HISTOPATHOLOGICAL STUDIES IN SELECTED ORGANS OF BOMBYX MORI EXPOSED TO PESTICIDES

8.1. INTRODUCTION:

Insect toxicology is becoming ever more relevant to insect pathology. For insects, which constitutionally can take “an unconscionable time dying” the final stages of morbidity involve any array of pathological conditions. These changes are relevant to the irreversibility that means death and so also are visible histological changes to the extent that they are consistent and peculiar insecticide (Brown, 1963). Symptoms of organophosphorous poisoning and ultimate cause of death is usually difficult to prove and still needs confirmation (Matsumura, 1985). The knowledge of insecticides in the insect body is therefore, a pre-requisite for devising and adapting control measures. It is also useful for selection, improvement and application of insecticides.

The susceptibility of animal tissues to different chemical agents may vary from animal to animal and also with in the same animal among different tissues and incorporation even at the very low concentrations of the parent and or their metabolites cause effect, in the vital tissues of organisms. The severity of damage depends on the toxic potentiality of a particular compound or pesticide accumulated in the tissue (Jayantha Rao et. al., 1985), although major advances have been made in recent years, pesticide toxicological studies, histology and histopathology of valuable insects are still to be studied when compared to other insects.

The information with regard to the effect of insecticides on the different parts of the insects, particularly of Indian valuable insects is scanty and has received less comparative attention. Therefore, there is a great need to screen insecticides and find out their effect on the different system of the body.

In the present study an attempt has been made to know the extent of damage to the general architecture of gut, silk gland and reproductive system of silk worm Bombyx mori due to oral administration of pesticides (Dichlorvos and Vijay neem pesticide) were presented.

8.2. MATERIALS AND METHODS:

For histopathological studies, control and experimental Bombyx mori instars (treated with different sublethal concentrations of Dichlorovos and Vijay neem pesticides) were dissected out and placed in plain saline water. The entire alimentary canal was taken out and cut in to fore gut, mid gut and hind gut. The tissues were preserved in 10% formalin solution and washed in tape water which were then dehydrated, stained with haemotoxylin and eosin and finally embedded with paraffin
at 65ºc (Luna 1968). After passing through usual microtomy process, the sections of 5µ thickness were obtained being stained by haematoxylin and eosin. The different functional or histological structures were examined and comparative physiological and anatomical changes in the gut were examined and the effect caused by pesticides was recorded by changes in the gross histological structure. Histopathological studies were also made in silk gland (Anterior, middle and posterior limb) and reproductive system in the same way.

For histopathological investigation of silk glands control and Experimental animals were selected for the study. The glands were fixed in Bouin’s fluid, a compound fixative. The water molecules were removed with the help of alcohol (Ethanol). The fixed tissues were passed through a series of increasing concentration of alcohol. The fixed tissues were passed through a series of increasing concentrations of alcohol such as 30% > 50 > 70% > 90% > absolute alcohol. After treated with absolute alcohol the materials were cleared with benzene.

After routine processing the cleared materials were embedded in paraffin wax for sectioning. Micro sections were cut a thickness of 6µ in rotary microtome. The ribbon strips were floated on water paved over aluminized slides. The sections were allowed to expand by gently warming the slide on a hot plate, maintained around 50ºC. When sections became flat and expanded, the water was drained. The slides were left over night on the hot plate maintained at constant temperature at 45ºC. The sections were stained with haemotoxylin a basic stain and eosin an acidic stain which stain the cells and cell components.

The slide containing sections were passed through a series of alcohol with decreasing concentrations such as absolute alcohol 90% > 70 > 50% > 30%. Finally
the sections were washed with distilled water to remove the excess stain. The stained sections were then passed through a series of alcohol with increasing concentrations such as 30% > 50% > 70% > 90% > absolute alcohol.

Then the materials were cleaned in xylene. The sections were identified by viewing through the Nikon-V-III multipoint sensor system, Japan under different magnifications and microphotographs were taken using Nikon FDX – 35.

