Türk. entomol. derg., 2011, 35 (2): 179-194
ISSN 1010-6960

Orijinal araştırma (Original article)

The influence of juvenile hormone analogue, fenoxycarb on the midgut remodeling in *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae) during larval-pupal metamorphosis[1]

Ebru GONCU[2*] Osman PARLAK[2]

Summary

During larval-pupal metamorphosis, midgut tissues of holometabolous insects undergo remodeling and this remodeling process involves two metamorphic events. Degeneration of larval midgut occurs via programmed cell death and pupal midgut develops from regenerative (stem) cells. All metamorphic events in insects are regulated by ecdysone and juvenile hormone. Molecular mechanisms of ecdysone are well known but action mechanism of juvenile hormone is not well understood. Fenoxycarb, 0-ethyl N-(2-(4-pheoxyphenoxy)-ethyl) carbamate has been previously shown one of the most potent juvenile hormone analogue against a variety of insect species and widely used for investigations the mechanisms of juvenile hormone. In this study, we aimed to analyze the metamorphic events in the midgut of *Bombyx mori* Linnaeus, 1758 and investigate the effects of fenoxycarb on this period. For this purpose, we applied two different doses of fenoxycarb (1ng/10µl and 10ng/10µl) topically on day 0 of fifth instar larvae. We followed the progression of remodeling processes with morphological observations and analyzed programmed cell death specific proteins, caspase 3 for apoptosis and acid phosphatase for autophagic cell death in control and experiment groups. Our results indicated that, programmed cell death of larval midgut has morphologic characteristics of apoptosis and caspase activation occurs during this period and juvenile hormone analogue fenoxycarb inhibits or delays remodeling process in dose dependent manner.

Key words: Apoptosis, autophagic cell death, *Bombyx mori*, fenoxycarb, midgut

Anahtar sözcükler: Apoptozis, otofajik hücre ölümü *Bombyx mori*, fenoksikarb, orta barsak

---

[1] This study was presented in the seventh International Workshop on Molecular Biology and Genetics of the Lepidoptera which was organized in August 23-29, 2009 in Crete, Greece and it was publıshed as summary.

[2] Ege University, Faculty of Science, Department of Biology, 35100, Bornova, Izmir/TURKEY

[*] Sorumlu yazar (Corresponding author) e-mail: ebru.goncu@ege.edu.tr

Alınış (Received): 10.03.2010 Kabul ediliş (Accepted): 08.09.2010

Introduction

Metamorphosis of insects requires some remodeling processes in digestive system. There are mainly two different events are involved during metamorphosis. Firstly, unnecessary larval tissues degenerate via programmed cell death process (PCD) (Lockshin & Beaulaton, 1974) then some of the tissues which are needed for adult life develop from imaginal cells. Midgut of holometabolous insects shows both of these events which are controlled mainly by two hormones: steroid hormone ecdysone and sesquiterpenoid juvenile hormone (JH) (Wigglesworth, 1972; Riddiford, 1975).

According to embryonic origins, insect digestive system consists of three different regions: foregut, midgut and hindgut. The midgut is composed of a single layered epithelium contains columnar cells, goblet cells, regenerative (stem) cells and endocrine cells. The epithelium is also covered by inner circular and outer longitudinal muscles. In the prepupal stage, regenerative cells proliferate and differentiate into pupal epithelium but the remaining larval cells degenerate via programmed cell death and fall into the lumen. The pupal midgut consists of thin and tall epithelial cells which are formed from the regenerative cells just before larval-pupal molt (Lehane & Billingsley, 1996).

Programmed cell death (PCD) is an active process and under tightly genetic control (Lockshin & Zakeri, 2004). Two main mechanisms of programmed cell death- autophagic cell death and apoptosis- have been well characterized (Saraste & Pulkki, 2000; Tsujimoto & Shimizu, 2005). The major features of the apoptotic cell death are membrane blebbing, proteolytic enzymes called “caspases” activation, DNA fragmentation and apoptotic body formation which is phagocytosed by the surrounding cells (Saraste & Pulkki, 2000).

Autophagy is a well known process for degrading long lived protein and it also plays a role as an adaptation of starvation and a lifesaver mechanism for both cells and organisms (Bowen et al., 1976). During autophagy, double or multimembrane bound structures are called as autophagosomes or autophagic vacuole. They are formed de novo to sequester cytoplasmic contents including mitochondria, endoplasmic reticulum and ribosomes (Tsujimoto & Shimizu, 2005). Autophagy is also involved in programmed cell death mechanism and called as autophagic programmed cell death. Especially the insect metamorphosis, whole tissues or organs have to degenerate for successful body construction in pupae or adult but there are not enough phagocytic cells for apoptotic cell death hence autophagy acts as programmed cell death process for complete degeneration of larval tissues (Goncu & Parlak, 2008). This cell death is characterized by the accumulation of autophagic vacuoles within the cells. One of the biochemical signs of autophagic cell death is appearance of lysosomes and the increase in lysosomal enzyme activities

(Sass et al., 1989; Zakeri et al., 1996). Acid phosphates is known as a lysosomal marker enzyme (Csikos & Sass, 1997). During degeneration of the larval organs such as silk glands (Goncu & Parlak, 2008) and flight muscles (Sahota, 1975), acid phosphatase activity was found abundantly and autophagic vacuoles were clearly demonstrated in dying anterior silk gland cells (Goncu & Parlak, 2008). In this study, we demonstrated the activation of lysosomal enzymes in *Bombyx* midgut as an indicator of autophagic cell death.

Caspases are cysteine proteases which are normally present in the cells as inactive forms. Following apoptotic signal, they cleave and activation occurs. Caspase activation during programmed cell death in larval salivary glands and midgut of *Drosophila melanogaster* has been demonstrated by Jiang et al., (1997) and Lee et al. (2002). Tettamanti et al. (2007) reported that caspase 3 activation was found in *Heliothis virescens* midgut during remodeling process. The role of caspase 3 in larval midgut degeneration of *Bombyx mori* and effects of fenoxycarb have not been studied so far. Therefore we aimed to analyze degeneration period of larval midgut in *Bombyx mori* and effects of juvenile hormone analogue, fenoxycarb. It has been reported that another juvenile analogoue methoprene treatment in *Aedes aegypti* delayed caspase expression during PCD of larval midgut (Wu et al., 2006).

