CONTENTS

➢ INTRODUCTION

Increased human population and expansion of agricultural production has led to augmented use of pesticides for agricultural and household practices. These pesticides are added in the environment for preventing, destroying, repelling or mitigating pests. Variety of pesticides of different chemical nature such as Organochlorine compounds, Organophosphates, Carbamates, Pyrethroids, Heterocyclic pesticides, Nitro compounds and amides are routinely used for crop production (Mavrikou *et al* 2008, Chowdhury 2012).

As stated by Abhilash and Singh (2009), out of two million tons of world wide annual consumption of pesticides, 24% is consumed in United States alone and 45% in Europe, and 25% in the rest of the world. Whereas, India accounts for the usage of more than 500 pesticide formulations with an annual consumption of 164,080 tons of active ingredients which average for 0.5 kg ha^{-1}.

Though major classes of pesticides, specifically insecticides like Organochlorine were developed and used between the period 1935 and 1950, but now ecofriendly pesticides like Organophosphates and Carbamates are being preferred to tide over the environmental persistence and bioaccumulation problems (Abhilash and Singh 2009).

Organophosphates Organophosphorus insecticides include derivatives of phosphoric acid, phosphorothioic acid, and phosphoric acid. Organophosphates (OPs) have found worldwide usage due to high insecticidal activity, low environmental persistence and moderate toxicity. They can be found easily in food and drinking water (Turgut 2003, John *et al* 2001, Galloway and Handy 2003).

The OPs toxicity has been reported in short and long term animal tests over the past several decades. They have been shown to cross the placental barrier and affect the developing fetus (Villeneuve 1972).

Organophosphates work by phosphorylating serine residue at the active site of acetylcholinesterase (AchE) and thus inhibiting this enzyme. This enzyme is further responsible for breaking down acetylcholine (Ach), an important neurotransmitter in the central nervous system (Eto 1974, Fukoto 1990, Weiss 1997, Gilbert 2004, Lazarnini *et al* 2004).As a result, Ach remains in the synaptic cleft and stimulates the postsynaptic cell. The over stimulation can result into paralysis, cessation of breathing, and even death (Weiss 1997, Centers for Disease Control and Prevention 2005).

Organophosphates being lipophilic in nature interact with the biological membranes and therefore enhance lipid peroxidation due to interaction with biomembranes. Production of Oxygen free radicals by OPs has also been reported to be the major cause of toxicity (Soltaninejad and Abdollahi 2009).Many of the OP compounds like Malathion, Methyl Parathion, Chlorpyrifos and Acephate have also shown to be genotoxic (Giri *et al* 2002, Underger and Basaran 2005, Mehta *et al* 2008, Ali *et al* 2008, 2009).

Besides this there are ample of experimental evidences demonstrating the teratogenic effects of Organophosphates in the developing embryo by Roger *et al* (1969), Greenberg and LaHam (1969), Walker (1971), Richert and Prahlad (1972), Meiniel (1977, 1978), Meneely and Charles (1989), Seifert and Casida (1981), Rao *et al* (1992), Sahu and Ghatak (2002), Harris *et al* (1998), Pourmirza (2000), Gilliland *et al* (2001), Uggini *et al* (2010), Alhifi (2011).

Widely studied OPs included Pirimiphosmethyl, Diazinon, Chlorfenvinphos, Dimethoate, Fenitrothion and Profenofos- Mdegela (2006), Malathion and Diazinon- Pourmirza (2000), Ramsey *et al* (2011), Dimethoate and Methidathion- Alhifi.(2011), RPR-V (Rao *et al* 1992), Choloropyrifos- Ahmad and Asmatullah (2007), Uggini *et al* (2010), Quinalphos -Dwivedi.*et al* (1998), Murphy (1980), Srivastava *et al* (1992), Ray *et al* (1992),.Rupa *et al* (1991), Vasilic *et al* (1992), Shukla *et al* (2000), Pant and Srivastava (2003) with number of animals species like chick embryos, rats, pigs, goats etc.

