshaw, 2000) by blocking its hydrolysis. Therefore, combination of these two insecticides was introduced in the agricultural market for the reason that together they show a synergistic effect and also could effectively control insects that developed resistance to either of the pesticides in isolation (Tiwari et al., 2008). A study reported by Wielgomas and Krechniak (2007) showed that rats on coexposure to cypermethrin and chlorpyrifos inhibited the hydrolysis of cypermethrin, which, in turn, caused an increase in cypermethrin content in the tissues. Earlier reports by Deacon et al. (1980), Gupta (1990), Muto et al. (1992), Roy et al. (1998), Farag et al. (2003), Tian et al. (2005), Ahmad and Asmatullah, (2007), and Slotkin et al. (2008) highlighted the teratogenic potential of chlorpyrifos or cypermethrin individually. But the teratogenic and embryotoxic potential of the combination of these two insecticides has not been studied so far with the avian embryonic model. Moreover, with regard to the fact that, in nature, the food chain is often contaminated by more than a single type of these toxicants due to their variable utility in agricultural fields and household, it was felt crucial to select combination pesticides for the study.

The other test chemical chosen was spinosad available in the market as 45% suspendable concentrate (SC). Spinosad is a new insect control agent that is derived from a fermentation product of a naturally occurring soil actinomycete bacterium, *Saccharopolyspora spinosa*. It comprises a mixture of spinosyns A and D and is the common name of the active ingredient that is present in *Tracer* Naturalyte (Mertz and Yao, 1990). It is effective against controlling a variety of insect pests (Sparks et al., 2001) by excitation of nervous system consistent with activation of nicotinic acetylcholine receptors, along with effects on γ-amino butyric acid receptor function (Hanley et al., 2002). The successful introduction of spinosad into the agricultural market place represents an important milestone in the use of natural products for commercial pest control (Crouse et al., 2001). Spinosad is classified as a reduced risk insecticide (EPA, 1997). Considering the fact that the xenobiotics at their

lowest level of exposure during critical windows of development may induce developmental defects and that studies on spinosad testing its embryotoxicity are very meager, a necessity was felt to evaluate the same.

MATERIALS AND METHODS

All experimental protocols were approved by Institutional Animal Ethics Committee according to Committee for the Purpose of Control and Supervision of Experiments on Animals, India. The toxicity test used strictly followed the procedures of the drugs and cosmetics rules, 2005, Appendix—III animal care standard.

Test Substance

The test substances used were two commercially available insecticides purchased from a local pesticide vendor. The first one was a combination insecticide (manufactured by AIMCO Pesticides Limited, Mumbai, India) *Anaconda 505*^{TM} (55% EC), which constituted of chlorpyrifos (50%), cypermethrin (5%), and a naturalyte insecticide *Tracer* (Spinosad, 45% SC), manufactured by Dow Agrosciences India Private Limited, Mumbai, India.

Test Organism

Fertile RIR eggs were obtained from the Intensive Poultry Development Unit of the government poultry farm at Vadodara, India. All eggs were cleaned with 0.5% povidone iodine to remove external contamination and blotted dry.

Doses and Intoxication

To assess the embryotoxicity of these insecticides, a preliminary dose range study was performed. Fifteen groups, each of 10 eggs, were dosed on day "0" of incubation with different doses of each insecticide, that is, 0.005, 0.001, 0.01, 0.05, 0.1, or 0.5 μg of combination insecticide and 1, 10, 50, 100, 500, 750, or 1000 μg of *Tracer* in volumes of 50 μL per egg. The dilutions were made in corn oil (Ashwin Vanaspati India Pvt Ltd, India) for the combination pesticide and 0.4% methyl cellulose (S.D. Fine Chemicals, Mumbai, India) for *Tracer*. The eggs were injected by the air sac method as per Blankenship et al. (2003), on day "0" of incubation. Based on the percent hatchability and rate of development, the toxicity of these two compounds was estimated, and three doses of insecticide, which had minimal, median, and sublethal effects, were chosen for further studies.

The Combination insecticide was dosed in concentrations of 0.01, 0.05, and 0.1μg per egg, whereas *Tracer* was dosed in concentrations of 100, 500, and 750 μg per egg. Two separate sets of controls were maintained for the two different groups of insecticides; that is, the corn oil (VC1) was injected into eggs to serve as vehicle control for combination insecticide treated groups, whereas 0.4% methyl cellulose (VC2) was injected into another set of eggs to serve as vehicle controls for spinosad-treated groups.

Egg Incubation

The eggs were incubated with their broad end up in an automated incubator (Scientific equipment works, New Delhi, India) and set at a temperature of 37°C ± 0.5°C and a humidity of 70–75%. The eggs were turned automatically every 1 h until the last 3 days before hatch. These eggs were candled every 4 days, and the unfertilized and dead embryos were culled out.

Study of Embryotoxicity and Teratogenicity

The rate of hatchability was calculated on the 21st day after the eggs of the different groups hatched. The developmental malformations wherever encountered, both in live hatchlings and dead embryos, were noted. For visualizing bone and cartilage development, the hatchlings as well as the unhatched/dead embryos of the various groups, collected on day 21, were deskinned and eviscerated and stained with alcian blue and alizarin red stains as per Lamb et al. (2003). The eggs were weighed before injecting the vehicles or insecticides. The hatchlings body weights were weighed after their feathers dried. The freshly excised livers and brains were gently bottled and weighed on a calibrated analytical balance (Sartorius, BS-223S). From the weights so obtained, the hatchling body weight relative to the initial egg weight and the relative weights of liver and brain was calculated.