Fenoxycarb is an insect growth regulator which possesses juvenile hormone activity. It is used to control a wide variety of insect pests like fire ants, fleas, mosquitoes, moths, scale insects and insect attacking vines, olives, cotton & fruit (Extension Toxicology Network, 1993; Miyamoto et al., 1993; Anonymous, 1991). Juvenile hormone analogues show similar effects as juvenile hormone but they are much more stable compounds (Matolcsy et al.,1988). Fenoxycarb was the first juvenile hormone analogue used to control agricultural pests (Miyamoto et al., 1993). It kills eggs and larvae of various insect species (Masner et al., 1987) Silkworm is extremely sensitive to this compound especially first three days of last larval instar which causes spinning inhibition and developmental arrest. The effect of fenoxycarb on progression of last larval instar in *Bombyx mori* has been studied in detail (Kamimura, 1995; Leonardi et al., 1996; Kamimura & Kiuchi, 1998; Dedos & Fugo, 1999; Monconduit & Mauchamp, 1999). Application of fenoxycarb in the last larval instar can induce a prolongation of the larval stage and inhibits larval-pupal differentiation dose and application time dependent manner (Kamimura & Kiuchi, 1998).

In this study, the morphological changes in the *Bombyx mori* midgut during larval pupal metamorphosis and differences between control and experiment groups were studied by using histological techniques. The expression patterns of cleaved caspase 3 and acid phosphatase activity were demonstrated for characterization of PCD in the larval midgut tissues and effects of JH analogue fenoxycarb were discussed.

Material and Methods

Animals

Hybrid races of the silkworm, *Bombyx mori*, were reared on fresh mulberry leaves at 25±1 °C. Immediately after the fourth larval ecdysis, the silkworms were topically treated with two different doses of fenoxycarb (1ng/10µl; 10ng/10µl) dissolved in acetone. Controls received only 10µl acetone. The final (fifth) larval instar lasts 10 days: 7 days for feeding, followed by 3 days of spinning to build the cocoon in control groups. Experiments were performed from day 6 larvae of fifth instar to 24 hr after pupation in groups which undergo larval-pupal metamorphosis; other groups were carried out until 14th day of fifth instar.

Light microscopy of *Bombyx mori* midgut

Control and experiment group's larvae were immobilized on ice. Dissected midguts were washed in Ringer solution then transferred to Bouin's fixative. Fixation was carried out for 6 hr at 4 °C. Dehydrated midguts by using ascending alcohol series were embedded paraffin. Paraffin sections (5µm) were cut and dewaxed using xylol for 15 min, rehydrated in graded series of alcohol (70 %, 96 % and 100 %). Hematoxylin&Eosin (Hem&E) staining was performed for morphologic evaluation of midguts. Histologic analysis was carried out in an Olympus BX51 microscope and photographed.

Determination of acid phosphatase activity

Lysosomal activation is important for autophagic cell death. Acid phosphatase has been used as the marker enzyme for lysosomes. Here we demonstrated the acid phosphatase activities in the midguts at the same time of each day of prepupal period and of the first two days of pupal stage. The measurements were repeated on three different series of animals. Acid phosphatase was measured using paranitrophenol phosphate (Sigma) as a substrate according to Bergmeyer et al. (1974). Midguts were homogenized in 0,5 % NaCl as a source of enzyme. They were incubated for 30 min at room temperature then 5ml of 0,1N NaOH was added to stop the reaction. The optical density of the liberated paranitrophenol read at 405nm using spectrophometre. Total protein amount in the samples were carried out by using Bradford Assay (Bradford, 1976). We expressed our data as phosphatase units (u) per mg protein. Student t test ($p \leq 0,05$) was used for evaluation of statistical significance according to Halaby et al. (1998)

Western blotting

Midguts were homogenized in 20 mM Tris-HCl (pH, 7,5) containing protease inhibitor coctail (Roche) and 0,5 % Nonidet P-40. The homogenate was centrifuged at 16000g for 10 min at 4°C. Total protein concentration was

determined with the bicinchoninic acid (BCA) protein assay kit (Pierce). Thirty micrograms of total proteins were separated by 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a gel running buffer (25 mM Tris, 192 mM Glycine, 0,1 % SDS, pH; 8,3) using Bio-Rad vertical electrophoresis. Proteins were electrotransferred onto a nitrocellulose membrane using Bio-Rad Transblot cell. Membranes were placed in blocking solution (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM EDTA, and 0,1 % Tween 20 (TBST) containing 5 % dried non-fat milk for 1 hour at room temperature. They were then incubated with cleaved caspase 3 antibody (Cell Signaling Technology, Asp175) in TBST containing 5 % dried non-fat milk (1:1000) during overnight at 4 °C followed by 1 hour incubation with horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, 7074). Detection was performed by chemiluminescence (Pierce, ECL western blotting substrate, 32106) according to manufacturer's instructions. Blots is scanned (Hp Psc 1315) and relative abundance of protein was calculated ImageJ software from the National Institutes of Health, USA (http://rsb.info.nih.gov/nih-image/).

Results and Discussion

During larval-pupal metamorphosis of *Bombyx mori*, morphological and biochemical changes occur depending on the fate of the cell or tissue. Remodeling tissues like midgut show both programmed cell death and cell differentiation at the same time (Parthasarathy & Palli, 2007). Fenoxycarb is an insect growth regulator that possesses JH activity and it is important for understanding to molecular mechanisms of juvenile hormone during insect life like other juvenile hormone analogues. Applications of fenoxycarb during larval life of *Bombyx mori* cause disruption of endocrine balance & abnormal development depending on the application time and dose (Kamimura & Kiuchi, 1998; Dedos et al., 2002).

