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Effects of *Bacillus thuringiensis kurstaki* on Midgut Cells of *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae), The Pine Processionary Caterpillar

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ABSTRACT

In this study effects of *Bacillus thuringiensis kurstaki (Btk)* on midgut cells of *Thaumetopoea pityocampa* larvae was investigated by electron microscopy. 3 mg/l *Btk* was given with food. 1 h, 3 h and 6 h after *Btk* administration no pathological changes in the midgut epitelial cells were observed. 12 h after *Btk* administration swelling of mitochondria in midgut epitelial cells were observed. Formation of vacuoles in cytoplasm was seen after 24 h. After 48 h decreasing of microvilli of midgut epithelial cell's and partial dissolving of cytoplasm, dissolving of nucleoplasm and chromatin clumps were observed. 60 h after *Btk* administration swelling and dissolving of mitochondria in midgut epithelial cells were detected. 96 h after *Btk* administration a membrane-body and dissolving in the cytoplasm clumps in midgut cells of *T. pityocampa* larvae were occurred. Present study shows that *Btk* is effective to *T. pityocampa* larvae.

Key Words: *Thaumetopoea pityocampa, Bacillus thuringiensis kurstaki, midgut, ultrastructure, transmission electron microscope.*

1. INTRODUCTION

Thaumetopoea pityocampa Den. & Schiff., Lep., Thaumetopoeidae, the pine processionary caterpillar, is the most important defoliator of pines in the Mediterranean region [1, 2, 3]. This caterpillar is the cause of considerable damage to forest and is also responsible for dermatitis, ocular lesions and, more rarely, respiratory signs and anaphylactic reactions in animals and humans [4]. The human health problems caused by the urticating hairs of the larvae [5]. *T. pityocampa* has a recently described hair protein: thaumetopoein [6]. Thaumetopoein, produces allergic reactions in men and animals [7, 8]. Thaumetopoein has a direct (non-IgE) effect on mast cells leading to their degranulation [9]. Kalender *et al.* [10] showed that.dermal mast cell degranulation in mice was observed 12 and 24 hours after exposure *T. pityocampa* larvae. Although there is an increasing demand for environmentally friendly alternative methods, efforts to control this lepidopteran insect involve mainly the use of chemical insecticides, particularly insect growth inhibitors [11]. Insect viruses

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(cytoplasmic polyhedrosis virus, nuclear polyhedrosis virus, and granulosis virus), fungi (*Beauveria bassiana*), pheromones parasites, and especially bacteria (mainly preparations based on *Bacillus thuringiensis* Berliner var. *kurstaki*) have been investigated for the biological control of *T. pityocampa* [9].

Bacillus thuringiensis Berliner (*Bt*) is a spore-forming gram-positive bacterium that produces insecticidal crystalline proteins (δ-endotoxins) when sporulating. Those toxic proteins are named crystal (Cry) proteins because of their abilities to auto-crystallize in the bacterial cytoplasm. Encoded by different cry genes from different isolates, different Cry proteins have different insecticidal specificities [12]. Although *Bt* is an insect pathogen, it is generally used as a bio-insecticide [13]. Strains of *Bt* have been found with δ-endotoxins that are active against a wide range of insect pests and some other organisms viz. Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Mallophaga, nematodes, mites, and protozoa [14-19].

The aim of the present study was to investigate the effects of *Bacillus thuringiensis kurstaki (Btk)* on the midgut cells of *T. pityocampa* larvae.

2. MATERIALS AND METHODS

2.1. Insects

Larvae of *T. pityocampa* were collected Kahramanmaras, Turkey. The larvae were fed with pine needles (*Pinus nigra*) in the laboratory. Larvae were individually reared in the laboratory at 25±1 ^{0}C, 60±10% r.h. under a 12:12 light-dark photoperiod.

2.1. *Bacillus thuringiensis*

The commercial preparation which was used is called MVP Bioinsecticide (Mycogen Corporation, USA). MVP is made of *Bacillus thuringiensis kurstaki* and has already been used for several years against Lepidoptera. Its concentration is 10000 IU/mg.