Analytical Methods

The data were analyzed statistically by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons using the statistical software GraphPad Prism (Version 5, San Diego, CA). Values were expressed as mean ± SE, and the differences between the control and treated groups were considered significant when the P value was less than or equal to 0.05. Unpaired Student's t test was performed between the VC1 and VC2 groups to analyze if there were any differences between the two different vehicle control groups used in the study.

RESULTS

Significant embryonic malformations in axial and appendicular skeleton were observed in all the three different dose levels of the combination pesticide selected. At a dose

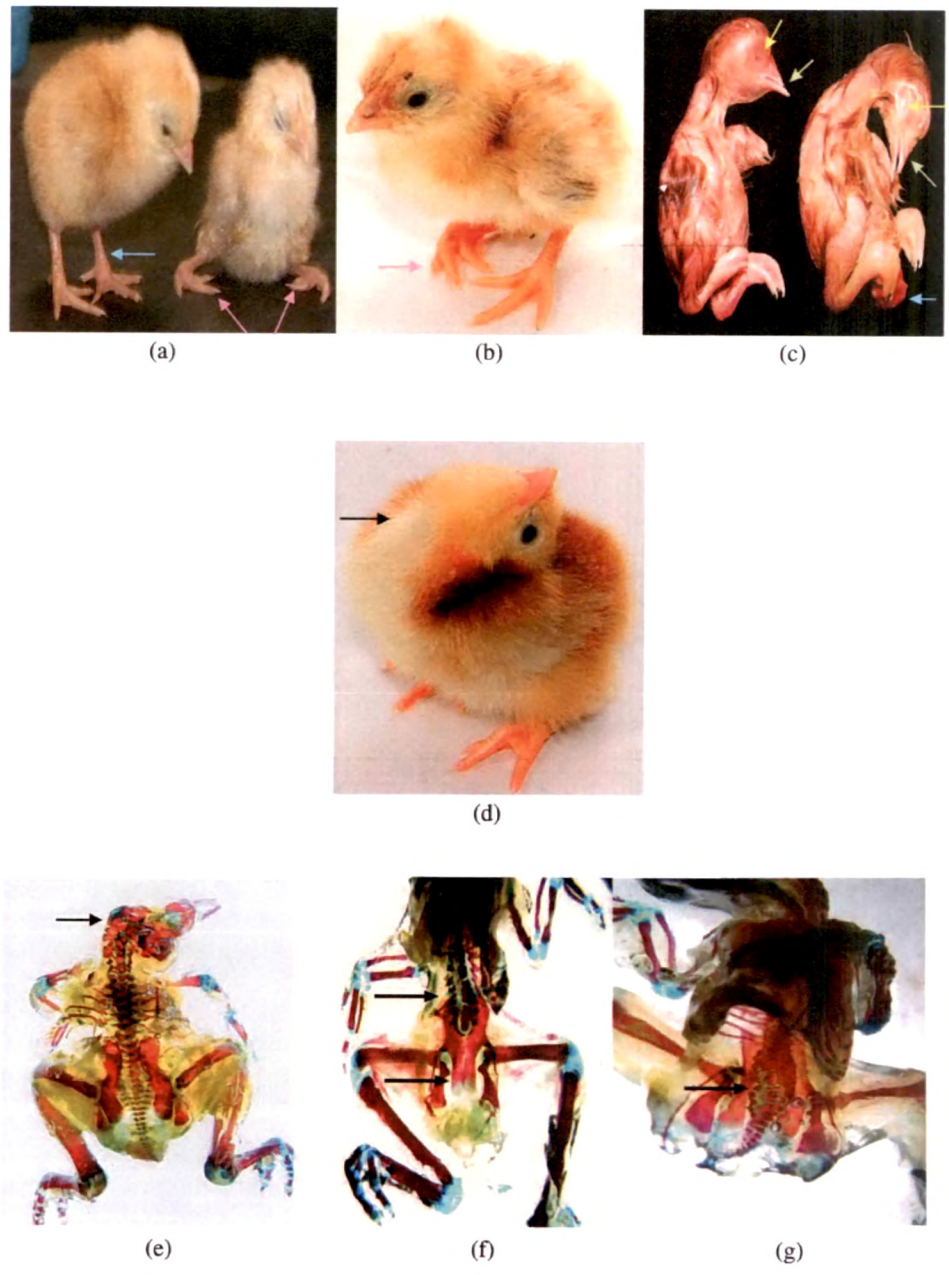

Fig. 1. (a) Control (blue ←), Cp dosed with crooked legs (pink ←); (b) crooked legs; (c) anophthalmia (yellow ←), beak defects (green ←), and umbilical hernia (blue ←); (d) wry neck; (e) craniorachischisis; (f, g) vertebral deformity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of 0.01 μg/egg, the effect was not very obvious morphologically, although an unsteady gait was observed occasionally. The abnormalities at 0.05 μg/egg were higher by the exhibition of 10% of the treated group with crooked legs, twisted phalanges [Fig. 1(a,b)], beak deformities, microphthalmia, and anophthalmia [Fig. 1(c)]. At 0.1 μg/egg, the hatchlings showed overt signs of teratogenicity in more than 20% of the treated group that include wry neck [Fig. 1(d)], craniorachischisis, in which the brain and the spinal cord remained open [Fig. 1(e)], beak defects, bilateral

anophthalmia, deformities in formation of sternum and ribcage, vertebral deformities [Fig. 1(f,g)], micromelia, missing phalanges, and umbilical hernia. Thus, the defects were more apparent as the dosage increased (Table I).