There are a few reports about midgut remodeling processes during larval-pupal metamorphosis in *Bombyx mori* and there is no information available regarding the effects of fenoxycarb. Therefore we wanted to provide new perspectives to the remodeling processes of midgut and to analyze the effects of juvenile hormone analogue fenoxycarb during this period.

The effects of juvenile hormone analogue fenoxycarb on larval-pupal development and morphology of midgut

Fenoxycarb was topically applied once on day 0 larvae of the fifth instar as two different doses (1-10 ng). The application doses were chosen according to previous studies (Kamimura, 1995; Kamimura & Kiuchi, 1998). Controls were treated with acetone only. In control group larvae ceased feeding end of the day 7 and gut purge occurred on this day. Larvae started to spinning behavior on

day 8 and 97 % of larvae showed larval-pupal ecdysis on day 11. In 1ng fenoxycarb treated group, the length of the last larval stage was prolonged about 4 days; spinning was seen on day 12 and 92 % of larvae showed spinning behavior. 10 ng of fenoxycarb application on day 0 caused inhibition of larval-pupal differentiation including spinning (only 2 % larvae showed spinning), gut purge and remodeling of midgut. This permanent larva called as dauer larvae.

Morphological characterizations of the midgut in control and experiment groups were performed by using histological techniques. Dominant cells in the larval midgut called columnar cells which act as absorption of digested products and secretion of digestive enzymes. They have microvilli on the apical region. The other midgut cell type, goblet cells, have large cavity and important for ionic homeostasis of midgut. Small regenerative cells are located randomly near the larval mature midgut cells. They are important for epithelium renewal of larval midgut and also pupal midgut construction (Lehane & Billingsley, 1996). Larval midgut degeneration started on day 8 of fifth instar and lasted until early pupal stage in control. Intact midgut morphology was maintained until day 8 of the 5^{th} instar. Columnar, goblet and regenerative cells were clearly distinguishable and tightly packed (Figs 1a). Columnar cells have round nucleus located in the apical region of the cell and brush border (Fig 1a). Goblet cells were found abundantly. They exhibits typical cavity which continues the midgut lumen. A few small regenerative cells were observed basal region of the mature cells. Degenerations in the apical region of columnar cells and nuclear condensation were observed after cessation of feeding (day 8) (Fig 1b). Apoptotic morphology became more obvious on day 9 (Figs 1c) and midgut cells seemed small and condensed probably due to shrinkage. In addition, columnar cell's nucleus stained intensively which demonstrated the condensation of nucleus. Intercellular area between the cells indicated cell detachment from each other and basal lamina. Vacuole structures were also seen on this day (Fig 1c). Proliferated regenerative cells located the basal region of epithelium (Fig 1d). Some strange and round shape cells like granulocytes were determined under the midgut basement membrane (Figs 1d). Degenerated larval cells discharged into the midgut lumen on day 10 which is called as yellow body and vacuole structures within the cells were observed (Figs 1e, 1f). Proliferation of larval regenerative cells was more obvious than before and formed as multilayered epithelium (Fig 1g). After larval-pupal ecdysis, regenerative larval cells differentiated into pupal epithelium which has tall and columnar shape (Fig 1h). Brush border also appeared in pupal epithelium. Formation of pupal midgut completed in 24 hr pupae (Fig 1i).

Two major events were observed during midgut remodeling. First, degeneration of larval midgut through PCD begun on day 8 and lasted until

early pupal stage; second, proliferation and differentiation of larval regenerative cells into pupal epithelium started just before pupation and proceeded early pupal period. One of the programmed cell death type, apoptosis, is characterized by nuclear condensation, cell shrinkage, membrane blebbing, DNA fragmentation, caspase activation and apoptotic body formation which is phagocytosed by the surrounding cells. (Saraste & Pulkki, 2000; Lockshin & Zakeri, 2004).

