TERATOLOGICAL AND BIOCHEMICAL EFFECTS OF A FORMULATION CONTAINING QUINALPHOS, AN ORGANOPHOSPHATE INSECTICIDE IN THE CHICK EMBRYO

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ABSTRACT

Present study was designed to investigate the toxic effect of Quinalphos 25% EC Flash, an organophosphate insecticide on chick embryos. The fertilized BV 300 eggs were immersed in low (15mg/l), median (31mg/l) and high (62mg/l) concentrations of the toxicant for one hour on embryonic day (ED) 4 of egg incubation. The teratogenecity was observed on ED 7 of incubation. For studying the biochemical parameters, whole embryo was taken. Level of total proteins and RNA was found to decrease significantly at all the dose levels while DNA was found to increase in a dose dependent manner, with highly significant increase at 62mg/l. However, Glycogen content showed insignificant change. Teratological changes observed in the present study included reduction in the size of brain and eyeballs; incomplete development of eyes, beak and wing buds; edema, gastroschisis, hematomas and hemorrhages. In some groups, eyes and in others beak was totally absent. The fresh body weight also showed significant reduction with increasing dose levels.


INTRODUCTION

Increased human population and expansion of agricultural production has led to an augmented use of pesticides for agricultural and household practices. Pesticides applied in the fields, persist on the plant body, in seeds or in the soil. During the application of pesticides, non target organisms also become susceptible to the adverse action of the toxicants. The pesticides are one such environmental stressor, which often interfere with the fundamental
developmental mechanisms, and eventually avert them from reaching their proper end points. Thus, pesticide contamination of the environment is a problem of global importance and data on the fate of pesticides in a living organism has become useful in evaluating their safety standards to non-target organisms.

Quinalphos (O, O-diethyl-O-quinoxalin-2-4 phosphorothioate) is lethal for the mites and insects and thus is used in large scale in India. It is sprayed on various crops, vegetables, cotton, tea, fruits and cereals to protect them from variety of pests.

Like other Organophosphate insecticides, Quinalphos has been studied for its role in inhibiting acetylcholinesterase (AChE) in the nervous tissue by Gallardo et al. (2006), in fetal brain and placenta by Srivastava et al. (1992), in brain of *Labeo rohita* by Das and Mukherjee (2000). Pant and Srivastava (2003) studied spermatoxic and testicular effects in rats while carcinogenic potential has been studied in mouse skin by Shukla et al. (2000).

Organophosphates may also cause damage to digestive, immune, and nervous systems and urinary-reproductive systems. It poses teratogenic threats to humans and experimental animals also (Hamm and Hinton 2000).

Chick embryo is a favorite experimental model for the purpose of developmental biology and toxicological studies. It is preferred over other models due to its merits like, easy availability, low cost and ease in handling. Besides this, due to the lack of maternal metabolism, considerably lesser amount of administered substances per embryo is required, which is mainly useful for testing rare and expensive compounds, or when the maternal toxicity is of concern (Petrova et al. 2009; Uggini et al. 2010). Moreover, egg with the growing embryo represents a complete set of growing morphological system and has benefits over *in vitro* systems, which have poor survival rates (Kotwani 1998). Avian systems are most susceptible towards any deflection in the environment. They are known to be the best biological indicators of pollutants. Since these chemicals (pesticides) are also known to disrupt endocrine system, therefore, hamper with reproductive cycles in higher animals, including birds. Several studies on the direct exposure of avian eggs to environmental pollutants have demonstrated that xenobiotics can cross the shell and its membranes and are subsequently taken up by the embryo (Hoffmann and Gay 1981; Martin 1990). The presence of pollutants in the growing avian egg has been shown to result in poor hatchability and high neonatal death (Hoffmann 1990).
There are ample of experimental evidences from the works of Roger et al. (1969), Walker (1971), Rao et al. (1992), Sahu and Ghatak (2002), Pourmirza (2000), Uggini et al. (2010) that demonstrates the teratogenic effect of Organophosphates in the developing chick embryo. However, the teratogenic and embryotoxic potential of Quinalphos 25% EC Flash has not been studied so far with the avian embryonic model. Although avian systems are different than mammalian systems but they are useful for screening xenobiotics widely used in the ecosystems. Also, Poultry is a source of nutrition for mankind; therefore, it is very necessary to trace the exposure in the poultry birds.