Furthermore, a dose-dependent decrease in hatchability (Fig. 2) was observed. The hatchability, which was 85% in the vehicle control, fell to 65% (0.01 μg/egg), 60% (0.05 μg/egg), and 35% (0.1 μg/egg). There was a significant decrease in hatchling body weight relative to initial egg weight as well as in the relative weight of liver at the dose of 0.1 μg. However, no significant changes occurred in hatchling body weight relative to initial egg weight, relative weights of liver or brain at 0.05 and 0.01 μg (Table II) when compared with the VC1 group.

Tracer, on the "other hand" dosed at much higher levels, that is, 100, 500, and 750 μg/egg showed no visible skeletal deformities in the hatchlings though the dead embryos seemed to be highly edematic (Fig. 3), and umbilical hernia was spotted at times (Table I). Nevertheless, a dose-dependent decrease in hatchability from 80% in vehicle control to 75% (100 μg/egg), 55% (500 μg/egg), and 60% (750 μg/egg) was observed (Fig. 4). The hatchling body weight relative to initial egg weight or the relative weights of livers and brains among the VC2 and spinosad-treated groups showed no significant difference.

Neither of the vehicle control groups, that is, the corn oil (VC1) or 0.4% methylcellulose (VC2)-treated groups showed any sort of malformations or developmental anomalies. Although there observed a significant difference in the egg weights and hatchling body weights between VC1 and VC2 groups, hatchling body weights relative to initial egg weights and also relative weights of liver and brain between the two control groups were found to be within the normal range.

TABLE I. Frequency of occurrence of abnormalities in vehicle controls and pesticide-treated groups

	Appendicular Deformities (Ap)		Axial Deformities (Ax)			
Dose	Ap1	Ap2	Ax1	Ax2	Others	Ax3
VC1	–	–	–	–	–	–
0.01CP	+	–	+	–	–	–
0.05CP	+	–	++	+	–	+[b]
0.1CP	+++	+	+++	+	+	+[a,b]
VC2	–	–	–	–	–	–
100Sp	–	–	–	–	–	–
500Sp	–	–	–	–	–	+[c]
750Sp	–	–	–	–	–	+[b,c]

$n = 30$.

Ap1, crooked legs, twisted phalanges and unsteady gait; Ap2, missing phalanges; Ax1, beak deformities; Ax2, defects in vertebral curvature, wry neck; Ax3, deformities in formation of skull, sternum and ribcage; CP, combination pesticide; others, anophthalmia[a], umbilical hernia[b], edema[c]; Sp, Spinosad; VC1, corn oil vehicle control; VC2, 0.4% methyl cellulose vehicle control; +, low incidence (≤5%); ++, moderate incidence (≤10%); +++, high incidence (≥20%).

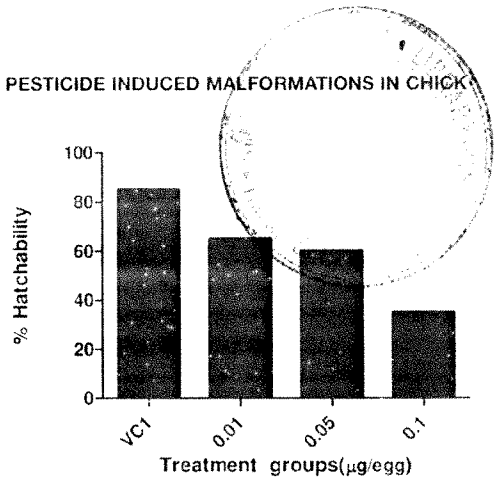

Fig. 2. Percent hatchability in the vehicle control and combination pesticide-treated group. n = 20; VC1 corn oil vehicle control.

DISCUSSION

A large body of evidences explains the susceptibility factors of developing embryo to various toxic substances. The pesticides are one such environmental stressor, which often interfere with the fundamental developmental mechanisms, and eventually avert them from reaching their proper endpoints. This study illustrates a similar instance.

Several types of axial and appendicular deformities with other types of malformations were encountered in the combination pesticide treated groups. Similar observations were reported by Rao et al. (1992) in hatchlings of white leghorns after dosing *in ovo* with RPR-V [E-2-butenoic acid 3-(diethoxyphosphinothionyl)ethylether], an organophosphate at 0.25, 0.5, and 1 mg/egg. Anwar (2003) observed severe teratological abnormalities in chick embryos at 100, 200, and 400 ppm of cypermethrin treatment. Xenobiotic-induced malformations were also reported by Ahmad and Asmatullah (2007), in fetuses of pregnant mice treated with chlorpyrifos at 18, 36, and 72 mg/kg b.wt., which included head and skeletal abnormalities such as, microcephaly, hydrocephaly, agnathia, anophthalmia, micromelia, hind limb twist, sacral hygroma, drooping twist, and kinky tail.

Organophosphates and pyrethroids are known to influence neurotransmission (Rose et al., 1999). The vertebral defects are long been attributed to decrease in acetylcholinesterase and the associated disruption of cholinergic system (Greenberg and La Ham, 1970; Landauer, 1975; Meiniel, 1978; Walker, 1971). This inhibition during the phase of embryonic development becomes more lethal, because acetylcholine is one of the transmitters that provide neurotrophic input, regulating the proliferation, differentiation, and migration of its target cells (Bachman et al., 1994; Hohmann, 2003; Hohmann et al., 1988, 1991). Thus, at an early stage of cell development, a given neurotransmitter signal may activate the genes required for replication of the target cell, whereas, at a later stage, the same transmitter and receptor signal may initiate the transition from replication