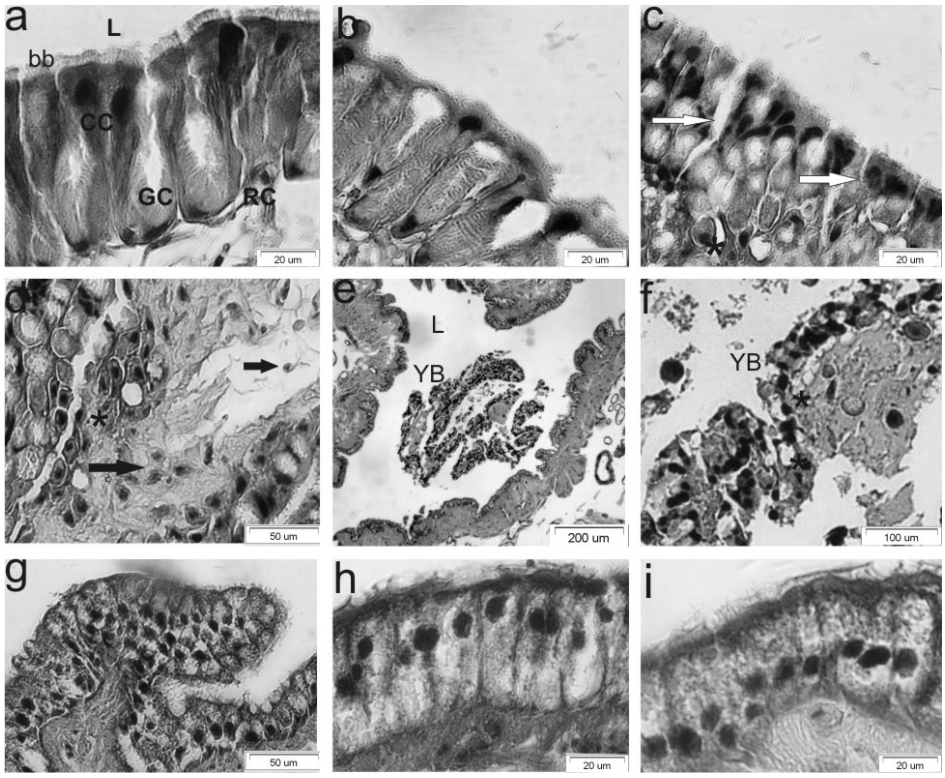

Figure 1. Larval midgut in control group during larval-pupal metamorphosis of *Bombyx mori*. L., 1758. Goblet cells (GC), columnar cells (CC) and their brush border were clearly seen on day 6. Regenerative cells (RC) were located in basal layer of the epithelium. Larvae were still feeding on this day (a). Degeneration in columnar and goblet cells were firstly detected on day 8(b). Larval epithelium looked unordered on day 9. Midgut cells became smaller, goblet cells were indistinguishable and columnar cell's nucleus stained intensively. Apoptotic morphology clearly observed. Asterisks indicate nuclear condensation. (c). Small groups of regenerative cells were seen. Arrows indicates that some round shape cells probably granulocytes were determined under the midgut basement membrane and stars show vacuoles (d) Just before pupation – day 10, larval epithelium completely detached from basal lamina (e) and called as yellow body (YB) and moved into the midgut lumen. Vacuole structures (asterisk) were observed within the cell (f). At the same time, regenerative cells (RC) proliferated for forming pupal epithelium (g). The new pupal epithelium consists of tall, columnar shape cells (h). Well developed basement membrane and brush border were observed on 24hr pupae (i) Lumen (L). (Original).

Some of the morphological changes of apoptosis were detected in larval midgut of *B. mori* (Fig 1). Loss of microvilli structure in columnar cells, nuclear condensation, cell shrinkage and detachment from basal membrane were obvious apoptotic changes especially after cessation of feeding. Similar results were reported in *Galleria mellonella* (Uwo et al., 2002), *Manduca sexta* (Hakim et al., 2001), *Drosophila melanogaster* (Micchelli & Perrimon, 2006) and *Heliothis virescens* (Parthasarathy & Palli, 2007; Tettamanti et al., 2007).

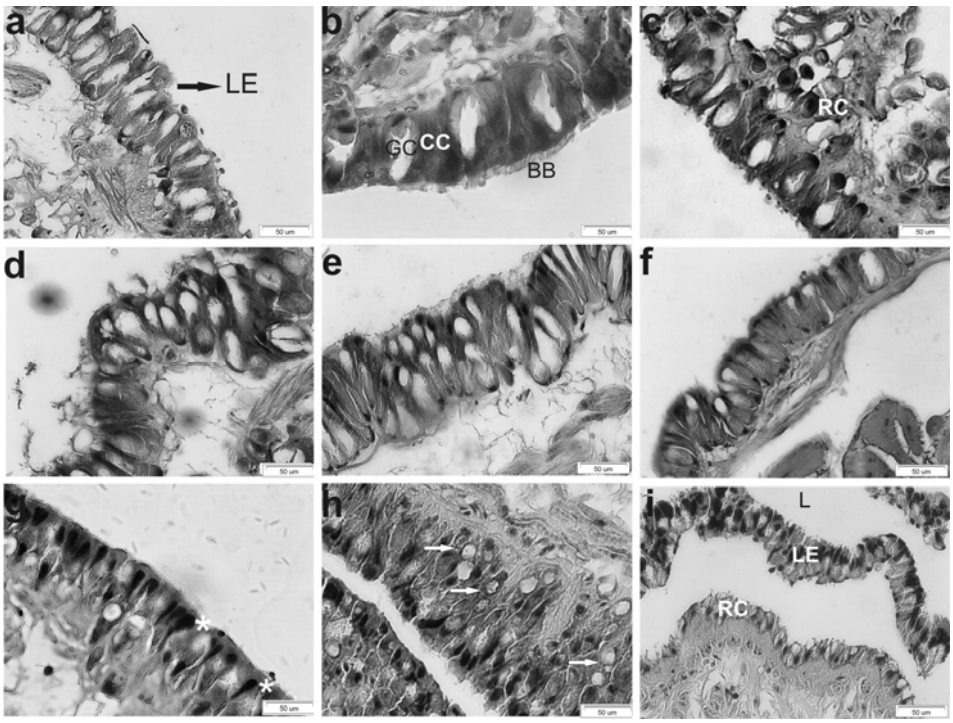

Figure 2. 1ng of fenoxycarb application on day 0 larvae was delayed midgut remodeling. Intact larval epithelium (LE) was maintained until day 12. Columnar cells (CC), goblet cells (GC) and regenerative cells (RC) were observed (a-f). Apoptotic morphology and vacuoles were seen on day 12 and day 13 (g-h) asterisks show nuclear condensation on day 12. Arrows indicates vacuole structure on day 13. Larval epithelium (LE) separated from basement membrane and proliferated regenerative cells (RC) was seen on day 14 (I). Lumen (L) (Original).