2.2. Treatment of Insects

Tests were done on fourth instar larvae of *T. pityocampa*. *T. pityocampa* larvae are active at night, therefore experiments were initiated at night. Larvae were put on a diet 2 days before the beginning of the experiments. Larvae were divided into a control and test groups. 50 larvae were present in each group. *Btk* was diluted in distilled water and 3 mg/l was given with food to larvae. Fresh *P. nigra* needles were dipped in a suspension of *Btk*, air dried and placed in 10 cm plastic dishes. Larvae were placed into the plastic dishes. Fresh *P. nigra* needles were given to the control group. 1, 3, 6, 12, 24, 48, 60, 96 h after *Btk* administration, larvae were dissected and prepared for electron microscopy.

2.3. Electron Microscopy

For electron microscopic examinations of tissues, primer fixation was made in 3% glutaraldehyde (Agar Sci. Ltd., Essex, England) in sodium phosphate buffer (200 mM, pH 7.4) (Merck, Alfred Paluka Co., Turkey) for 3 h at 4°C. Materials were washed with the same buffer and postfixed in 1% osmium tetroxide (Agar Sci. Ltd., Essex, England) and in sodium phosphate buffer pH 7.4 for 1 h at 4°C. Tissue samples were washed with the same buffer for 3 h at 4°C, and were dehydrated in graded ethanol series (Agar Sci. Ltd., Essex, England) and were embedded in Araldite (Agar Sci. Ltd., Essex, England). Thin sections were cut with Reichert OM U3 (Leica Co., Austria) ultramicrotome. Samples were stained with 2% uranyl acetate and lead citrate. The sections were viewed and photographed on a Jeol 100 CX II transmission electron microscope (TEM) (Jeol Ltd, Japan) at 80 kV.

3. RESULT

In our electron microscopic examinations, the midgut of *T. pityocampa* larvae was lined by columnar epithelial cells. The apical surfaces of midgut epithelial cells have abundant and long microvilli. Midgut epitelial cells contain numerous mitochondria and rough endoplasmic reticulum (Figure 1). 1 h, 3 h and 6 h after *Btk* administration no pathological changes in the midgut epitelial cells were observed (Figure 2-Figure 4). Swelling in mitochondria of midgut epitelial cells occurred at 12 h after *Btk* treatment at 3 mg/l**.** The mitochondrial matrix lost its electron-dense structure. Large and small vacuoles observed in cytoplasm of midgut epitelial cells (Figure 5). Numerous vacuoles in the cytoplasm of midgut epitelial cells were observed at 24 h after bacterial treatment. Vacuoles have residual bodies in it (Figure 6). Swelling of mitochondria, dissolving of nucleoplasm and chromatin clumps in midgut epitelial cells of *T. pityocampa* larvae were noticed at 48 h after *Btk* treatment (Figure 7). 60 h after *Btk* administration swelling and dissolving of mitochondria in midgut epitelial cells were detected (Figure 8). 96 h after *Btk* administration a membrane-body and dissolving in the cytoplasm in midgut epitelial cells of *T. pityocampa* larvae were occurred (Figure 9).