TABLE II. Egg, Hatchling, Liver, and Brain Weight in Control and Treated Groups

	Attribute				
Treatment (μg)	IE.Wt (g)	H.Wt (g)	H.Wt/IE.Wt (%)	R.L.Wt	R.B.Wt
VC1	61.87 ± 1.23[a]	42.63 ± 0.84	68.96 ± 0.94	2.38 ± 0.06	2.12 ± 0.08
0.01CP	63.07 ± 1.44	43.67 ± 1.11	69.27 ± 0.97	2.19 ± 0.07	2.10 ± 0.06
0.05CP	59.15 ± 1.27	40.37 ± 1.33	68.12 ± 1.11	2.31 ± 0.09	2.22 ± 0.05
0.1CP	61.30 ± 0.89	39.17 ± 0.99	64.01 ± 1.80[b,*]	1.96± 0.19[b,*]	2.34 ± 0.07
VC2	55.48 ±2.00[b,*]	37.03 ±1.90[b,*]	66.99 ± 1.83	2.52 ± 0.09	2.40 ± 0.10
100Sp	61.00 ± 1.53	40.31 ± 1.07	66.09 ± 0.65	2.42 ± 0.12	2.44 ± 0.07
500Sp	57.90 ± 1.29	38.15 ± 0.75	65.22 ± 1.76	2.52 ± 0.05	2.37 ± 0.05
750Sp	61.30 ± 2.10	38.24 ± 1.17	67.10 ± 1.98	2.44 ± 0.14	2.26 ± 0.10

[a] Values are expressed as mean ± SE; *P value $\leq$ 0.05.

[b,] Marked on the VC2 row refers to the significant difference compared to VC1 after unpaired t-test.

n = 12.

CP, combination pesticide; H.Wt, hatchling body weight; H.Wt/IE.Wt(%), hatchling body weight relative to initial egg weight; IE.Wt, initial egg weight; R.B.Wt, relative brain weight; R.L.Wt, relative liver weight; Sp, Spinosad; VC1, corn oil vehicle control; VC2, 0.4% methyl cellulose vehicle control.

to differentiation (Slotkin, 2005). Hence, any hindrance to the functioning of AChE during early embryonic development would mean debilitation much severe than just neurotoxicity. This could be the reason for the presently observed malformations in the combination pesticide-treated embryos.

One of the malformations craniorachischisis, observed in this study is an indication of failure to initiate closure of neural tube at the start of neurulation. Similar observations made by Murdoch et al. (2001) while explaining the neural defects in loop tail (*Lp*) mutant mouse give credence to the present notion. Vertebrate neurulation involves a precisely orchestrated set of morphogenetic movements within the neural plate itself (intrinsic processes) and also within neighboring tissues (extrinsic processes) (Smith and Schoenwolf, 1997). This process is complex and is regulated by many genetic and environmental factors. Murdoch et al. (2001) proposed that role of *Lpp1* (a novel gene) in neurulation may be to restrict the lateral extent of differentiation of the floor plate, thereby allowing precisely controlled midline bending of the neural tube closure. They also opined that *Shh* (sonic hedgehog) acts as a negative regulator of *Lpp1* expression. Therefore, it is logical to hypothesize that a flaw in the expression of either of these genes might have resulted in the neural tube defect like the currently observed craniorachischisis.

In this study, it is likely that the teratogenic propensity of the combination pesticide involves more than one kind of biochemical/molecular/cellular lesions, which may include an altered or interrupted cell signaling, inappropriate apoptosis, and/or defective closure of neural tube other than being just a cholinesterase inhibitor. And hence, there observed a variety of anomalies right from a decrease in hatchability and hampered growth to more serious conditions of skeletal malformations.

Tracer, on the other hand, exhibited a toxicological profile relatively benign. Under the highest dose tested, that is, 750 μg/egg, only hydrocephaly and edematic condition could be noticed. No axial or appendicular deformities

Fig. 3. Edema. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

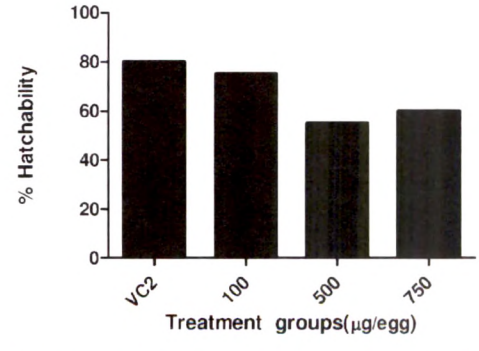

Fig. 4. Percent hatchability in the vehicle control and spinosad treated groups. n = 20; VC2, 0.4% methyl cellulose vehicle control.

were observed at the tested dose levels of spinosad. No published references were found regarding avian *in ovo* studies dealing with spinosad toxicity, although developmental toxicity studies were conducted on mammalian models. An 8-week study on male albino rats by Mansour et al. (2008b), a commercial formulation of spinosad dosed corresponding to Acceptable Daily Intake (0.30 and 0.02 mg a.i.kg $^{-1}$ b.w.), No Observed Adverse Effect Level (29.0 and 9 mg a.i.kg $^{-1}$ b.w.), and 1/100 LD50 (13.75 and 37.38 mg a.i. kg $^{-1}$ b.w.) lead to the inhibition of serum acetylcholinesterase. In this investigation, low levels of AChE inhibition or inhibition at a later stage might have occurred and possibly waived off the impediment to skeletal development. Investigations by Breslin and coworkers (2000) on CD rats and New Zealand white rabbits showed that spinosad at 200 mg/kg/day and 50 mg/kg/day, respectively, showed no maternal toxicity or developmental toxicity. Moreover, they opined that the overall incidence of external, visceral, or skeletal malformations in rat fetuses was incidental and not treatment related.

