ABSTRACT
Pesticides are major contaminants of our environment and pose a serious threat to non target organisms. The present experimental design aims to study the toxic effect of Flash on the embryo of chick treated at the post organogenesis stage of its developmental period. Fertilized eggs of pure breed (BV 300) of chick were immersed in low, moderate and high concentrations of the toxicant for one hour on day 7 of incubation and recovered on day 15 to observe skeletal development. The treated group’s revealed malformations at all doses. The mortality rate was found to increase with significant decrease in wet body weight with increase in the dose. The pesticide caused overall reduction in ossification of skeleton; with significant malformations at only high dose, and in thoracic and caudal vertebrae. Present findings show that Flash is a potent teratogenic with reference to avian species.


INTRODUCTION
Pesticides are one of the major contaminants of our environment and many of them persist in the environment. Although, they play an important role by fulfilling the demands of the population by protecting crops from pest attack; they pose great threats to the health of humans and non target organisms. The organophosphate (OP) insecticides are one of the most widely applied groups of pesticides accounting for 50% of the global insecticidal use. Exposure of OPs in agricultural field is an important occupational hazard. [1] In humans, OPs
have been detected in amniotic fluid [2] and are known to cross the placenta, [3], [4] posing a threat to the unborn child during period of brain development. Moreover, several nonspecific OP exposures has been reported to be associated with abnormal neonatal reflexes, mental deficits and developmental disorders and attention deficit/ hyperactivity disorder indicator at age of 5 years.[5],[ 6], [7], [8],[ 9] Teratogenic effects of OP insecticides are of two types: type I from KFase inhibition creating a block in amino acid metabolism; type II from acetylcholinesterase (AChE) inhibition disrupting nerve function. The structural features of the OP inhibitor and the enzyme target determine the type of teratogenic effect which is induced. [10], [11], [12] Toxicity of organophosphate pesticides results in negative effects on many organs and systems such as the liver, kidney, nervous system, immune system and reproductive system. The skeletal system has also been known as the potential target of pesticide toxicity. [13], [14], [15], [16], [17], [18], [19], [20], [21] Their toxicity is important because of accidental exposure to humans and non target organisms during their widespread use in farming. Organophosphates have proven mechanism of action, i.e. inhibition of acetyl cholinesterase (AchE). [22], [23] In the present study chick model was used to screen the toxic action of Quinalphos 25% EC (Flash), as it is cheep, easy to handle and helps in eliminating the variables related to maternal physiology. Agents can be administered directly to the medium surrounding the embryo, without maternal mediation.[24]

MATERIAL AND METHODS

In nature, eggs of avian species are usually exposed externally to pollutants which enter through the shell; therefore, the immersion technique was used, which mimics the exposure related to agricultural practices and with immersion technique the embryos get exposed for a longer period of time. [25] The toxicant Quinalphos 25% EC Flash (Organophosphate) is a commercially available insecticide and was purchased from the registered trader of pesticides from Jaipur, Rajasthan, India. The pure lines of fertilized BV 300 eggs were obtained from the Poultry farm at Ajmer, Rajasthan, India.

The doses were calculated according to the recommended dose (31.25mg/l) used for field application. The dilutions were made in distilled water (DW). The eggs were immersed for one hour duration on day 0 of incubation. The toxicity of the compound was estimated on the basis of the number of viable embryos obtained after the treatment. Three doses of insecticides, which had low, moderate and sub-lethal effects, were chosen for further studies.
In the present study five groups (20 fertile eggs, each) were randomly selected. On day 7 of incubation the eggs of first three groups were immersed individually, for one hour, in Low-15.62mg/l, Median-32.50mg/l and High- 62.50mg/l doses, respectively. The fourth group was treated with distilled water for the same period and served as control group and the fifth group, was left untreated and labeled as normal group.

After immersion, the eggs were kept in an incubator at 37.5°C with relative humidity 65-70%. Eggs were candled before immersion and the unfertilized eggs were discarded from the experiments.

The batches of eggs were opened on day 15 to study the skeletal development. The gross skeletal abnormalities were visualized and the images of the deformities were captured digitally.

**Skeletal staining**-On embryonic day 15 embryos were processed for staining with a whole mount double cartilage and bone staining technique described by Inouye. [26]

**Statistical Tests**- Statistical analysis were performed to calculate the wet body weight using student’s t-test, significance of differences were attributed at P<0.05, P<0.01 and P<0.001.