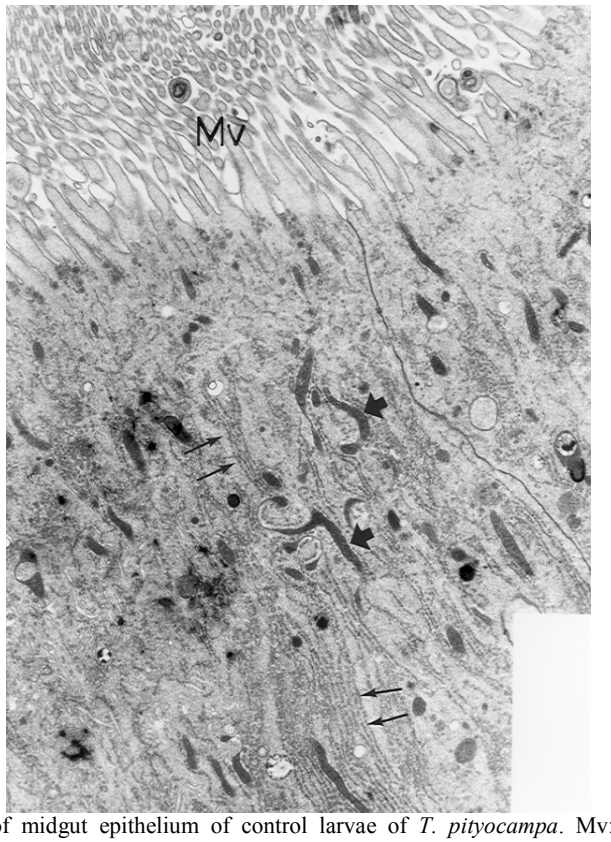

Fig. 1. Electron micrograph of midgut epithelium of control larvae of *T. pityocampa*. Mv: microvilli, →: endoplasmic reticulum, ➡: mitochondria. X6750

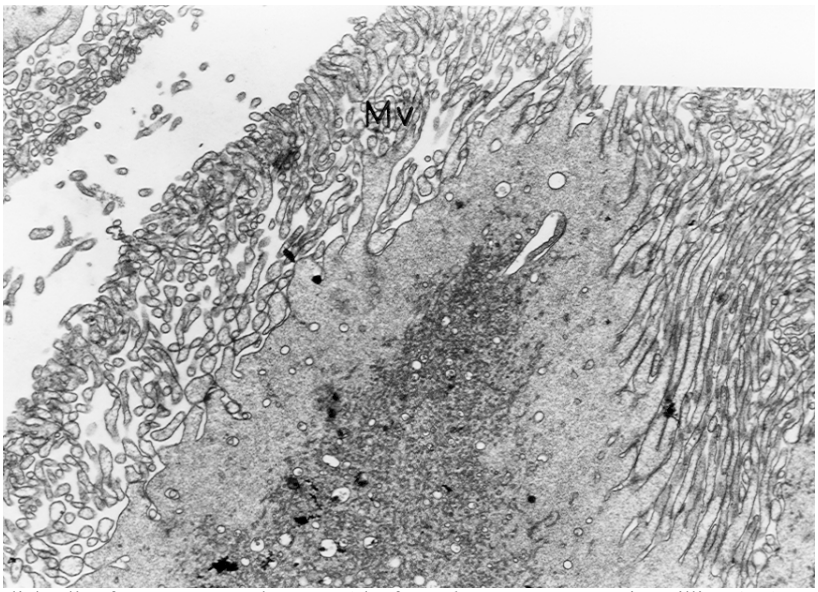

Fig. 2. Midgut epithelial cells of *T. pityocampa* larvae at 1 h after *Btk* treatment. Mv: microvilli. X6750