**MATERIALS AND METHODS**

The aim of the present research work was to assess the teratogenic effect of formulations of insecticide applied on the farmland. Various studies have demonstrated that the inert ingredients in the pesticide formulation enhance the toxicity of active ingredients, therefore, their environmental monitoring should include full assessment of “formulations” (Cox and Surgan 2006; Mansour et al. 2008; Uggini et al. 2010). In the present study, dose was computed on the basis of recommended levels of formulations; applied by farmers in the cropland against sucking and chewing insect pests of crops such as cotton, tea, citrus fruits etc.

The toxicant Quinalphos 25% EC Flash (Organophosphate) is a commercially available insecticide and was purchased from the authorized wholesale market. The experimental model; fertilized pure breed BV 300 eggs were procured from the poultry farm at Ajmer, Rajasthan.

Quinalphos was administered into the egg through immersion technique. In nature, avian eggs are usually exposed to pollutants externally through the shell; therefore, the experimental protocol of Arias (1988) was used in the study of chick embryo development. Immersion method is preferred over the injection method as it reflects field exposure resulting into environmental contamination. Moreover, by immersion method the embryo is exposed to the pesticide for a longer time (Varga et al. 2002).

To assess the embryotoxicity of the insecticide, a preliminary dose determining experiment was conducted. For this, six groups with 10 eggs in each were treated on day “0” of incubation with different doses of the insecticide 15, 23, 31, 46, 62 and 78mg/l. The doses were set according to the recommended dose (31mg/l) used for field application. The
dilutions were made in Double Distilled Water (DDW). The eggs were immersed for one hour duration on day “0” of incubation. Based on the percent hatchability and rate of development, the toxicity of the compound was estimated, and three doses of insecticides, which had minimal, median and sublethal effects, were chosen for further studies.

Five groups with 30 fertile eggs in each were randomly selected. On Embryonic Day (ED) 4 of incubation, the eggs of first three groups were immersed in three different concentrations of Low-15mg/l, Median-31mg/l and High- 62mg/l doses respectively for 60 minutes. The fourth group was treated with DDW for the same period and served as control I and the fifth group, was left untreated to study the background toxicity and labeled as Control II.

After immersion, the eggs were dried, kept in an incubator at 37.5°C with relative humidity 65-70% and were rotated once every day. Before that eggs were candled and the unfertilized eggs were discarded.

The embryos were recovered on day “7” of incubation period. Gross morphology was studied using dissecting microscope and various teratogenic features were noted and the images of the animals were captured by camera. Mortality, normal viability, malformed viable embryos were categorized and fresh body weight was recorded. Thereafter, various biochemical tests were performed to assess the embryotoxicity of the compounds administered on day “4”. The whole embryo was homogenized for estimation of biochemical parameters- Protein (Lowry et al.1951), and DNA and RNA (Schneider 1957) using diphenylamine and orcinol reagents and Glycogen (Montgomery 1957).

Statistical analysis was performed using Student’s t-test, significance of differences were attributed at P<0.05 (less significant), P<0.01 (significant) and P<0.001(Highly significant).

RESULTS AND DISCUSSION
On comparing the two control groups; no significant difference was obtained. No mortality occurred in this group. The fresh body weight in gm was recorded as 1.18±0.1 (Control I) with less significant decrease in (Control II) 1.18±0.02 (Table-1). Malformed embryos were 3 (10%) out of 30 survivors in Control I and 4 (13.3%) in Control II (Table-2, Fig-1, A).

Treatment with 15mg/l caused 6.7% mortality and change in the fresh body weight was found to be insignificant. Malformed embryos obtained were 6 (21.4%) out of 28 embryos.
At moderate dose level (31mg/l) 16.7% mortality occurred and change in the fresh body weight was found to be insignificant. Malformed embryos were 9 (36%) out of 25 embryos. Treatment with high dose of 62mg/l caused 26.7% mortality and very highly significant reduction in the fresh body weight was observed (0.097 ±0.01) (Table-1). Malformed embryos were 13 (56.09%) out of 22 embryos (Table-2).