CONCLUSION

This study revealed that the combination pesticide induced a more pronounced teratological manifestations that include morphological and skeletal malformations, decline in hatchability, hatchling body weight, and the liver weight, when compared with that of *Tracer*. In the light of the present investigation, it could be construed that the commercial combination formulation of chlorpyrifos and cypermethrin is a potential teratogenic and embryotoxic compound, whereas *Tracer* under the present experimental conditions seems to be relatively less toxic to the embryonic development. In conclusion, notwithstanding the hazardous effects, a complete ban on pesticides would not be a wise decision. Rather, in the light of observations of this study, it is highly recommended that the usage of hazardous chemical pesticides should be limited and use of safer, target species specific alternate class of pesticide like spinosad should be encouraged.

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Original Article

EVALUATION OF PESTICIDE INDUCED DEVELOPMENTAL IMMUNOTOXICITY IN RIR CHICKS

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Abstract: *The present study is an attempt to evaluate immunotoxicologic potential of pesticides in the developing RIR chicks. Two commercial insecticide formulations, Anaconda 505 (Combination of 50% Chlorpyrifos and 5% Cypermethrin) and Tracer (Spinosad 45% SC) were injected separately into fertilized RIR eggs. Seven days after hatching, the chicks were tested for nonspecific immune response and phagocytic ability by conducting the bacterial clearance test and NBT salt reduction test. The chicks subjected to combination insecticide showed an apparent reduction in bacterial clearance as evident by high number of bacterial colonies in the plates inoculated with blood, liver and spleen homogenate compared to that of the controls. However, spinosad evoked a lag in bacterial clearance only at the highest tested dose. The phagocytic activity of splenic macrophages too was found reduced in chicks exposed to in ovo combination insecticide, while spinosad intoxicated chicks showed no significant change in the said phagocytic activity. Moreover, the combination insecticide treated chicks showed a reduction in the absolute body weight and relative weight of thymus and spleen at the highest dose tested. However, spinosad treated chicks showed no significant variations in the body as well as the lymphoid organ weights. Therefore, from the present study it could be construed that the combination insecticide induces far more immunotoxic afflictions to the developing embryo than the naturalyte pesticide spinosad.*

Key words: Immunotoxicity, Developing chick, Pesticides

INTRODUCTION

Many toxic chemicals have become an integral part of the ecosystem and pesticides are one among them. With the introduction of many pesticide regulation acts and the environmental protection agencies, there has been an increased awareness regarding the pros and cons of the pesticide usage. Although efforts are being made to make the newer class pesticide more selective to insects and less toxic to non-targeted organisms, they still pose serious health hazards like immunosuppression that often remain elusive to common regulatory toxicological screening. The immune system is both target and mediator of environment-induced injury [1]. Several reports showed that pesticides can lead to immune suppression. The humoral and cell mediated immune responses were depressed in murines treated with various pesticides like fenitrothion, fenthion, diazinon [2], dimethoate [3], phosphamidon [4], and chlorpyrifos [5]. The phagocytic function was reported to be diminished in fish by the chlorpyrifos treatment [6,7].

The developing organism may be at greater risk because chemicals may alter their organ systems with effects that are more persistent and/or more severe than those observed in adults, or they may alter these systems at lower doses than in adult animals [8]. Considering specific organ systems, the nervous and immune systems have been identified as possibly exhibiting a unique susceptibility during development that may not be apparent if toxicological data are only acquired in adult animals [9-11]. Several investigators reported the immunotoxic effects of the pesticides in the adult animals [2-7], however

developmental immunotoxic studies, on avain models, specially on commercial animals, like chick, are scanty and need more attention. Therefore, the present study was designed to evaluate the non specific immune response in the chicks after an initial *in ovo* pesticide treatment. Two distinct commercially available formulations of pesticides were chosen for the present study. The first one was a widely used combination of chlorpyrifos and cypermethrin, under the trade name *Anaconda 505*. The second test article was a new generation naturalyte class of insecticide, spinosad traded as *Tracer*.

MATERIALS AND METHODS

Chick Embryos: Fertile eggs of Rhode Island Red hens were obtained from the Intensive Poultry Development Unit, Vadodara. Eggs were wiped clean with povidone iodine and randomly allotted to control or treatment groups. Each egg was weighed, injected the appropriate test article and set to incubation in the incubator (Scientific equipment works, New Delhi) regulated to a temperature of 37.5 ± 0.5 ^{0}C and 75-80% relative humidity, for 21 days. The eggs were manually turned over an angle of 180° for seven times a day till 3 days prior to hatch. After hatching, the control and pesticide treated hatchling groups were housed in separate pens. Layer starter mash and water were provided *ad libitum*.

Pesticide inoculation: The eggs were injected through the air sac method [12] before setting to incubation. The limits of the air space on the egg were marked with a pencil by viewing through a Candler. The marked surface was then wiped with a 70% alcoholic swab. Using a sharp and sterile piercing tool a small hole was drilled at the centre of the air chamber. Holding the egg horizontally, the appropriate dose was then injected through this hole by a sterile syringe with 36 gauge needle. The hole was sealed with molten paraffin wax immediately, and transferred to the incubator. The above process of egg injection was carried out in a sterile laminar hood.

The LD_{50} was calculated in an earlier study by injecting the pesticide on day '0'of incubation and observing the hatch on 21st day. For observing the immunotoxicity of these insecticides, they were dosed in concentrations of LD_{50}, $LD_{50}/2$, $LD_{50}/10$ i.e. 0.1, 0.05 and 0.01µg/egg of combination insecticide; 1.5, 0.75 and 1.5mg/egg of spinosad in volumes of 50µl/egg. The combination insecticide was diluted in corn oil, while spinosad was diluted in 0.4% methyl cellulose. Two vehicle control groups were kept, one received corn oil and the other 0.4% methyl cellulose in volumes of 50µl/egg.