Quinalphos, an Organophosphate pesticide, is found to be effective against both biting and sucking pests on vegetables, cotton, tea, fruits and other cereals. Like other Organophosphate insecticides, Quinalphos acts by inhibiting acetylcholinesterase (AChE) in the nervous tissue (Gallardo *et al* 2006).Technical Quinalphos has been reported to cause significant inhibition of acetylcholinesterase activity in fetal brain and placenta (Srivastava *et al* 1992).

Carbamates Carbamates are linked with organophosphates in terms of the mode of action; inhibiting acetylcholinesterase (Brimijoin and Koenigsberger 1999). The alkaloid physostigmine was the first representative of this group followed by insecticides like propoxur and others. In contrast to organophosphates, the inhibition of AChE is reversible and mild to moderate severity of toxicity is seen by Alvares (1989).There are several cases of human poisoning associated with exposure to various carbamates, in particular carbaryl by Cranmer (1986) and Propoxur by Hayes (1982).

Some of the commonly studied Carbamates are Fenoxycarb, Propamocarb and Propoxur -Schmuck and Mihail (2004), Carbosulfan- Renzi and Krieger (1986), Giri *et al* (2002), Ksheerasagar *et al* (2011), Carbaryl- Pant *et al* (1996), Munglang *et al* (2009), Kang *et al*

(2010), Diuron, Thiram, Mancozeb, Carbofuran- Seth *et al* (2000) in animals like chick embryo, rats, fish etc.

Carbosulfan is active against caterpillars, green leaf hopper, brown plant hopper, gall midge, stem borer and leaf folder of paddy and white aphids of chillies. Carbosulfan was in the priority list for toxicological evaluation by the joint FAO/WHO Meeting on pesticide residues in 2003.

Although, both the acetylcholineestarase inhibitors-Quinalphos and Carbosulfan have been reported immunotoxic, genotoxicant, mutagenic, carcinogenic, hepatotoxic etc. However, their role in causing teratogenicity has very few implications.

The present study has been designed on chick, as it has been the most favourite experimental model for developmental biologist undertaking the teratogenicity, neurobehavioral teratogenicity and the related studies to screen xenobiotics. The avian model is subjected to teratogenicity testing by injecting the compound directly into the egg causing defined exposure of the toxicant.

Karnofsky (1965) has stated that the chick embryo is the best model in developmental biology because in his view, it makes the examination of drugs easier, as the embryo shows sensitivity to both chemical and physical agents. Moreover, Gancedo *et al* (1982), Puchkov *et al* (1981), Repetto *et al* (1984) have stated that morphological and functional development of chick embryo parallels that of mammalian embryos. The most important charm of using the chick embryo is the minimal xenobiotic biotransformation Repetto *et al* (1984), Kotwani (1988).

Pesticides, besides being selective for their intended target species, can also cause adverse health effects on non-target species, including humans. Moreover, the acknowledgement

that pesticides standards are compounded on healthy adults, and thus may not be protective for children has opened new areas for research and regulations NRC (1993), Colborn (2006).

➢ OBJECTIVE

Assessment of teratogenic potential of Quinalphos and Carbosulfan in chick embryo.

- ✓ Identification of biochemical / morphological parameters for screening of chemicals for their teratological potentials in mammals.
- ✓ Study the morphological malformations and skeletal deformities caused by pesticides at various dose levels.
- ✓ Study the changes in biochemical and histopathological parameters in exposed embryos.

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➢ REVIEW OF LITERATURE

A large body of evidences explains the developmental malformations in the embryos due to various toxic substances. The pesticides are one of the many reported environmental stressor, which interfere with the normal developmental processes.

An array of studies has been carried out on pesticides. The substances penetrate into the organism through skin, respiratory system, alimentary canal, resulting in poisoning, damage to liver, kidney, cardiac muscle, damage to central nervous system and peripheral nervous system (Greenberg and LaHam 1969). Some have an embryotoxic effects, affecting viability of chicks (Romanoff and Romanoff 1972, Kang *et al* 2010).