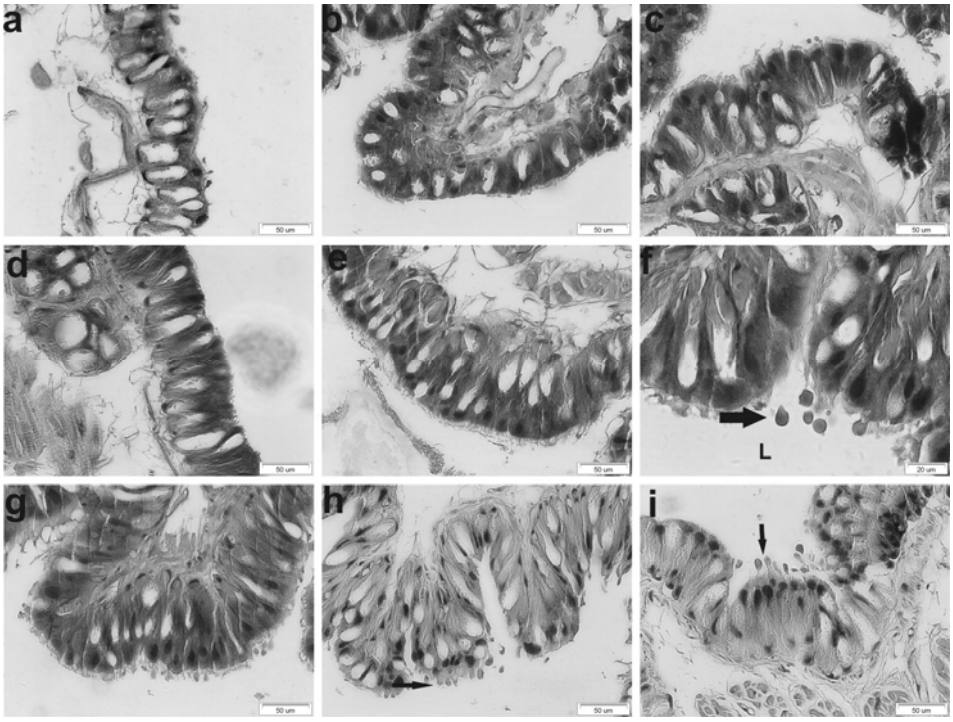

Figure 3. 10 ng of fenoxycarb application on day 0 larvae was inhibited midgut remodeling. Healthy larval epithelium was observed during experiment period. Secretion vesicles (arrows) which contain digestive enzymes were found until day 14 (a-ı). Lumen (L) (Original).

We observed cell groups under the basement membrane on day 9 of 5th instar. They were small and round shape cells (Fig 1d). Their morphologic features resembled one of the hemocyte type, granulocytes. It has been reported that granulocytes and plasmatocytes are dominant hemocyte type during larval period and they are the only phagocytic cells in *Bombyx mori*. Granulocytes also involved in the secretion of basal membrane components of Lepidoptera (Nardi et al., 2001). Shinohara et al. (2008) reported that granulocytes were seen in midgut lumen of *B. mori* after occurrence of apoptotic bodies. According to previous studies (Adachi et al., 2005; Shinohara et al., 2008) and our results, we propose that round shape cells might be granulocytes and they may involve both phagocytosis of apoptotic bodies and rearrangement of pupal basal membrane.

In 1 ng fenoxycarb treated group, healthy larval midgut maintained until day 12. Columnar and goblet cells were clearly distinguishable during these days (Fig 2a-f). Nuclear condensation in columnar cells, degeneration in goblet cells and many in number regenerative cells were seen on day 12 and 13 (Figs 2g, h). Apoptotic body formation began on day 14. Dying larval midgut cells lost

its intercellular junctions and detached from its cellular environment and basement membrane, then they collected in midgut lumen (Fig 2i). In 10ng fenoxycarb treated group, intact larval midgut was observed until end of the experiments on day 14 of fifth instar (Fig 3). We did not find any signs of programmed cell death in this group midgut. Secretion vesicles which contains digestive enzyme were found even on day 14 (Fig 3i, arrow) which indicated the feeding activity during experiments. Parthasaraty & Palli (2007) reported that another juvenile hormone analogue methoprene application to *Heliothis virescens* caused delaying or inhibition of midgut remodeling in dose dependent manner.

Acid phosphatase activity

Lysosomal enzymes are marked for autophagic cell death process during degeneration of intersegmental muscles (Lockshin & Williams, 1965a, b), salivary glands (Aidells et al., 1971) and silk glands (Goncu & Parlak, 2008). Zakeri et al. (1996) have estimated lysosomal activity by measuring acid phosphatase (AP). We determined the AP activity during metamorphosis as an indicator of autophagic cell death. AP activity was found low on day 6 but after cessation of feeding, total activity increased slightly in control. Rising activity became more obvious just before pupation (Fig 4A). In contrast to control group, 1 ng of fenoxycarb treated group showed rising AP activity after at the end of the feeding period and only one peak on day 14 of fifth instar. Low AP activity maintained during experiment period in 10 ng fenoxycarb treated group's midgut (Fig 4B). Constant acid phosphatase activity was determined in all groups but we did not determine significant differences between control and experiment groups ($p \leq 0,05$). According to Cavalcante & Cruz-Landim (2004), these results may arise from enzyme content in the digestive juice during larval period but when considered the experiment days, feeding activity was completely finished and gut contents removed after day 6 in control group; and after day 11 in 1 ng fenoxycarb treated group. Therefore high AP activity after this day of control group cannot be explained with this early report. Increasing in AP activity after cessation of feeding likely result of autophagic activity in control group midgut. Similar results obtained from 1 ng fenoxycarb treated group. Both of these results suggest that autophagic activity begun at the end of feeding. In 10 ng fenoxycarb treated group, feeding activity lasted until end of the experiments. Therefore relatively high AP activity in 10 ng fenoxycarb treated groups may be the reflection of AP content of digestive juice.

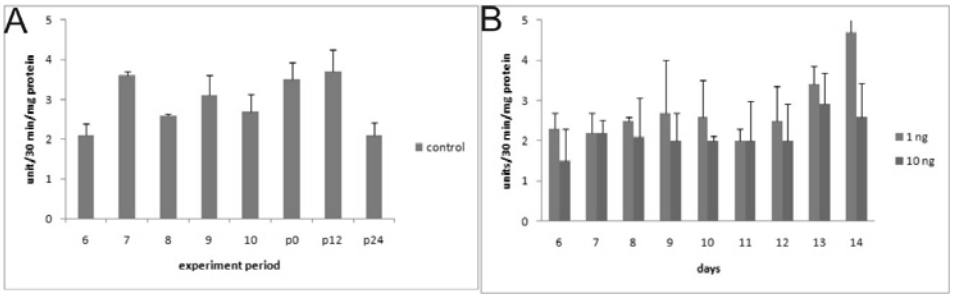

Figure 4. The pattern of acid phosphatase activity in the midgut homogenates during larval-pupal development. Control (A), Numbers show days of 5th instar, p0, at pupation; p12, 12 hrs after pupation; p24, 24 hrs after pupation. Fenoxycarb treated groups (B). The vertical bars repress ent the mean ±SE (N=10).