Bacterial clearance assay: The assay [13] was performed on 7 day old chicks. *Escherichia coli* were obtained from the microbiology department of the Baroda Medical College and diluted in sterile physiological saline. Inoculums in volumes of 0.2 ml, which had 3 x 10^6 cfu (colony forming unit) were injected into the brachial vein. Blood samples (0.2ml) were drawn after 10, 20, 40, 60 and 80 min post injection. After 110 min, the birds were euthanized; livers and spleens were removed aseptically. Spleens were homogenized each in 1 ml sterile PBS, while 0.1g of liver was weighed and homogenized in 1 ml sterile PBS. 200 µl of the blood or tissue homogenate thus collected were plated onto separate Mac Conkey agar plates. The plates were incubated in a laminar flow cabinet overnight at room temperature. The *E. coli* colony-forming units were then enumerated on a colony counter.

NBT test: NBT test [14] was done on one week old chicks. Spleen was dissected and impression smears of spleen were taken on a glass slide and kept in Petri dish. NBT salt solution was poured on the smear and incubated for 25 min. Smears were examined for NBT positive macrophages having formazan deposit in their cytoplasm. Two hundred cells were counted per slide.

Body weight and relative weight of lymphoid organs: Terminal body weight and the weight of freshly excised thymus, spleen and bursa of Fabricius were taken on calibrated electronic balances (Sartorius). The relative weights of the lymphoid organs were subsequently calculated.

Statistical Analysis: The data were expressed as mean ± SE and were analyzed by one way analysis of variance followed by Dunnett's multiple range tests using the software GraphPad Prism (version 5, San Diego California, USA). The results were considered significant ($P \leq 0.05$).

The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

RESULTS

The absolute body weight and also the relative weight of thymus and spleen were found to be significantly low in 0.1µg/egg combination insecticide treated chicks while the relative weight of bursa showed no variation compared to control. The other treatment groups i.e. 0.01 and 0.05µg/egg of combination insecticide and 0.50, 0.75 and 1.50mg/egg of spinosad did not lead to any significant variation in the body weight or relative weights of thymus, spleen or bursa of Fabricius, respective to the vehicle control groups (Table 1).

A viable bacterial load which was significantly higher than the controls was retrieved from the blood samples of 0.1µg/egg collected at 20, 40, 60 and 80 min after inoculation and the greatest disparity was observed at 80 min. In chicks treated with 0.05µg/egg combination insecticide, bacterial clearance was significantly delayed at 60 min and later. In chicks treated with 0.01µg/egg the clearance was comparable to the controls (Fig. 1). The liver and spleen homogenates in 0.05 and 0.1µg/egg combination insecticide treated chicks showed significantly lowered bacterial clearance (Fig. 2). Spinosad treated chicks revealed a lag in bacterial clearance ability only at a dose of 1.5mg/egg, however the clearance rate was delayed only towards the end of the observation period (Fig. 3), although the bacterial counts from the liver and spleen were comparable to the control (Fig. 4). The other two tested doses of spinosad did not show any significant changes in phagocytic functions in the blood, liver or spleen.

The NBT test revealed a decreased number of active phagocytic cells in the spleen at 0.1µg/egg and 0.05µg/egg doses of combination insecticide treatment, while 0.01µg/egg showed no changes. None of the tested spinosad doses induced changes in the phagocytic activity (Table 2).

Treatment	Parameters			
	Body weight (gm)	Relative weight of Thymus	Relative weight of Spleen	Relative weight of Bursa
VC1	62.84 ± 0.83@	0.314 ± 0.009	0.067 ± 0.004	0.207 ± 0.012
0.01 µg/egg	65.01 ± 1.67	0.321 ± 0.018	0.065 ± 0.004	0.201 ± 0.016
0.05 µg/egg	59.11 ± 1.12	0.339 ± 0.026	0.068 ± 0.002	0.223 ± 0.013
0.10 µg/egg	57.96 ± 1.54*	0.233 ± 0.025m*	0.055 ± 0.002*	0.201 ± 0.014
VC2	63.74 ± 1.14	0.332 ± 0.014	0.069 ± 0.003	0.219 ± 0.015
0.15 mg/egg	64.60 ± 1.16	0.343 ± 0.019	0.066 ± 0.003	0.203 ± 0.012
0.75 mg/egg	62.68 ± 0.92	0.314 ± 0.029	0.063 ± 0.002	0.205 ± 0.014
1.50 mg/egg	63.24 ± 1.17	0.346 ± 0.017	0.065 ± 0.002	0.204 ± 0.014

Table 1: Body weight and relative weights of lymphoid organs of controls and treated chicks. @ Values are expressed as Mean ± SE; n=10; *p value < 0.05, VC1: corn oil vehicle control,VC2: 0.4% methyl cellulose vehicle control