The teratological observations were analyzed by using Mann-Whitney U-test. The calculations were done using SPSS and significance was judged at $\alpha=0.05$ levels.

**RESULTS**

Treatment of the eggs with different concentrations of the toxicant exposed on day 7 was found to be teratogenic for young chick embryos at only high doses. Mostly all skeletal defects were found to occur at all the concentrations tested. However, significant defects were seen only at high doses and in thoracic and caudal vertebrae only. Treated embryos exhibited one or more types of malformations in each treatment group (Figure-1). Skeletal defects were recorded in all the embryos irrespective of the concentration used-reduced ossification of ribs, cervical vertebrae, metacarpus and digits, short kinked caudal vertebrae and reduced pygostyle, flexed digits, shortness of humerus and scapula, small sized skull, short beak and abnormally formed frontals and parietals (Table-2). The embroyolethality was i found to increase with increase in the concentration of the toxicant (Table 1).Mortality at 62.50mg/l was 35% as compare to 25% at 31.25mg/l of the toxicant used. Body weight
showed marked reduction at each dose level. However, highly significant reduction was recorded at moderate (31.25mg/l) and high dose (62.50mg/l).

Table 1: toxicity of flash in the chick embryos on 15\textsuperscript{th} day of incubation (toxicant exposure-4\textsuperscript{th} day)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of eggs/Treatment</th>
<th>Mortality (%)</th>
<th>Number of Surviving embryos</th>
<th>Surviving embryos with Malformations (%)</th>
<th>Surviving embryos with Malformations (N)</th>
<th>Wet Body weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control I (Untreated)</td>
<td>20</td>
<td>5</td>
<td>19</td>
<td>10.52</td>
<td>2</td>
<td>13.921± 0.375</td>
</tr>
<tr>
<td>Control II (Treated)</td>
<td>20</td>
<td>15</td>
<td>17</td>
<td>17.64</td>
<td>3</td>
<td>13.22 ±0.620</td>
</tr>
<tr>
<td>15.62mg/l</td>
<td>20</td>
<td>10</td>
<td>18</td>
<td>27.77</td>
<td>5</td>
<td>13.605 ±0.272</td>
</tr>
<tr>
<td>31.25mg/l</td>
<td>20</td>
<td>25</td>
<td>15</td>
<td>26.66</td>
<td>4</td>
<td>11.417± 0.358*</td>
</tr>
<tr>
<td>62.50mg/l</td>
<td>20</td>
<td>35</td>
<td>13</td>
<td>46.15</td>
<td>6</td>
<td>10.173±0.240***</td>
</tr>
</tbody>
</table>

* Each value represents Mean±Standard error. *= p≤0.05 (Significant), **=p≤0.01 (Highly Significant), ***=p≤0.001 (Very Highly Significant).

Table 2: Skeletal malformations in chick embryo on 15\textsuperscript{th} day of incubation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Skeletal Malformations (Mean Ranks + Z Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skull</td>
</tr>
<tr>
<td>Untreated</td>
<td>18.89</td>
</tr>
<tr>
<td>Treated</td>
<td>18.06</td>
</tr>
<tr>
<td>Z Value</td>
<td>-.496</td>
</tr>
<tr>
<td>15.62mg/l</td>
<td>19.06</td>
</tr>
<tr>
<td>Z Value</td>
<td>-0.056</td>
</tr>
<tr>
<td>31.25mg/l</td>
<td>16.79</td>
</tr>
<tr>
<td>Z Value</td>
<td>-.763</td>
</tr>
<tr>
<td>62.50mg/l</td>
<td>16.18</td>
</tr>
<tr>
<td>Z Value</td>
<td>-.402</td>
</tr>
</tbody>
</table>

Mean Ranks and Z Values computed by Mann-Whitney Test using SPSS

*Significance at 0.05 level of significance.