8.3. RESULTS AND DISCUSSION:

8.3.1. HISTOLOGY OF SILK GLAND:

8.3.1. a. Control worm:

Silk gland in Bombyx mori is a protein factory specialized in silk production during the larval growth and development. It is bilaterally symmetrical paired structure situated on the ventrolateral sides of the body from the 4th to 8th segment and it is well differentiated in the fourth and fifth instar larvae. It accounts for about 50% of the weight of the larvae. The silk glands are cylindrical tubular organs of exceedingly variable length with characteristically branched nuclei. There are three distinct regions in the silk gland differing in structure and function. A thread like anterior part, a swollen middle part and a tubular crooked and curved posterior part. The anterior parts of the paired ducts unite and open in the final outlet called spinneret. The middle region of the silk gland is the most important region and it is the storehouse of liquid silk it is folded into a W-shaped structure and hence has three limbs Viz; Fore, mid and hind limbs. The posterior region is highly folded and the folds lie in the midst of the dermo-visceral muscles. They secrete the major protein of the silk, namely fibroin. A pair of small glands named filippis or Lyonnetts gland is situated at the junction of two anterior ducts.
A viscous fluid of unknown function is secreted by this gland. Silk is secreted by the silk gland in the form of fibrinogen which undergoes denaturation of extrusion to form a tough, elastic protein, Fibroin and is surrounded by an outer layer of water soluble gelatinous protein known as sericin. The glands are dermally derived and modified labial glands. The silk glands are tubular in nature and the diameter of the tube varies in the three distinct regions of the silk gland.

Histologically the silk gland is formed of three layers. The outer tunica propria of uniform thickness, the middle glandular layer and the inner tunica intima of varying thickness. The tunica intima is very thick in the anterior region, and is shed at each moult and in other regions it is thin and not shed at each moult. Tunica intima is followed by lumen. Anterior region of the silk gland has a narrow lumen with a thin stream of silk. Secretory cells are less. The tunica intima is wide in this region. (Fig. 8.3.1.a.1)

The posterior silk gland of the normal silk worm has a prominent secretory role. Secretory cells have rich secretory granules. The secretory layer is wide when compared to tunica propria. The tunica intima is narrow. The lumen of the posterior silk gland has a central mass of silk protein and a wide gap is seen between central mass of silk and tunica intima. The silk content in the posterior part is ensheathed by a boundary and this gives a definite package to the silk content. Inside the package the silk mass shows uniform density. (Fig 8.3.1.a.2)

The middle silk gland is ‘W’ shaped with three distinct regions anterior, middle and posterior limbs. It is a store house of liquid silk. In the normal worms the middle silk glands shows distinct tunica propria, glandular layer, tunica intima and lumen. (Fig 8.3.1.a.3)
The lumen is engorged with liquid fibroin which is surrounded by a darkly pigmented sericin or liquid silk. The glandular cells are secreting sericin and it is poured into lumen is quiet evident from photo micrographs. Tunica propria and tunica intima are narrow and glandular layer predominates the wall of the middle silk gland. In the lumen fibroin masses are present as individual units and these units were surrounded by sericin. In the middle limb of the mid silk gland the secretory activity is highly prominent. Secretions are poured in to the lumen as a stream of granules. Immediately after tunica intima, sericin layer is present. Sericin encircles fibroin mass. Haemocytes are seen in large numbers in the glandular zone. Two types of secretory cells are seen in glandular layer. Fibroin secretions are also poured in to the lumen from the glandular layer. (Fig 8.3.1.a.4)

8.3.1. b. Treated worm:

Histological arrangement in the anterior region of the silk gland speaks about poor secretory role of the anterior region. In treated worms (0.0005%) marked variations were noticed in the cellular arrangement. Secretory cells and columnar cells are hypertrophied and lumen has less dense silk indicating poor presence of silk in this region. (Fig 8.3.1.b.1)

The middle silk gland of treated worms showed many changes in histology. Secretory cells in the glandular layer showed poor secretory activity. Secretory cells are filled with amorphous substances, which are darkly stained. Secretory granules are loose in composition in between sericin and fibroin layer. The gap is seen between sericin and fibroin layer which receive loose granular secretory particles; and the border of fibroin layer is brush like. The secretory cells are vacuolated and large
vacuoles appear in the sericin mass. In the sericin layer of the lumen vacuolated spots are seen. (Fig 8.3.1.b.2)