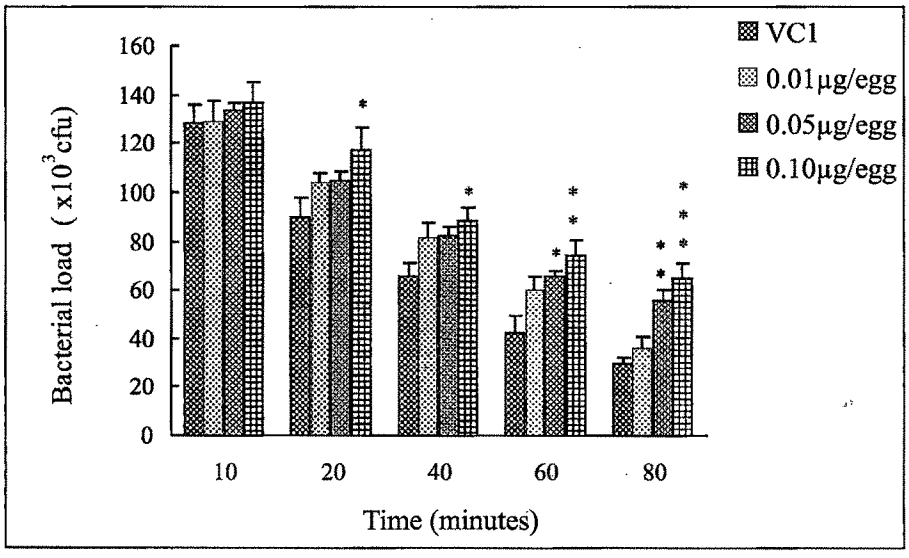

Fig. 1: Bacterial load in blood of control and combination insecticide treated chicks.

*p value < 0.05

**p value < 0.001

*** p value < 0.0001

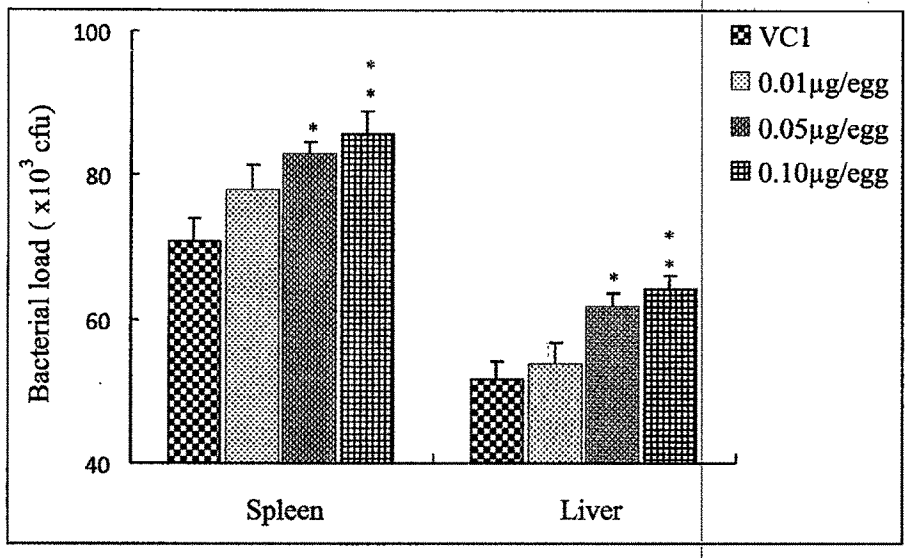

Fig. 2: Bacterial load in spleen and liver of control and combination insecticide treated chicks

*p value < 0.05

**p value < 0.001

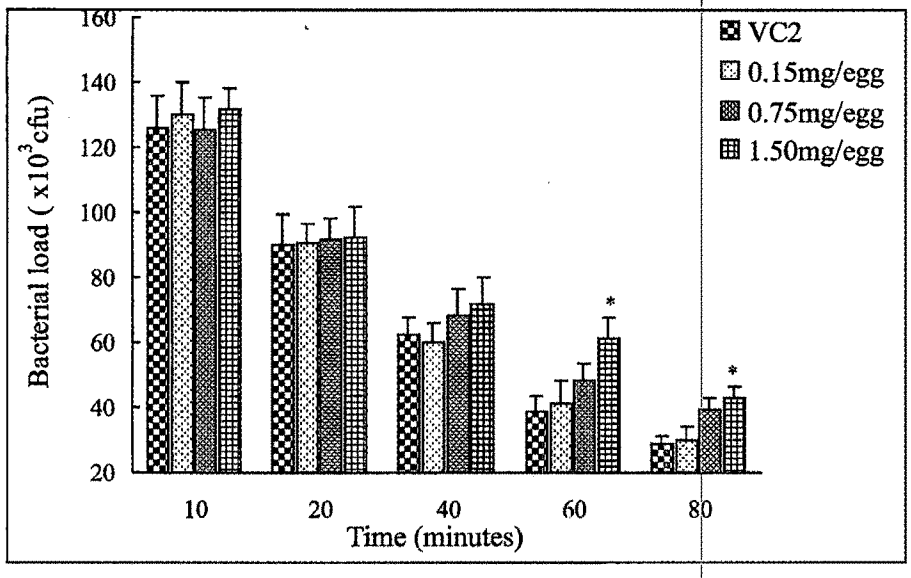

Fig. 3: Bacterial load in blood of control and spinosad treated chicks

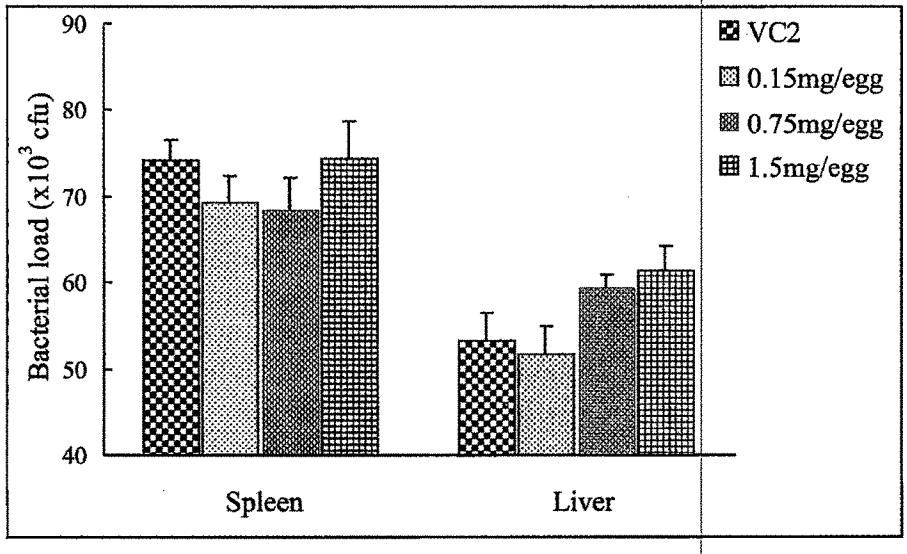

Fig. 4: Bacterial load in spleen and liver of control and spinosad treated chicks