Figure 1 Photographs showing dorsal views of 15 day old chick embryos –untreated, treated (DW) and treated (Flash). Untreated embryo an untreated embryo has following parts: (1)
Premaxilla (2) Lower jaw, (3) Skull (4) Cervical vertebrae (5) Wing, (6) Ribs, (7) Femur,(8) Tibia, (9) Metatarsus , (10) Pelvic girdle, (11) Caudal vertebrae, (12) Pygostyle, (13) Digits. **Treated embryo** an embryo treated with DW shows (1) unossified hind parts of frontals and pterygoid, and (2) Reduced ossification of vertebrae (3) Kinky caudal vertebrae. **Low dose**- (1) reduced ossifications of the frontals and paritals, (2) short limbs (3) reduced pygostyle. **Moderate dose**- (1) incomplete ossification of frontals, parietales and palatine, (2) reduced fusion of atlas and axis (3) short humerus, radius and ulna on right and (4,5) reduced ossifications thoracic vertebrae. **High dose**- (1) incomplete ossification of frontals, parietales and palatine, (2) bent vertebrae (3) short humerus, (4) reduced radius and ulna, and (5) reduced ossification of caudal vertebrae.(6) curled toes.

*Malformed embryos exhibited one type or 2-4 types of malformations.*
DISCUSSION

The general morphology of the chick embryos on day 15 were confirmed with the normal stages in chick development studied and reported by Hamburger and Hamilton.[27] High mortality rate and reduced body weight at increasing concentrations of the toxicant was recorded with chick embryos. This report is in accordance with many researchers who have worked on chick embryos and with different other pesticides. [17], [21], [28], [29], [30], [31], [32], [33]

The growth retardation effect of Quinalphos on the developing chick embryo could be due to its intervention on metabolism suppression of gluconeogenesis or disruption of the retinoid signaling pathways and incapability to utilize yolk by growing embryo. [21], [32], [33], [34] Moreover, metabolic products formed by the parent compound might have shown high inhibiting effects on the Cholinesterase. Bird embryos are exposed to metabolic products as they remain inside the egg during whole incubation period and are not excreted. [35]

Liver is the main detoxicating organ which develops and starts functioning only after 4th-5th day of incubation.[36]Immersion of eggs on day 7 of incubation might regulate the toxicant metabolism to less toxic compounds at lower doses by conjugating with glutathione but at higher doses glutathione level in the tissue drops and is unable to detoxify the toxic compounds. Hence, a very negative effect of all the toxicants occurs during the first few days of the development. [37]

The skeletal system is the potential target of pesticide toxicity and Organophosphates have been reported to cause reduction in bone formation. [38], [39] Therefore, treatment of embryos on day 7 of incubation affects the skeletal development as has been found that in the studies done by Osdoby and Caplan where they found that mineralization of the long bones in the chick embryo hind limbs begins by day 8 of development.[40]

Similarly skeletal abnormalities like hernia, limb defects, and incomplete ossification of the skeletal system in chick embryos treated with Lufenuron, an insect growth regulator has been reported by Wagh et al.[33]

Beak defects, curled toes; reduced ossifications of the skeleton have been reported with RPRV, an organophosphate. [17]
The observed beak abnormality could be due to impairment of chondroitin sulfate, through interference with an NAD-dependent process.

The curled toes and flexed digits might have been the cause of muscular tension in the feet at the time of toe bones ossification. This might be due to the failure of the embryo to receive some esterase-metabolized products from the yolk sac. [17], [19] Severe shortening, contortion of body vertebral axis and tibiotararsals, rib and sternum defects have been reported in embryos of bobwhite quail injected with organophosphates diazinon and parathion in chick embryos. [41]

Misawa et al reported inhibited growth of femur, tibia, metatarsals and digits in chicks injected with an organophosphate dicrotophos. [42]

Anwar, Abou-Egla et al, Mobarak and Al-Asmari have also reported various skeletal abnormalities in chick embryos treated with cypermethrin, methomyl and endosulfan insecticides. [32], [37], [43] The skeletal defects are linked with Acetyl-Cholinesterase inhibition, disruption of cholinergic system and influx of calcium across the cell membranes. [19], [37], [44] Acetylcholine inhibition affects proliferation, differentiation and migration of target cells and any hindrance to the functioning of acetylcholinesterase (AchE) during early development would cause harmful effects. [45]

Hence, the potential of Flash an organophosphate insecticide in causing overall skeletal malformations may include disruption in signaling pathways, unutilization of the yolk content, inhibition of the cholinesterase and impairment of chondroitin sulfate.

It is therefore, concluded that Quinalphos 25% EC, Flash disrupts embryonic development in the chick and causes teratogenic effects which is in support with other teratogenic studies with many organophosphates on chick embryo. In the light of these observations it is clear that Flash should be used with caution as it can cause hazard to non target organisms.

ACKNOWLEDGEMENT
We thank The IIS University, Jaipur in providing all the necessary facilities to carry forward above study.
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