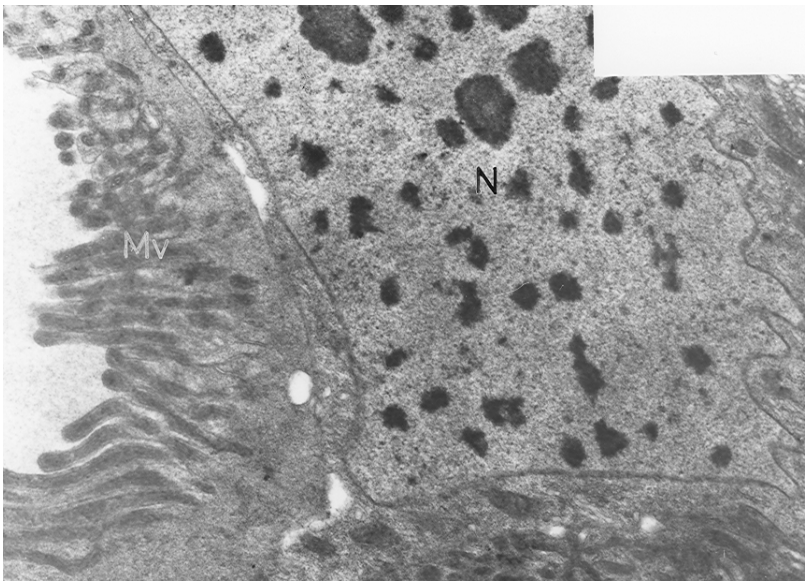

Fig. 3. Midgut epithelial cells of *T. pityocampa* larvae at 3 h after *Btk* treatment. Mv: microvilli, N: nucleus. X10000

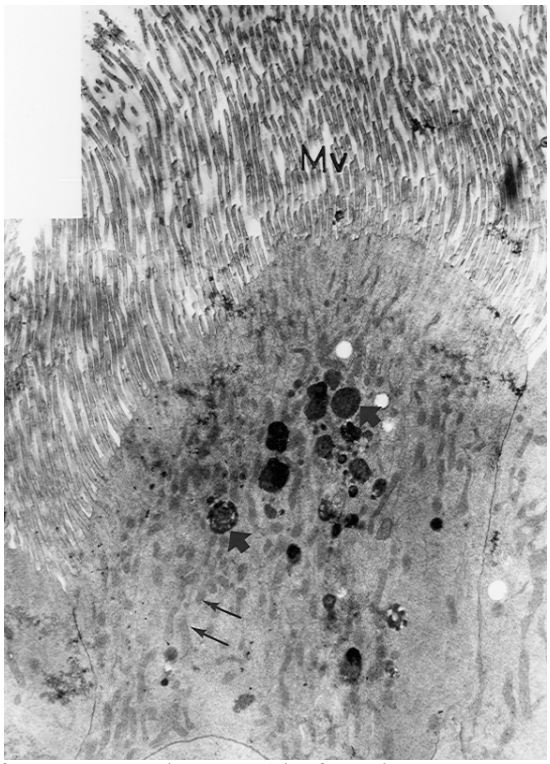

Fig. 4. Midgut epithelial cells of *T. pityocampa* larvae at 6 h after *Btk* treatment. Mv: microvilli, →: mitochondria, ➡: mitochondria. X5000

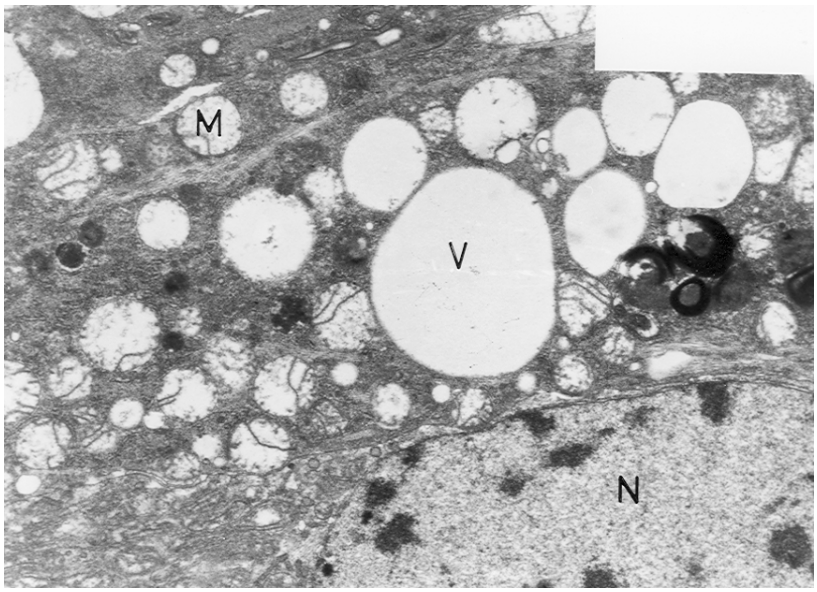

Fig. 5. Swelling of mitochondria (M) in midgut epithelial cells of *T. pityocampa* larvae at 12 h after *Btk* treatment. N: nucleus, V: vacuole. X10000