Table 2: NBT salt reduction test . @ Values are expressed as Mean ± SE, n=10; *p value ≤ 0.05; ** p≤ 0.001; VC1: corn oil vehicle control, VC2: 0.4% methyl cellulose vehicle control

Treatment	Parameters	
	No. NBT positive cells	% NBT positive cells
VC1	99.16 ± 3.37@	49.58
0.01 µg/egg	93.83 ± 3.84	46.91
0.05 µg/egg	84.00 ± 3.91*	42.00
0.10 µg/egg	76.00 ± 4.76 **	38.00
VC2	101.6 ± 3.08	50.83
0.15 mg/egg	100.6 ± 2.55	50.33
0.75mg/egg	95.33 ± 3.73	47.66
1.50mg/egg	98.66 ± 2.99	49.33

DISCUSSION

The immune response in birds is highly regulated and breakdown in regulation often results in immunodepression [15]. The developing organisms are more vulnerable to a vast majority of known immunotoxicants than do the adults [16]. Therefore, diagnosis and prevention of pesticide intoxication in terms of developmental immunotoxicty is of considerable importance.

The defense mechanisms in a vertebrate evoke an appropriate immune response whenever they identify a foreign antigenic invasion. These responses of the immune system are quite sophisticated and function on an intricate balance. The first step of host defense against bacterial invasion is phagocytosis and degradation of phagocytic cells. The mononuclear phagocytic system takes up the function of phagocytosis in the form monocytes in blood and macrophages in the tissues like spleen, liver and lymph nodes. The macrophages are unique in that they are crucial players in both innate and adaptive immune responses [17]. The heterophils which offer an innate immune response are also important phagocytic cells against microbial pathogens [18].

The present investigation was designed to study and compare the immune responses evoked in two groups of *in ovo* pesticide intoxicated chicks by employing an *E. coli* challenge into their blood streams. The results revealed significantly high recovery of viable bacterial colonies from the blood streams of combination insecticide treated group of chicks. Further, the liver and spleen homogenates too showed higher numbers of uncleared bacterial colonies. This indicates a compromised bacterial clearance, which might be a consequence of diminished phagocytic and lytic potential of the monocytes and/or heterophils in the blood and macrophages in the liver and spleen. NBT-salt reduction test was performed to assess the functional ability of splenic macrophages of pesticide-intoxicated RIR chicks. The percentage of active macrophages in the intoxicated chicks was significantly lower than that of control only in combination pesticide treated chicks. The impaired phagocytosis might also be due to the adversely modulated immunogenic potency of lymphocytes.

The immune function is programmed during the fetal and neonatal life and is under close coordination by neural input and therefore, agents or drugs that interfere with the development of the nervous system elicit corresponding immunologic deficits [19]. Through different mechanisms, the organophosphates [20-22] and also the pyrethroids [23,24] are known to induce developmental neurotoxicity. Exposure of rats to chlorpyrifos during a developmental period in which this organophosphate pesticide is known to produce lasting changes in neural function, elicits corresponding, long term deficits in immune competence [19]. It is, therefore, likely that the immune suppression in the combination pesticide dosed chicks could be due to disturbances in neural development. Chlorpyrifos and cypermethrin as a combination were shown to have enhanced insecticidal effect [25]. And this could be the reason for the combination insecticide being toxic at levels as low as 0.1 and 0.05µg.

With the spinosad treatment of 1.5mg/egg, there was a decrease in bacterial clearance at 60 and 80 min blood sampling, however the liver and spleen showed no significant variations. The other lower doses nevertheless, showed no significant variation in bacterial clearance when samples from the blood, liver and spleen were tested. The NBT test showed no significant difference in the number of active macrophages in the spleen for all the three tested doses of spinosad. Spinosad was reported to be non-neurotoxic to rats in acute, sub chronic, or chronic toxicity studies and had shown no developmental effects [26]. Due to its low toxicity and perceived low impact on the environment, EPA registered spinosad as a reduced-risk material [26,27]. The present results are also in agreement with the earlier reports.

The weights of thymus, bursa of Fabricius and spleen can be used to assess the relative immune status in poultry [28]. In the present study the absolute body weight and also the relative weight of thymus and spleen were significantly low at 0.1μg/egg of combination insecticide treated chicks, while the relative weight of bursa showed no variation compared to control. These lowered weights might be associated to a direct necrotic effect of the insecticide on the lymphoid tissue and leading to poorer numbers of T cells and macrophages, and thereby causing a deficit in antigen recognition and phagocytosis. At 0.01 and 0.05 μg/egg neither the body weight nor the relative weight of lymphoid organs showed any significant changes. However, in none of the spinosad treated groups changes relating absolute body weight or relative lymphoid organ weight were observed.

Comparision of rate of bacterial clearance in the circulating blood, liver and spleen; and the splenic phagocytic activity in both the pesticide treated groups, a clear conclusion can be drawn that the combination pesticide is far more potentiated to induce immunotoxicity in developing chick at quite lower doses; while spinosad was relatively mild in terms of inducing developmental immunotoxicity in chicks.

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