The occurrence of embryonic death and decrease in wet body weight with increasing concentrations of the toxicant has been reported by several researchers -Rao *et al* (1992), Pourmirza (2000), Sahu and Ghatak (2002), Slotkin *et al* (2008), Petrova *et al* (2010), Kang *et al* (2010).

Petrova et al (2010) studied the effect of single dose of Bendiocarb, a Carbamate insecticide in chick embryo and found body weight to decrease on embryonic day (ED) 5 and 10 as compared to ED 2, 3 and 4. General growth retardation along with defects of body wall, microphthalmia, anophthalmia, cleft beak was observed in the treatment groups.

Research by Khera and Bedock (1967), Rao *et al* (1992), Pourmirza (2000), Sahu and Ghatak (2002), Misawa *et al* (2004), Petrova *et al* (2010), Uggini *et al* (2010) has revealed that many of them caused increased mortality of embryos, decreased hatching rate and had teratogenic effects.

Khera and Bedock (1967) in their study used Parthion or Diazinon in the pre-incubated and 4-day incubated eggs and they reported skeletal defects in all the chick embryos that survived acute lethal effects. The earliest sign was a brachymorphic neck in 7-day old embryos. At 19 days of age the axial length of the insecticide treated embryos was one half of the normal. The head was compressed anterio-posteriorly, the neck was short and limbs were micromelic. Thus, leading to total teratogenicity.

In (1985), Garrison and Wyttenbach treated white leghorn chicken eggs with Dicrotophos (Bidrin), an Organophosphate and observed general developmental retardation as well as unilateral retardation of the cranial sense organs. All the defects observed were associated with those structures which were undergoing initial or early morphogenesis.

Rao *et al* (1992) reported teratogenic potential of yet another insecticide known by the name RPR-V, an Organophosphate on the chick embryos injected on day 4 of incubation. He found the dose dependent decrease in the hatchability and increasing incidence of deformities. The number of deformed chicks increased to 87% at the highest dose with the dose dependent reduction in body weight. Malformations like wry neck, beak defects, foot deformities, under developed sternum, exposed viscera due to malformed sternum as well as curled toes were reported to increase with increasing doses of RPR-V.

Exposure of Organophosphate and Carbamate insecticides in young children have shown to inhibit cholinesterase activity by Gamlin *et al* (2006). Symptoms of cholinesterase inhibition were diarrhea, vomiting, bronchial hypersecretion, excessive sweating, hypothermia, salivation and abdominal pain. The most troublesome complication was respiratory failure. However, Carbamate poisoning was found to be less severe than

Organophosphate exposure because of the rapid hydrolysis of the Carbamyl-acetylcholinesterase intermediate to regenerate an active enzyme.

Uggini *et al* (2010) used a combination of Chlorpyrifos and Cypermethrin and reported marked alteration of the embryonic growth and development. He observed significant malformations in axial and appendicular skeleton. Crooked legs, twisted phalanges, beak deformities, micropthalmia and arophthalmia, wry neck, cranioschischisis, in which brain and spinal cord remained open, deformations in formation of sternum and rib cage, vertebral deformities, micromelia, missing phalanges and umbilical hernia were observed in the treatment groups. However, defects were found to be more apparent at the increasing dose levels only.

Effect of Carbaryl, a Carbamate insecticide was studied by Kang *et al* (2010) on *Bombina orientalis* (amphibian) and was found to be embryotoxic and teratogenic. Carbaryl was found to be detrimental for embryonic survival and caused axial skeletal defects in the embryo.

Sahu and Ghatak (2002) studied the effects of Dimecron, an Organophosphate insecticide on developing chick embryo. The insecticide was administered at two different doses (25µg and 35µg) into the egg yolk at day 0 of incubation. Liver and Kidney were found to be severely affected by the pesticide at both the doses tested. Weight of brain, spinal cord, liver and kidney were found to be decreased remarkably. Various malformations were observed like one eye, short hind limbs, crossed beak, abnormally exposed brain and internal organs. Liver histopathology studied on embryonic day 8 and 14 showed non-nucleated and vacuolated cells, different in shape and pycnotic in nature, ruptured cell membranes of liver cells, and obliteration of sinusoids.