In Vijayneem treated worms the posterior silk gland is much affected histologically. The general rigidity seen in the normal worm is quiet absent in the posterior gland of treated worms. Some of the secretory cells are empty. The lumen also has lost its normal structure. In the silk mass vacuolations were seen with concentrated silk mass. Demarcation between secretory and columnar cells is poor. There was reduction in quantity of silk due to Dichlorvos and Vijay neem. (Fig 8.3.1.b.3)

8.3.2. HISTOPATHOLOGICAL CHANGES IN ALIMENTARY TRACT:
8.3.2.a Control worm

The alimentary canal is a convoluted epithelial tube with three distinct regions, viz, fore gut, mid gut and hind gut.

The fore-gut arises as an anterior ectodermal invagination and forms buccalcavity, pharynx and oesophagus. Histologically, the fore gut is made up of a cuticular coat, which is continuous with the cuticle of the body wall, epithelial layer having basement membrane bounding the outer surface, longitudinal muscles, circular muscles and connective tissue sheath. (Fig 8.3.2.a.1)

The mid gut in the larvae is the main organ involved in digestion and absorption. It is a straight and long tube occupying the major part of the alimentary tract. Histologically, a stratum of enteric epithelium, the outer ends of whose cells rest upon a basement membrane, lines the mid gut. The latter is followed by an inner layer of circular muscles and an outer layer of longitudinal muscles. The outer most coat of
the mid gut is a thin peritoneal membrane. Both muscles are composed of striated fibers and their positions are the reverse to what obtains in the fore gut. The enteric epithelium of mid – gut is devoid of cuticle, but have a delicate detached sheath called peritrophic membrane. It is produced by the delamination of thin sheets from the surface of the cells throughout the length of the mid gut. The mid gut cells are chiefly of two types, columnar cells and secretory or goblet cells. In addition regenerative cells replacing the destroyed epithelial cells during moulting are also present. The inner margin of the epithelial layer bordering the lumen is wrinkled with microvilli or gastric diverticula so as to augment the surface area for digestion and absorption. (Fig 8.3.2.a.2)

The columnar (cylindrical) cells of the mid gut are active functional cells, whose inner brush border projecting into the lumen promotes secretion and absorption. Goblet cells (calcyform) are small secretory cells interspersed among columnar cells. The goblet cells have reduced cytoplasm and their striated border has deep invagination to form cavity. Among the active epithelial cells there are small basal, embryonic or replacement cells called regeneration cells.

As epithelial cells destroyed through secretion or degenerated during moulting, the basal regenerated cells grow to replace them. The regenerative cells are scattered singular along the gut. In the mid gut some of the cells are vacuolated and these cells are secretory and vacuolation also indicate emptiness after absorption of material in that cells. (Fig 8.3.2.a.3)

The undigested material goes straight to the hind gut where water absorption and faeces formation and elimination occur. The hind gut is morphologically subdivided into pylorus, ileum, colon and rectum. The malphigian tubules open into
the pylorus. Histologically the hind gut is made up of the same layers as in the foregut, but the epithelial cells are larger. The cuticle is thin. In the hind end the epithelial cells are highly folded. In the rectal region the tunica intima layer develop thorny projections which are shed at each moulting. The circular muscle layer is continuous and longitudinal muscles are bundled. The hind gut of control worm is distinguished into ileum, colon and rectum. The propria intima or cuticular lining of the ileum and colon is thrown into numerous folds and provided with spinous projections. The rectum is a pyriform chamber provided with several invading projecting papillae. It consists of single layer of tall epithelial cells with a hollow cavity. The longitudinal muscles are in bundles. The circular muscles form a continuous layer. Malphigian tubules are inserted into the musculature of the rectum. Malphigian tubules have a lumen and the perimeter of the lumen has a single layer of epithelial cells. The epithelial layer is surrounded by a peritoneum.