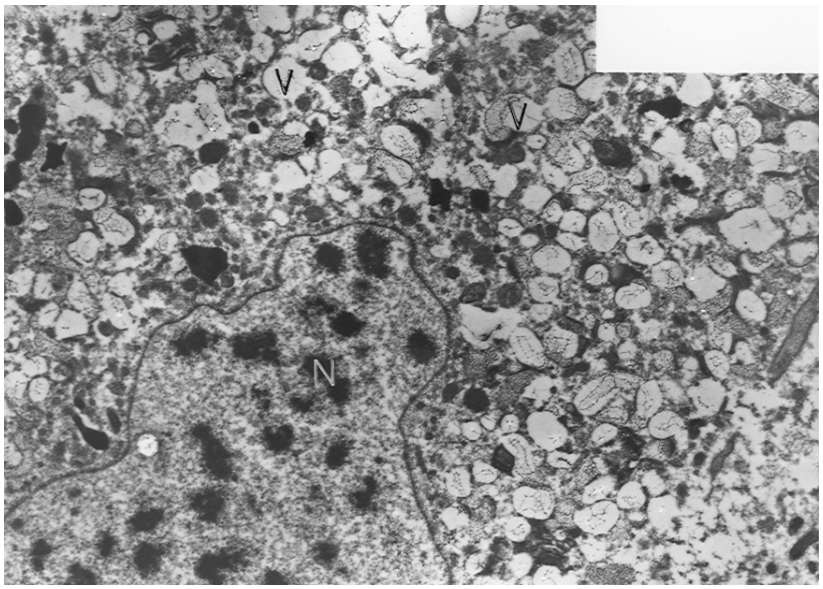

Fig. 6. Vacuoles (V) in cytoplasm in midgut epithelial cells of *T. pityocampa* larvae at 24 h after *Btk* treatment. N: nucleus. X7250

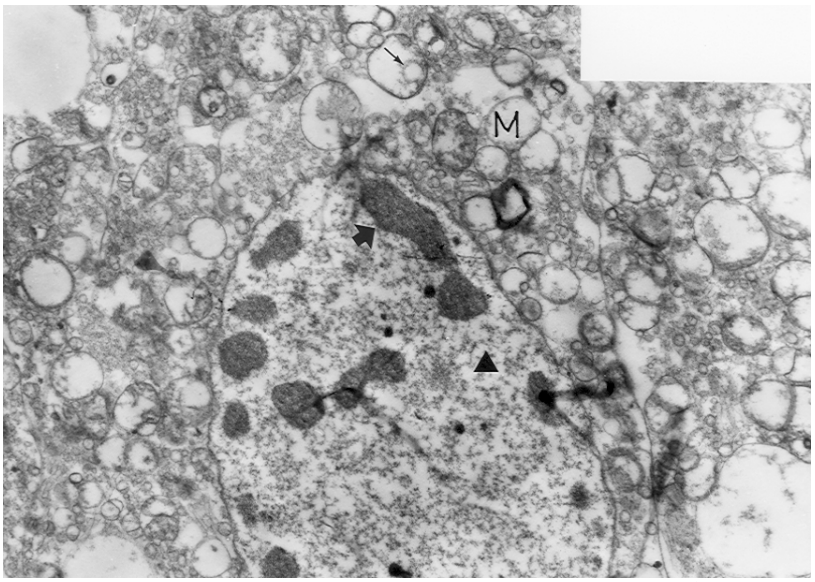

Fig. 7. Swelling of mitochondria (M), dissolving of nucleoplasm (★) and chromotin clumps (➡) in midgut epithelial cells of *T. pityocampa* larvae at 48 h after *Btk* treatment. X7250