AChE inhibition by the two classes of insecticides, organophosphates and carbamate has been reported as the main cause of toxicity and teratogenicity among the organisms. Some of the widely studied acetylcholine inhibitors and their role in causing teratogenicity and embryotoxicity are reported with following insecticides Diazinon -Khera and Bedok (1967), El Mazoudy *et al* (2001), Misawa *et al* (2004), Ducolomb *et al* (2009), Dicrotophos -Garrison and Wyttenbach (1985), Chlorpyrifos- Slotkin *et al* (2008), Colombo *et al* (2005), Uggini *et al* (2010), Carbosulfan -Ksheerasagar *et al* (2011, Giri *et al* (2002), Quinalphos -Pant and Srivastava (2003), Shukla *et al* (2000), Das and Mukherjee (2000), Dikshith *et al* (1980, 1982) in their respective work on species like rats, chicks, pigs, goats etc.

Although, Carbamates and Organophosphates, the new world pesticides are commonly referred to as environment friendly but researchers in their studies have found their deleterious effects in number of species belonging to invertebrate and vertebrate groups. Cholinesterase inhibiting insecticides such as Monocrotophos, Quinalphos, Carbosulfan, Dimecron, Diazinon, Chlorpyrifos, Dicrotophos, Bendiocarb, Parathion, Flupyrazofos etc.have been shown to cause genotoxicity, teratogenicity, fetotoxicity, embryotoxicity, immunotoxicity, neurotoxicity, carcinogenicity, and toxicity to reproductive systems.

One of the insecticide which is also chosen to be used for the present research work is Quinalphos, an Organophosphate insecticide. It has been reported toxic in female guinea pigs, rats, and goats by Dikshith *et al* (1980, 1982). Goats intoxicated with 0.5mg/kg of Quinalphos for a period of 110 days indicated body tremors, profuse salivation, weakness and diarrhea and reduced food consumption. Liver showed fatty degenerative changes in the parenchyma. Hepatocytes of the centrolobular area appeared vacuolated and carried granular cytoplasm. The AChE activity was significantly inhibited in guinea pigs and

goats. Das and Mukherjee (2000) also reported 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 levels were shown to be elevated.

Pant and Srivastava (2003) studied spermatoxic and testicular effects of Quinalphos in rats. The symptoms of Quinalphos toxicity-lethargy, staggering during locomotion, weight loss and hyperirritability leading to death was observed at highest dose level tested.

Shukla *et al* (2000) evaluated carcinogenic potential of Quinalphos in mouse skin and found out about its tumor initiating activity which was higher in magnitude in the multiple dose initiated animals.

Ksheerasagar *et al* (2011) reported that carbosulfan, a carbamate insecticide has adverse effects on the kidney functioning leading to physiological impairment in albino mice. Carbosulfan has been reported as potent genotoxic agent in mice by Giri *et al* (2002). Carbofuran, Structural analog of Carbosulfan has been reported to be teratogenic and embryotoxic by Gupta (1994).

Carbosulfan, as with other Carbamates is reported by Renzi and Krieger (1986) to be extremely toxic to mammals and its toxicity is mediated through inhibition of AChE.

Munglang *et al* (2009) also studied morphological and morphometric changes in the liver produced by the insecticide Carbaryl, a Carbamate. Liver hepatocytic plates were found to be disheveled and the hepatocytes singly placed. Various signs of hepatocellular degeneration were noticed.

Thus, the present research work has been designed to evaluate the teratogenic potential of Quinalphos and Carbosulfan, an anticholinesterase inhibiting toxic insecticides; with proven records which confirm their toxicity in wide varity of organisms including humans.

➢ MATERIAL AND METHODS

A. TEST MATERIAL

Quinalphos 25% EC (Flash), an Organophosphate insecticide: It is used as an emulsifiable concentrate containing 25%w/w active ingredients; balance emulsifier and solvent. It is considered to be broad spectrum, contact and stomach insecticide, effective against sucking and chewing insect pests.