Cleaved caspase 3 determination by using western blotting

Caspases are important regulators of apoptosis and they are responsible for proteolytic cleavage of important cell proteins. They are normally present within the cells as an inactive form. Following apoptotic signal, caspase 3 cleaves into p12 and p17 fragments and activation occurs. Previous studies in the *Lepidopteran Heliothis virescens* (Parthasaraty & Palli, 2007) & *Spodoptera littoralis* (Vilaplana et al., 2007) reported to reveal mammalian caspase 3 homologues in insects. In addition, Ahmad et al. (1997) demonstrated that *Lepidopteran* caspase 1 shows high homology to mammalian caspase 3. We reported the caspase 3 activation during midgut remodeling in *Bombyx* for the first time by using western blotting. Fig 5A demonstrates the results of western blot analysis of cleaved caspase 3 (17 kDa). We detected cleaved caspase 3 on day 8, day 9 and day 10 of control. Relative abundance of the protein was determined as 8.94 %, 8.84 % and 82.22 %, respectively. Maximum density was seen on day 10 just before pupation (Fig 5B) and similar result was reported in *Galleria mellonella* (Uwo et al., 2002), and *Heliothis virescens* (Parthasarathy & Palli, 2007). In the 1 ng fenoxycarb treated group, cleaved caspase 3 was detected after day 12. Relative abundance was determined 21.8 % on day 12; 43.5 % on day 13 and 34.7 % on day 14 (Fig 5B). We didn't find caspase activation in 10 ng of fenoxycarb treated group (Fig 5A).

Our morphologic observations and estimated caspase activity indicated that larval midgut degeneration starts as apoptotic cell death, but after pupal ecdysis it is proceeded predominantly by autophagic cell death. Degenerated larval midgut cells which pushed later to the lumen have vacuoles (Figs 1c, 1h) which are probably autophagic. Besides these results, pupal midgut epithelium cells have absorptive cell features like having brush border although no feeding activity. We suggest that nutrients from larval midgut which is provided by autophagic cell death are resorbed by pupal midgut epithelium.

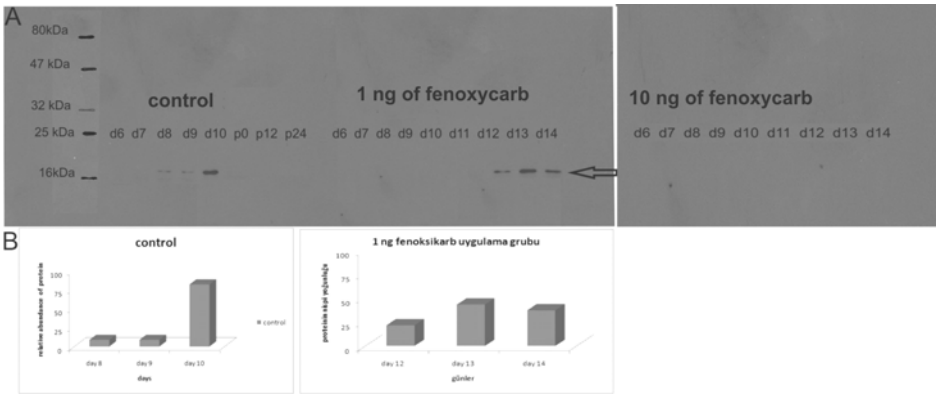

Figure 5. Detection of cleaved caspase 3 protein by Western blotting during larval-pupal development of *Bombyx mori* midgut (A). Numbers show days of 5th instar, p0, at pupation; p12, 12 hrs after pupation; p24, 24 hrs after pupation, the molecular weight of cleaved caspase was found 17 kDa by Western blot, and arrows indicate cleaved caspase 3. The average intensities of each band and relative abundance of cleaved caspase 3 were estimated (B) (n=10).

It is well known that developmental events in holometabolous insects are strictly controlled by endocrine systems. Steroid hormone ecdysone triggers metamorphic events and juvenile hormone acts as a modulator of ecdysone secretion but there is also some evidence that JH can regulate cellular response to ecdysone (Hiruma et al., 1999). According to ecdysone release timing during last larval instar of *B. mori*, two ecdysone peaks occur: on day 6 and just before larval-pupal ecdysis. We suggested that the beginning of larval midgut degeneration occurs in response to rising ecdysone level after day 6 of fifth instar and differentiation of regenerative larval cells occurs in response to second ecdysone peak. Previous studies reported that small ecdysone rising in the absence of JH on day 3 of fifth instar is needed for pupal commitment of tissues like midgut (Sakurai et al., 1998; Riddiford et al., 1999; Takaki & Sakurai, 2003). Therefore we believed that fenoxycarb application on the beginning of fifth instar prevented or delayed rising of ecdysone. Kamimura & Kiuchi (1998) reported that 1ng of fenoxycarb application delays ecdysone rising in the hemolymph. Hence, the results of experiments may arise from inhibition effect of fenoxycarb on prothoracic gland.

Özet

Bombyx mori' L., 1758 (Lepidoptera: Bombycidae) de larval-pupal metamorfoz süresince orta barsaktaki yeniden şekillenme sürecine juvenil hormon analoğu fenoxycarb'ın etkisi

Larval-pupal metamorfoz boyunca, tam başkalaşım geçiren böceklerin orta barsakları yeniden şekillenme sürecine girer ve bu süreç 2 metamorfik olayı içerir. Larval orta barsağın dejenerasyonu programlanmış hücre ölümü ile meydana gelirken pupal orta barsak rejeneratif (kök) hücrelerden oluşur. Böceklerdeki tüm metamorfik olaylar

ekdizon ve juvenil hormon tarafından kontrol edilir. Ekdizonun moleküler mekanizması gayet iyi bilinmekle birlikte juvenil hormonun moleküler mekanizması henüz tam olarak anlaşılamamıştır. Fenoksikarb, O-ethyl N-(2-(4-pheoxyphenoxy)-ethyl) karbamat'ın pek çok böcek türünde etkili juvenil hormon analoglarından birisi olduğu ortaya konulmuştur ve juvenil hormonun mekanizmasına ilişkin çalışmalarda sıklıkla kullanılmaktadır. Bu çalışmada, *Bombyx mori Linnaeus, 1758*'nin orta barsağındaki metamorfik olayların analiz edilmesi ve bu süreçte fenoksikarb'ın etkisinin araştırılması amaçlanmıştır. Bu amaçla, beşinci larval döneminin 0. gününde iki farklı dozda fenoksikarb (1ng/10µl and 10ng/10µl) topikal olarak uygulanmıştır. Yeniden şekillenme sürecinin ilerleyişini morfolojik gözlemler ve programlanmış hücre ölümüne spesifik proteinlerin - apoptosis için kaspaz 3 ve otofajik hücre ölümü için asit fosfataz- kontrol ve deney gruplarında analizi ile takip edilmiştir. Sonuçlar larval orta barsakta programlanmış hücre ölümünün apoptozisin morfolojik karakterlerine sahip olduğunu, bu period boyunca kaspaz aktivasyonunun meydana geldiğini ve juvenil hormon analoğu fenoksikarb'ın doza bağlı olarak yeniden şekillenme sürecini geciktirdiğini veya engellediğini göstermiştir.