Table 1:- Immersion day “4” and recovery day “7”: Fresh body weight, survivals with malformations, mortality rate and biochemical parameters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Treated</th>
<th>Untreated</th>
<th>Toxicant- “Quinalphos”</th>
<th>High 62mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control I</td>
<td>Control II</td>
<td>Low 15mg/l</td>
<td>Moderate 31mg/l</td>
</tr>
<tr>
<td>No. of eggs</td>
<td></td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>No. of surviving embryos</td>
<td></td>
<td>30</td>
<td>30</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>Survivals with malformations</td>
<td></td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>6.7</td>
<td>16.7</td>
</tr>
<tr>
<td>Fresh body weight in (gm)</td>
<td></td>
<td>1.18±0.1</td>
<td>1.18±0.2*</td>
<td>1.02±0.4</td>
<td>1.04±0.2</td>
</tr>
</tbody>
</table>

BIOCHEMICAL PARAMETERS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Treated</th>
<th>Untreated</th>
<th>Toxicant- “Quinalphos”</th>
<th>High 62mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/gm)</td>
<td></td>
<td>15.1±0.57</td>
<td>12.6±0.59*</td>
<td>7.9±0.49***</td>
<td>4.3±0.6***</td>
</tr>
<tr>
<td>Glycogen (mg/gm)</td>
<td></td>
<td>2.56±0.34</td>
<td>2.6±0.36</td>
<td>2.23±0.67</td>
<td>2.19±0.50</td>
</tr>
<tr>
<td>DNA (mg/100gm)</td>
<td></td>
<td>0.08±0.023</td>
<td>0.06±0.01</td>
<td>0.12±0.018</td>
<td>0.14±0.011</td>
</tr>
<tr>
<td>RNA (mg/100gm)</td>
<td></td>
<td>0.2±0.018</td>
<td>0.18±0.01*</td>
<td>0.12±0.018*</td>
<td>0.10±0.018*</td>
</tr>
</tbody>
</table>

# Each value in (fresh body weight in (gm) and biochemical parameters) represents Mean±Standard error. *= p<0.05 (Less Significant), **=p≤0.01 (Significant), ***=p≤0.001 (Highly Significant) computed on Microsoft Office Excel 2003 using Student’s t-test at degrees of freedom (df): 4.

Various malformations noticed were categorized into skin defects- edema, hematomas, haemorrhages; head defects–brain and eye defects (microcephaly, macrocephaly, delay in brain development, microphthalmia, macrophthalmia, exophthalmia; neck defects-crooked neck, narrow neck; limbs-short limbs, absence of limbs, and the lower body defects-gastrochisis and runt (Table-2).
Table 2: Immersion day “4” and recovery day “7”: Incidence of various types of malformations in malformed embryos.

<table>
<thead>
<tr>
<th>Treatment (30)</th>
<th>Control</th>
<th>Toxicants Quinalphos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated Control I</td>
<td>Untreated Control II</td>
</tr>
<tr>
<td>SURVIVALS WITH MALFORMATIONS</td>
<td>3 (10%)</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td>TYPES OF MALFORMATIONS</td>
<td># INCIDENCE OF MALFORMED EMBRYOS (X)</td>
<td></td>
</tr>
<tr>
<td>SKIN DEFECTS</td>
<td>(1)</td>
<td>(2)</td>
</tr>
<tr>
<td>Edema</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hematomas</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Haemorrhages</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Adermia</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HEAD DEFECTS</td>
<td>(0)</td>
<td>(1)</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Macrocephaly</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Microphthalmia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Macrophthalmia</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Exophthalmia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anophthalmia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BEAK DEFECTS</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| BEAK DEFECTS/mouth defects/jaw defects | 0 | 0 | 1 | 2 | 3 |

| NECK DEFECTS | (2) | (1) | (1) | (2) | (2) |
| Crooked neck/twisted neck | 1 | 1 | 0 | 0 | 2 |
| Narrow neck | 1 | 0 | 0 | 2 | 1 |
| LIMB DEFECTS | (1) | (1) | (2) | (4) | (4) |
| Absence of Tail/tail bud | 0 | 1 | 1 | 2 | 1 |
| Short limbs | 1 | 1 | 0 | 2 | 3 |
| Absence of limbs | 0 | 0 | 1 | 0 | 1 |
| LOWER BODY | (1) | (3) | (5) | (5) | (7) |
| Gastrochisis | 0 | 1 | 1 | 2 | 3 |
| Runt | 1 | 2 | 4 | 4 | 5 |

# INCIDENCE OF MALFORMED EMBRYOS (X) = NUMBER OF MALFORMATIONS.
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**B. QPL** (Quinalphos low dose-62mg/l)