8.3.2. b. Histopathological studies in the gut of Pesticide treated worms:

The digestive system of silk worm larvae is highly functional to utilize the large quantity of mulberry leaves the worms consume. The pesticides induce toxication in the alimentary tract. Hence the architectural changes in the gut histology could expose the magnitude of the disease. The damage to gut architecture changes the colour of the worm. In the present investigation marked variations were observed in the gut histology.

Fore gut:

1. In the fore gut of neem pesticide treated worms large undigested pieces of mulberry leaves were seen indicating poor digestive function (Fig 8.3.2.b.1 &8.3.2.b.2)
2. The epithelial cells were completely shriveled and reduced in size in the larvae treated with Vijay neem pesticide and intimal layer and epithelial cells were disintegrated and spread into the gut lumen, circular muscles also ruptured.

3. Circular muscles, epithelial cells and intimal layers were heavily damaged and spread into the gut lumen of fore gut, and some epithelial cells were disintegrated in the larval instars treated with Vijayneem (Fig 8.3.2.b.3)

Mid gut:

The mid gut of both Dichlorovos and neem pesticide treated larvae of silkworm *Bombyx mori* the histological organization and holocrine secretion got affected.

In the present study much histological changes have been observed in the mid gut of both Dichlorovos and Vijay neem pesticide treated instars of *Bombyx mori*.

**Dichlorovos treated worms: (Fig 8.3.2.b.4)**

1. The mid gut epithelium gave dehydrated appearance with the elongation of its cells and their disintegration into the lumen of the gut.

2. Rounded or club shaped segment of cytoplasm with and with out nuclei were seen pinching off from the tip of the epithelial cells. The inner ends of some of the cells were broken and cytoplasmic granules were given out.

3. The nuclei got elongated and chromatins of the nuclear granules were observed of larger size.

4. The epithelial cells were detached from the basement membrane and fell in to the lumen.
5. Peritrophic membrane was partially or completely damaged and was not closely lying to the epithelial cells. Cytoplasmic vesicles were found between epithelium and peritrophic membrane.

**Neem pesticide treated worms: (Fig 8.3.2.b.5)**

- The cell contents were entirely emptied and boundaries were lost.
- The nuclei were in the advanced stage of degeneration and the nuclear membranes of most of the nuclei were broken and their contents were emptied.
- The nuclei were in the advanced stage of degeneration and the nuclear membrane of peritrophic membrane was completely obliterated.
- Disintegration of regenerative cells and thinning of circular muscles observed
- Glandular hyperactivity has been observed throughout the length of the mid gut.
- The nucleus was condensed at the basal region of the goblet cells and granular in columnar cells.

**Discussion**

The silkworm larvae are voracious feeders and the efficiency of feeding, digestion, absorption and feed type decides the economics of sericulture. Minimizing the feed intake and break down in feed conversion interrogates the energy budget and ends in poor quality cocoon formation. Hence the digestive system needs much attention in silkworm larvae.

As the result of pesticide treatment, epithelium of oesophagus was completely obliterated. Mahabir Singh (1990) also reported the same effect. Fore gut was more affected than the hind gut. The same effect was reported by Sharma (1966) while
studying the effect of parathion on the alimentary canal of male *Poecilocerus pictus* F, Lakshman Lal et. al., (1970) reported as the result of feeding of Dichlorovos to the fourth instar larvae of tobacco caterpillar intima was not affected, whereas disintegration, degeneration, vacuolization, disappearance of cell of a poor degree and shedding of cytoplasm was observed in the oesophagus and crop.

In the mid gut of Vijay neem treated instar larvae, the epithelial cells were disintegrated and spread into the gut lumen. The peritrophic layers were degraded. Earlier, Ramarethinum et. al., (2000) also observed similar results in *Spodoptera litura* due to the pesticide nimbicidine. Also fore gut epithelial cells were completely shBoterlled and reduced in size and in mid gut circular muscles and epithelial cells collapsed in the larvae. Circular muscles epithelial cells and intimal layers were heavily damaged and spread into the gut lumen of foregut and mid gut in the Dichlorovos treated instars. Also the epithelial cells were detached from the circular muscles. Circular muscles were broken here and there.