Acknowledgements

The research was supported by Ege University (2009 Science 021).

References

Adachi, T., M. Tomita & K. Yoshizato, 2005. Synthesis of prolyl 4-hydroxylase a subunit and type IV collagen in hemocytic granular cells of silkworm, *Bombyx mori:* involvement of type IV collagen in self-defense reaction and metamorphosis. **Matrix Biology, 24**: 136 - 154.

Ahmad, M., S. M. Srinivasula, L. Wang, G. Litwack, T. Fernandes- Alnemri & E. S. Alnemri, 1997. *Spodoptera frugiperda* caspase-1, a novel insect death protease that cleaves the nuclear immunophilin FKBP46, is the target of the baculovirus antiapoptotic protein p35**. The Journal of Biological Chemistry, 272**: 1421–1424.

Aidells, B., R. A. Lockshin & A. M. Cullin, 1971. Breakdown of the silk glands of *Galleria mellonella* - acid phosphatase in involuting glands. **Journal of Insect Physiology, 17**: 857-869.

Anonymous, 1991. The Pesticide Manual: A World Compendium*.* Ninth Edition. Unwin Brothers Limited. The British Crop Protection Council, Great Britain, 1141 pp.

Bergmeyer, H. U., 1974. Reagents for enzymatic analysis. pp 495-496. In: Methods of Enzymatic Analysis Vol I. (Eds: Bergmeyer HU, Gawehn K) Verlag Chemie, Weinheim.

Bowen, E. D., T. A. Ryder & C. Dark, 1976 The effects of starvation on the planarian worm Polycelis tenuis Iijima. **Cell Tissue Research, 169**: 193–209.

Bradford, M. M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding **Analytical Biochemistry, 72**: 248-254.

Cavalcante, V. M. & C. Cruz-Landim, 2004. Electrophoretic protein pattern and acid phosphatase activity in the midgut extracts of *Apis mellifera* L. (Hymenoptera: Apidae) during metamorphosis. **Neotropical Entomology, 33** (2): 169-171.

Csikós, G. & M. M. Sass, 1997. Changes of acid phosphatase content and activity in the fat body and the hemolymph of the flesh fly Neobellieria (Sarcophaga) bullata during metamorphosis. **Archives of Insect Biochemistry and Physiology, 34**: 369–391.

Dedos, S. G. & H. Fugo, 1999. Induction of dauer larvae by application of fenoxycarb early in the 5th Instar of the silkworm, *Bombyx mori.* **Journal of Insect Physiology, 45** (8): 769-775.

Dedos, S. G., F. Szurdoki, A. Székács, T. Shiotsuki, B. D. Hammock, J. Shimada & H. Fugo, 2002. Fenoxycarb levels and their effects on general and juvenile hormone esterase activity in the hemolymph of the silkworm, *Bombyx mori.* **Pesticide Biochemistry and Physiology, 73** (3): 174-187.

Extension Toxicology Network, 1993. (Web page: http://ace.orst.edu/cgi-bin/mfs/01/pips/methopr. (Data accessed: 10.03.2010)

Goncu, E. & O. Parlak, 2008. Some autophagic and apoptotic features of programmed cell death in the anterior silk glands of the silkworm, *Bombyx mori*. **Autophagy, 4** (8)**:** 1069-1072.

Halaby, R., M. L. Martinez,R. Lockshin & Z. Zakeri, 1998. 20-hydroxyecdysone induces apoptosis in the labial gland of *Manduca sexta*. **Journal of Research on the Lepidoptera, 37**: 3-10.

Hakim, R. S., K. M. Baldwin & M. Loeb, 2001. The role of stem cells in midgut growth and regeneration. **In Vitro Cellular & Developmental Biology – Animal, 37**: 338-342.

Hiruma, K., T. Shinoda , F. Malone & L. M. Riddiford,. 1999. Juvenile hormone modulates 20-hydroxyecdysone-inducible ecdysone receptor and ultraspiracle gene expression in the tobacco hornworm, *Manduca sexta.* **Developmental Genes and Evolution, 209** (1):18-30.

Jiang, C., E. H. Baehrecke, & C. S. Thummel, 1997. Steroid regulated programmed cell death during *Drosophila* metamorphosis **Development, 124** (22): 4673-83.

Kamimura, M., 1995. Effects of juvenile hormone analogue, fenoxycarb, on larval growth of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). **Applied Entomology and Zoology, 30**: 487-489.

Kamimura, M. & M. Kiuchi, 1998. Effects of a juvenile hormone analog, fenoxycarb, on 5th stadium of the larvae of the silkworm, *Bombyx mori* (Lepidoptera:Bombycidae). **Applied Entomology and Zoology, 33** (2): 333-338.

Lee, C. Y., B. A. K. Cooksey & E. H. Baehrecke, 2002 Steroid regulation of midgut cell death during *Drosophila* development. **Developmental Biology, 250**: 101-111.

Lehane, M. & P. Billingsley, 1996. Structure and Ultrastructure of the Insect Midgut. In: Biology of the Insect Midgut (Eds: M. Lehane & P. Billingsley) Springer; First Edition, 504 pp.