**C. QPM** (Quinalphos Moderate dose-31mg/l): 1. Hematomas,

**D. QPH** (Quinalphos High dose- 62mg/l)
1. Micophthalmia, 2. Microcephaly, 3. Edema on Head,

**Fig-1:**-Immersion day “4” and recovery day “7”: Image of gross-morphological changes in embryos of A) Control II-Untreated, B) QPL low dose-15mg/l, C) QPM Moderate dose-31mg/l and D) QPH High dose-62mg/l.
According to Romanoff (1949), two phases of deaths in an embryo were marked as comparable points; one was during initial embryonic life between embryonic day “3” and “5” and the other just before the hatching date.

Mortality during the first critical phase of development might be due to maladjustments in respiration (Riddle 1930). Important respiratory surfaces like area vasculosa and chorioallantois form during first three to four days of embryonic incubation. Moreover, waste produced is mainly ammonia; which further adds to increase the toxic effects (Brody 1972).

In the present study decline in protein levels under the influence of insecticidal action; have shown marked influence on the body weight and death rate in embryos immersed on ED “4” of incubation. The role of nutrients during the development of an embryo is indispensable and thus poor nutritional levels during mid point of the growth phase could be another possible cause for the observed death rate and decrease in body weight (Romanoff 1949) (Table-1).

Incidence of gross morphological malformations was found to increase with rise in the dose concentration. However, the defects observed were not uniform in nature however were quite severe at moderate and high dose levels. All the reported defects were found across the groups and one animal was seen with one or more than one defect.

Occurrence of edema is a general feature noted with Organophosphate caused tissue insult. It could be due to decrease in protein levels and inadequate vascular sheath which results into increase in surface area and leaky vasculature (Garrison and Wyttenbach 1985) (Fig-1, D).

The teratogenic propensity is known to elevate during critical periods of organogenesis i.e. “0” and “4” day of incubation. The defects of brain, eyes and neuronal structures are observed during this phase of treatment and the incidence of abnormalities parallels with increase in dose concentration (Fig-1). Development of organ is dependent on the embryonic metabolism and any hindrance in their pathways during the critical growth period would deliberately affect the overall formation of an organism (Sahu and Ghatak 2002).

The morphological malformations of the embryo recorded in the present study is mainly due to decrease in protein concentration which is essential for organ development and overall growth during developing period and is in agreement with Aronzon et al. (2010) who
reported similar results while working on the embryos of toads intoxicated with a herbicide-
2,4-Dichlorophenoxyacetic.

However, among the biochemical parameters studied; less significant reduction was observed
only in the levels of protein (12.6±0.59) and RNA (0.18±0.01) in Control II (untreated) when
compared with Control I (vehicle treated-DW).

At 15 mg/l, less significant reduction was observed in RNA (0.12±0.018) and highly
significant reduction in protein (7.9±0.49) was recorded. At moderate dose of 31 mg/l, less
significant reduction was noticed in RNA (0.10±0.018) levels and highly significant
reduction in protein (4.3±0.6) concentration; when the values were compared with control I
values.

Similarly, significant reduction in RNA values (0.06±0.011) was observed on treatment with
62 mg/l of Quinalphos; significant reduction was seen in protein (3.6±0.30) level, as
compared to DNA (0.20±0.017) which showed highly significant increase in its level (Table-
1) when compared with control I values.

In the current study made on embryos of chick, decline in Protein and RNA levels and rise in
DNA concentrations; corresponds to the observations made with Quinalphos treatment in
female guinea pigs and goats by Dikshith et al. (1980 and 1982). The AChE activity was
shown to be markedly inhibited in guinea pigs and goats. Das and Mukherjee (2000) also
noticed inhibition of AChE activity in the brain of *Labeo rohita* by Quinalphos. Among the
biochemical parameters in *Labeo rohita*, the protein and RNA levels were found to decrease
and DNA and acid phosphatase concentrations were shown to elevate. Hence, there is a
direct correlation between RNA and protein. Disturbance in the formation of any type of
RNA would have its affect in the failure of protein synthesis. Thus, the toxicant might
decrease the levels of protein if RNA formation is declined from DNA. The gradual decrease
in the protein level of intoxicated embryos suggests the disruption of carbohydrate, protein
and protein synthesizing machinery and inhibition of ATP production (Thenmozhi et al.
(2010).

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