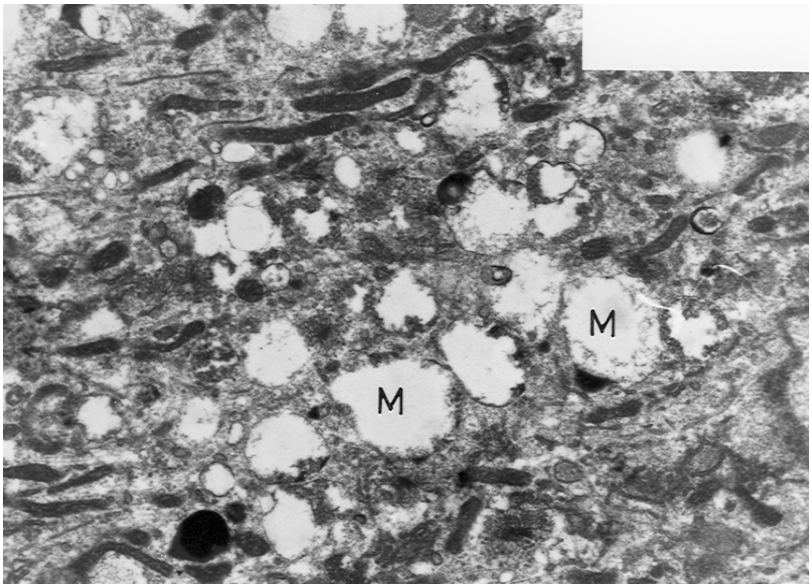

Fig. 8. Swelling and dissolving of mitochondria (M) in midgut epithelial cells of *T. pityocampa* larvae at 60 h after *Btk* treatment. M: mitochondria. X10000

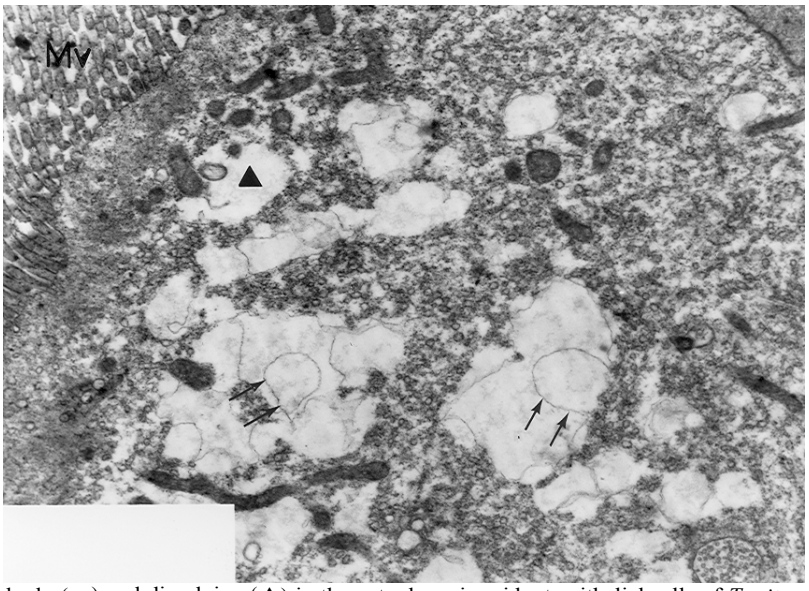

Fig. 9. A membrane-body (→) and dissolving (▲) in the cytoplasm in midgut epithelial cells of *T. pityocampa* larvae at 96 h after *Btk* treatment. Mv: Microvilli. X10000