Leonardi, M. G., S. Cappellozza, P. Ianne, L. Cappellozza, P. Parentis & B. Giordina, 1996. Effects of the topical application an insect growth regulator (fenoxycarb) on some physiological parameters in the fifth instar larvae of the silkworm *Bombyx mori*. **Comparative Biochemistry and Physiology, 113B** (2): 361-365.

Lockshin, R. A. & J. Beaulaton, 1974. Programmed cell death. **Life Sciences, 15**: 1549-1566.

Lockshin, R. A. & C. M. Williams, 1965a. Programmed cell death. I. Cytology of degeneration in the intersegmental muscles of the pernye silkmoth. **Journal of Insect Physiology, 11**: 123-133.

Lockshin, R. A. & C. M. Williams, 1965b. Programmed cell death. V. Cytolytic enzymes in relation to the breakdown of the intersegmental muscles of silkmoths. **Journal of Insect Physiology, 11**: 833-844.

Lockshin, R. A. & Z. Zakari, 2004. Apoptosis, autophagy and more. **The International Journal of Biochemistry** & **Cell Biology, 36**: 2405–2419.

Masner, P., M. Angst & S. Dorn, 1987. Fenoxycarb, an insect growth regulator with juvenile hormone activity: A candidate for *Heliothis virescens* (F.) control on cotton. **Pesticide Science, 18**: 89-94.

Matolcsy, G., M. Nadasy & V. Andriska, 1988. Pesticide Chemistry: Studies in Environmental Science, Elsevier. New York, 800 pp.

Micchelli, C.A. & N. Perrimon, 2006. Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. **Nature, 439**: 475–479.

Monconduit H. & B. Mauchamp, 1999. Fenoxycarb, a potent contaminant of the silkworm *Bombyx mori* L., does not influence its juvenile hormone titer. **Archives of Insect Biochemistry and Physiology, 40**: 141-149.

Miyamoto, J., M. Hirano,Y. Takimoto & M. Hatakoshi, 1993. Insect growth regulators for pest control with emphasis on juvenile hormone analogs: present status and future prospects. **Pest Control With Enhanced Environmental Safety, 524**: 144-168.

Nardi, J. B., C. Gao & M. R. Kanost, 2001. The extracellular matrix protein lacunin is expressed by a subset of hemocytes involved in basal lamina morphogenesis. **Journal of Insect Physiology, 47**: 997–1006.

Parthasarathy, P. & S. R. Palli, 2007. Developmental and hormonal regulation of midgut remodeling in a Lepidopteran insect *Heliothis virescens.* **Mechanism of Development, 124**: 23–34.

Riddiford, L. M., 1975. Juvenile hormone-induced delay of metamorphosis of the viscera of the cecropia silkworm. **The Biological Bulletin, 142**: 310-325.

Riddiford, L. M., K. Hiruma, Q. Lan & B. Zhou, 1999. Regulation and role of nuclear receptors during larval molting and metamorphosis of Lepidoptera. **American Zoologist, 39**: 736-746.

Sahota, T. S., 1975. Effect of juvenile hormone on acid phosphatases in the degenerating flight muscles of the douglas- fire beetle, *Dendroctonus pseudotsugae*. **Journal of Insect Physiology, 21**: 471–478.

Sakurai, S., M. Kaya & S. Satake, 1998. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, *Bombyx mori*: Role of low ecdysteroid titer in larval-pupal metamorphosis and reappraisal of the head critical period. **Journal of Insect Physiology, 44**: 867-881.

Saraste, A. & K. Pulkki, 2000. Morphologic and biochemical hallmarks of apoptosis. **Cardiovascular Research, 45** (3): 528-537.

Sass, M., L. Kőműves, G. Csikós & J. Kovács, 1989. Changes in the activities of lysosomal enzymes in the fat body and midgut of two Lepidopteran insects (*Mamestra brassicae* and *Pieris brassicae*) during metamorphosis. **Comparative Biochemistry and Physiology, 92** (3): 285-289.

Shinohara, Y., N. Ishii, S. Takahashiand & T. Okazaki, 2008. Appearance of apoptotic cells and granular cells in the silkworm midgut lumen during larval-pupal ecdysis. **Zoological Science, 25**: 139–145.

Takaki, K. & S. Sakurai, 2003. Regulation of prothoracic gland ecdysteroidogenic Activity leading to pupal metamorphosis. **Insect Biochemistry and Molecular Biology, 33**(12): 1189-99.

Tettamanti, G., A. Grimaldi, M. Casartelli, E. Ambrosetti, B. Ponti, T. Congiu, R. Ferrarese, M. L. Rivas-Pena, F. Pennacchio & M. Eguileor, 2007. Programmed cell death and stem cell differentiation are responsible for midgut replacement in *Heliothis virescens* during prepupal instar. **Cell and Tissue Research, 330** (2): 345-59.

Tsujimoto, Y. & S. Shimizu, 2005. Another way to die: autophagic programmed cell death. **Cell Death and Differentation, 12**: 1528-34.

Uwo, M.F., K. Ui-Tei, P. Park & M. Takeda, 2002. Replacement of midgut epithelium in the greater wax moth, *Galleria mellonella*, during larval–pupal molt. **Cell and Tissue Research, 308**: 319–331.

Vilaplana, L., N. Pascual, N. Perera & X. Belle´ s, 2007. Molecular characterization of an inhibitor of apoptosis in the egyptian armyworm, *Spodoptera littoralis*, and midgut cell death during metamorphosis. **Insect Biochemistry and Molecular Biology, 37**: 1241–1248

Wigglesworth, V. B., 1972. Digestion and Nutrition, 476–552. In: The Principles of Insect Physiology. Chapman & Hall, London, 827 pp.

Wu, Y., R. Parthasarathy, H. Bai & S. R. Palli, 2006. Mechanisms of midgut remodeling: juvenile hormone analog methoprene blocks midgut metamorphosis by modulating ecdysone action. **Mechanism of Development, 123**: 530-547.

Zakeri, Z., D. Quaglino, T. Latham, K. Woo & R. A. Lockshin, 1996. Programmed cell death in the tobacco hornworm, *Manduca sexta*: alterations in protein Synthesis. **Microscope Research and Technique, 34**: 192-201.