Lakshman Lal et. al., (1970) observed similar results in *Spodoptera litura* Fab due to the Endosulfan, Diazinon and Dichlorovos which supports the above findings. Chandbourne and Rain water, (1953) reported similar observations when calcium arsenate was fed to the larvae of boll worm, *Heliothis armigera* Hubner. Insecticides caused damage to the epithelial cells of the mid gut of *Orthreis maternal* (Chitralekha Deshmukh 2009).

Degeneration, dehydration and vacuolization of mid gut epithelium, liberation of nuclei and cytoplasmic fragments into the lumen clumping of chromatin material and degeneration of regenerative cells were noticed in treated worms. The same effect was reported by, Hussanein and Khalil (1969) in the Metasystox poisoned larvae of
Coccinella _undecim Punctata_, Toppozoda et. al., (1968) in parathion poisoned _Spodoptera littoralis_, Mukherji and Mistra (1971) in Malathion and parathion poisoned _Hieroglyphus baion_.

Elongation of mid gut was observed in the present finding. Elongation of epithelial cells of mid gut was observed also by Singh (1990) in ethyl parathion treated _Chrotogonous trachypterus_ and Toppozoda et. al., (1968) who considered such elongation as a result of hypersecretory activity. The same effect was noticed by Dubey and Awasthi, (1995) in organophosphorous treated phytophagous insects.

Glandular hyperactivity is also evident in pesticide treated silk worm _Bombyx mori_ instars, where at places the cellular proliferation from the tip of epithelial cells has been observed resulting into pinching off of rounded or club shaped cellular fragments into the lumen as has also been observed in _A.mellifera_ (Salkeld, 1950, 1951), _P.americana_ (Dutta and Das 1971), phytophagous insects (Dubey and Awasthi, 1995), _Mactra violacea_ (Syed Mohamed shah et. al., 2003), Toppozada et. al., 1968, Rizvi and Khan, 1973; Deshmukh and Tambhare, 1998, Chitralekha – Deshmukh et. al., (2009).

In the present study clumping of nuclear chromatin and condensation of nuclei has been observed which appeared deeply stained similar to the findings of Lal et. al., (1970) and Dutta and Das (1971), Toppozoda et. al., (1968), Deshmukh (2006), Chitralekha Deshmukh (2009).

Mistra (1981) and Mistra and Mukharji (1972) reported shrinkage and even complete degeneration of nuclei in insects treated with organophosphorous insecticides. Nuclear elongation has also been observed by Hopp (1953) in carbon
tetra chloride poisoned *Pediculus humanus* and by Singh (1990) in ethylparathion treated *C.\textit{trachypeterus}* respectively.

Hypersecretory activity was noticed. The insecticides may cause rapid secretion of the stored enzymes in the mid gut tissue (Eguchi et. al., 1972; Chanda and Roy 1986; Deshmukh and Tembhare, 1998). Insecticides interact with a variety of neurochemical process and are likely to be involved in the disruption of nerve function (Soderlund and Bloom quirt 1989) which might have caused the initial hypersecretory activity and cytological changes.

Imbalance in enzyme substrate complex and inhibition of peristaltic movement of the gut might have inhibited the enzyme activity in the treated insects (Senthil Nathan et. al., 2005). Insecticides may interfere with the production of certain types of proteins (Smile et. al., 1996) and digestive enzyme activity (Eguchi et. al., 1972; Huang et. al., 2008). Vacuolization of the mid gut epithelium was also reported after the treatment of arsenites (Rizvi and Khan, 1973) and organophosphates (Deshmukh and Tembhare, 1998) Carbaryl and r – BHC (Chitralekha Deshmukh, 2009). Apical swelling and blabbing of large cytoplasmic vesicles by the columnar cell nuclei in vesicles in to the gut lumen and lysis of the gut epithelium (Blackburn et. al., 1998) Mainly the mid gut epithelium is affected by treatment with Tannic acid in aquatic Diptera, Muramorosch (2002).

Thus, when silk worm larvae is happened to expose to pesticides they cause irrepairable architectural changes in the vital organs like the gut and making the animal less fit for better survival. These histopathological changes can alter various physiological activities of silk worm larvae such as release of various enzymes and consequently metabolism is affected. Both the pesticides are well competent with a short period due to their quick mode of action at cellular level.