Carbosulfan 25%EC (Marshal), a Carbamate insecticide: This is also a broad spectrum and contact insecticide based on the active ingredient, Carbosulfan.This formulation is used for the control of caterpillar and sucking pests of rice and chillies.

These formulations are available with the registered suppliers of pesticides of Jaipur city, Rajasthan,India.

B. TEST ANIMAL

The present research work will focus on the impact of above mentioned pesticides on pure bred Fertilized BV 300 eggs which will be procured from poultry farm at Ajmer, India. Eggs kept in an incubator at 37◦C with 65-70% relative humidity will be turned manually uptill 16^{th} day of incubation. The embryo development in eggs will be examined with the help of the Candler every day for ensuring proper growth and viability

C. EXPERIMENTAL DESIGN

Toxicity tests of Quinalphos 25% EC Flash (Organophosphate) and Carbosulfan 25% EC Marshal (Carbamate) will be conducted for:

- Teratological features
- Certain biochemical parameters
- Skeletal deformities.

For studying the aforementioned parameters the test animal will be grouped into five categories:

- Group I- Normal
- Group II- Control-immersed in DW
- Group III- Low dose-half of the recommended dose used in field
- Group IV- Moderate dose-recommended dose used in field
- Group V- High dose-Twice of recommended dose used in field.

There Plans have been proposed to study the aforementioned parameters-

1. "0" day immersion- embryos will be recovered on 4^{th} and 7^{th} day to study the teratology and biochemistry of whole embryo. Prehatched embryos on day 15^{th}-16^{th} will be recovered for histopathological study of liver and to examine skeletal growth. Specimens will be stained with Alcian Blue and Alizarin Red to study the skeletal system. Biochemistry will be performed using tissues of the liver and brain of 15^{th} -16^{th} day old embryo.

2. "4th" day immersion- embryos will be recovered on 7th and 10th day to study the teratology and biochemistry of whole embryo. Prehatched embryos on day 15th-16th will be recovered for histopathological study of liver and to examine skeletal growth. Specimens will be stained with Alcian Blue and Alizarin Red to study the skeletal system. Biochemistry will be performed using tissues of the liver and brain of 15th-16th day old embryo.
3. "7th" day immersion-. Prehatched embryos on day 15th-16th will be recovered for histopathological study of liver and to examine skeletal growth. Specimens will be stained with Alcian Blue and Alizarin Red to study the skeletal system. Biochemistry will be performed using tissues of the liver and brain of 15th-16th day old embryo.

D. EXPERIMENTAL PLAN

➲ **Plan I**

Eggs incubated on day "0"
(All the eggs will be incubated)

Eggs will be immersed separately in Quinalphos and Carbosulfan for 60 minutes in low, moderate and high dose.

25% of the total embryos will be recovered after 4^{th} day of incubation to study the biochemistry and teratology.

25% of the total embryos will be recovered after 7th day of incubation to study the biochemistry and teratology.

50% of the total embryos will be recovered on 15^{th}-16^{th} day of incubation to study biochemistry, histopathology and skeletal growth.

➲ **Plan II**

Eggs incubated on day "4th"
(All the eggs will be incubated)

Eggs will be immersed separately in Quinalphos and Carbosulfan for 60 minutes in low, moderate and high dose.

25% of the total embryos will be recovered after 7th day of incubation to study the biochemistry and teratology.

25% of the embryos will be recovered after 10th day of incubation to study the biochemistry and teratology.

50% of the total embryos will be recovered on 15th-16th day of incubation to study biochemistry, histopathology and skeletal growth.

➲ **Plan III**

Eggs incubated on day "7th"

(All the eggs will be incubated)

Eggs will be immersed separately in Quinalphos and Carbosulfan for 60 minutes in low, moderate and high dose.

All the embryos will be recovered on 15th-16th day of incubation to study biochemistry, histopathology and skeletal growth.