4. DISCUSSION

The pine processionary moth *Thaumetopoea pityocampa* (Den.&Schiff.) (Lep., Thaumetopoeidae) is one of the main pine pests in countries of the Mediterranean region where it causes sometimes complete defoliation [20]. This defoliation seldom causes death of adult infested trees [21]. In addition of being an important defoliator, it is also harmful to people and animals, due to the urticating hairs released by the caterpillars [6, 22]. The allergenicity of the *T. pityocampa* larvae has been demonstrated in previous studies [23, 24, 25]. *T. pityocampa* larvae is able to elicit cutaneous and ocular lesions and more rarely, respiratory symptoms and anaphylactic reactions [4, 6]. But so far, no death caused by caterpillars has been recorded [12]. Two persons in our team revealed allergic reactions during our study. Blisters were observed especially on their hands, arms and necks and hence they were kept in hospital for two days. In addition, the same types of allergic reactions were detected even on their family members. This occurred because hair of larvae can be transferred with clothes. For this reason this insect should be controlled. *T. pityocampa* larvae are gregarious throughout development and pass through five instars [26, 27]. The L3-L4 larval stages are mainly responsible for the pine forest defoliation [9], as well as erucism and allergies caused to humans and animals [28, 29]. Therefore, in this study the L4 larval stage of *T. pityocampa* was used.

Insect viruses (cytoplasmic polyhedrosis virus, nuclear polyhedrosis virus, and granulosis virus), fungi (*Beauveria bassiana*), pheromones parasites, and especially bacteria (mainly preparations based on *Bacillus thuringiensis* Berliner var. *kurstaki*) have been investigated for the biological control of *T. pityocampa* [9]. There are studies investigation the effects of *Bt* on the midgut of insects [30, 31]. The digestive tract of insects is considered an effective physical and chemical barrier against potentially invasive pathogens that are ingested with food [32]. The gut is a simple tube connecting mouth and anus. The alimentary canal can always be divided into three regions; foregut, midgut and hindgut. The foregut is cuticle-lined, and as impermeable as the external surface of insect. So, while the foregut may be considerable importance in storage and digestion, it does not play a significant role absorption. The hindgut, although it also has cuticular lining, is far more permeable [33], and serves to finally "reclaim" useful substances before they are lost to the insect in faeces. However, the midgut is by far the most permeable region of the alimentary canal. It follows that the midgut must play a central role in the transport processes by which an insect interacts with its environment [34].

Bt is preferred because of its specific toxicity spectrum, it does not affect beneficial insect, plants, or animals, including humans. *Bt* is a microbial insecticide are commonly used as environmentally-safe alternatives to synthetic pesticides [35-38]. *Bt* constitutes a large family of bacterial subspecies highly specialized as insect pathogens found in many different habitats. *Bt*, a gram-positive bacteria, produces a proteinaceous parasporal crystalline inclusion during sporulation. More than 14 distinct crystal protein genes are described, and recently additional insecticidal proteins have been identified [39, 40]. Upon ingestion by insects, this crystalline inclusion is solubilized in the midgut, releasing proteins called δ-endotoxins. These proteins (protoxins) are activated by midgut proteases. The active toxin fragment binds to specific membrane receptors on the apical border of the midgut epithelium columnar cells [41]. After binding, the toxin or part of it, insert into the cell membrane [42],

leading to the formation of lytic pores [43, 44], which disrupt midgut ion gradients and the transepithelial potential difference. This disruption is accompanied by an inflow of water that leads to cell swelling and eventual lysis, resulting in paralysis of the midgut and subsequent larval death [45]. *Bt* endotoxin has been shown by light microscope immunofluorescence staining to bind to cell's of the insect midgut and Malpighian epithelium [40].

Researches have investigated the histopathological changes, employing light microscope and electron microscope; the important changes produced in the cells are reported to be hypertrophy of cells, swelling of the plasma membrane at the free surface and the disruption of microvilli, vacuolation of cytoplasm, changes in the cisternae of the endoplasmic reticulum, loss of ribosomes, and changes in the configuration of mitochondria [33, 34, 46, 47, 48]. Our findings have similarity to those of the other studies. Death, connected with toxin administration, was observed early. *Btk* that was used in this study is available commercially at a concentration of 10000 IU/mg. The excessive effect is observed 24 h after treatment. Ultrastructural changes were observed 12 h after. In conclusion, *Btk* causes pathologic changes the midgut epithelium of insects, It disturbs the mechanism of oxidative phosphorilation and causes the dissolution of nucleoplasm, disordering of microvilli, swelling of mitochondria. *Btk* could be used reliably on *T. pityocampa* larvae control.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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