E. PARAMETERS

Teratogenicity includes biochemical, histopathological, gross morphological and skeletal parameters. The chick embryos will be subjected to following parametric studies for evaluation of teratogenic response in the animal.

➲ **Biochemistry-** Biochemical tests will be performed for:

Protein	Lowry *et al* (1951)
Cholesterol (Libermann-Burchard's method)	**Henry and Henry (1974)**
Glycogen (Rex Montogomery's method)	**Montgomery (1957)**
Nucleic acids (DNA & RNA)	**Schneider (1957)**
Enzymes-	1) GSH -**Moron *et al* (1979**), 2) AkP,AcP- **Kind and King** (**1954**), 3) GPT & GOT-**King (1965*)***, 4) AChE -**Ellman *et al* (1961**)

➲ **Histopathology**- The animals after being sacrificed will be dissected to expose the liver lobes. The tissues after being washed in the saline will be fixed in 10% formalin. The liver lobes will be dehydrated by passing through alcohol and processed in paraffin for sectioning with microtome. Staining will be done with haematoxylin and eosin. The slides will be examined and various histopathological changes will be observed.

➲ **Skeletal staining**-On Embryonic Day (ED) 15-16, embryos will be processed for staining with a whole mount double cartilage and bone staining technique described by Inouye. (1976).The embryos will be recovered from their egg shell and will be washed with Distilled Water (DW).

1. The animals will be eviscerated and fixed in 95% ethanol for 5 days.
2. To remove the fat and for making the specimens firm the embryos will be kept in acetone for 2 days.
3. Animals will be stained in freshly prepared staining solution at 37˚C:0.3% alcian blue in 70% ethanol-1 volume; 0.1% alizarin red in 95% ethanol-1 volume; acetic acid- 1 volume; 70% ethanol-17 volumes.
4. The specimens will be washed in distilled water and feathers will be easily removed with the help of scalpel.
5. The specimens will be kept for 48 hours in 1% KOH in summers and 2%KOH in winters to clear to clear the tissues.
6. Clearing will be done through 20%, 50%, and 80% glycerine/1% aqueous KOH solutions: 20%-1 volume glycerine/4 volumes 1% KOH; 50%-1 volumeglycerine/1 volume 1% KOH; 80%-4 volume glycerine/1 volume 1%KOH.
7. Specimens will be preserved in 100% Glycerol.

    - Skeletal defects like delayed or reduced ossification of (ribs, cervical vertebrae, metacarpus and digits), short kinked caudal vertebrae and reduced pygostyle, flexed digits, shortness of humerus and scapula, shortness of beak, pelvic girdle and skull, abnormally formed frontals and parietals etc will be studied.

➲ **Gross Morphology**-Embryos recovered on ED 4, 7, 10 and 15 will be observed for studying gross morphology. Number of alive, dead and alive with malformations will be noted. The living embryos will be wet weighed and examined for the presence of external malformations (of head, limbs, body and tail) under dissecting microscope. The abnormalities to be recorded are-Crooked neck, absence of beak, eye and tail, microcephaly (disproportionately small head) and delay of brain development, Microphthalmia (reduced size of the eye), Edema (swelling due to abnormal accumulation of fluid beneath the skin), Haemorrages (bleeding under skin), Hematomas (blood patches), runt (half the actual size), gastrochisis (Herniating organs) etc. Comparison will then be done with the series of stages in the development of the chick embryo given by Hamburger and Hamilton (1951).

F. TECHNIQUES

i. **Differential staining of cartilage and bone**- Alizarin Red and Alcian Blue.

ii. **Biochemistry**-Manually

iii. **Spectrophotometer**- UV-visible, Nano-spectrophotometer

iv. **Microtomy**- Tissues preserved in Formalin will be mounted in wax and thin sections will be cut using microtome. Slides will be prepared by passing through the alcohol series.

v. **Photography-**Digital Camera

vi. **Analytical method**- Student's t-test, Mann-Whitney U test using SPSS software.

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