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Research Article

Programmed Cell Death in the Digestive Canal of *Bombyx mori* (Lepidoptera: Bombycidae) during Prepupal Period[1]

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Abstract

In silkworms, like other holometabolus insects, larval digestive canal degenerates via programmed cell death (PCD) during larval-pupal metamorphosis. PCD plays a key role in developmental biology. It generally occurs with two major mechanisms; apoptosis and autophagic cell death. In this study, we aimed to determine the metamorphic events in the digestive canal of *Bombyx mori* during the prepupal period. The general morphological changes were studied by using histological techniques; the degenerative cells were determined using H&E (Hematoxylen&Eosin) and PAS (Periodic Acid Schiff) staining. We have used TUNEL (terminal transferase-mediated dUTP nick end-labeling) assay for determination of apoptotic cell nuclei. Also, the acid phosphatase activity was calculated to determine the autophagic cell death. In conclusion; our observations indicated a co-occurrence of apoptosis and autophagy observed during the cell death of the digestive canal.

Keywords: *Bombyx mori*, apoptosis, digestive system, autophagy, TUNEL.
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Prepupal Periyod Sürecinde *Bombyx mori* (Lepidoptera: Bombycidae) Sindirim Kanalında Meydana Gelen Programlanmış Hücre Ölümü

Özet

Tam metamorfoz geçiren ipek böceklerinde, larval-pupal gelişim sırasında sindirim kanalı programlanmış hücre ölümü yoluyla dejenere olmaktadır. Gelişim biyolojisinde programlanmış hücre ölümü anahtar rol oynamaktadır. Programlanmış hücre ölümü genel olarak, Apoptotik ve otofajik hücre ölümü olarak isimlendirilen iki ana mekanizma ile gerçekleşmektedir. Bu çalışmadaki amacımız, larval-pupal metamorfoz sırasında, Bombyx mori sindirim sisteminde meydana gelen metamorfik olayların gösterilmesidir. Histolojik teknikler kullanılarak morfolojik değişimler gösterilmiştir; H&E ve PAS boyama yöntemleri kullanılarak dejeneratif hücreler belirlenmiştir. Apoptotik hücre nukleuslarını belirlemek için TUNEL yöntemi uygulanmıştır. Otofajik hücre ölümünü göstermek amacıyla ise Asit Fosfataz aktivite hesaplamaları gerçekleştirilmiştir. Sonuç olarak; bulgularımız, sindirim kanalında meydana gelen ölümde apoptoz ve otofajinin birlikte rol oynadığını göstermiştir.

Anahtar kelimeler: *Bombyx mori*, apoptoz, sindirim sistemi, otofaji, TUNEL.

[1] This study is part of PhD thesis.

Introduction

Silkworms are holometabolous insects and they pass through complete metamorphosis from egg to adult stage through two intermediate stages of larva and pupa (Nijhout 1891; Parlak 2001). The endocrine system by hormones is quite important in metamorphosis.

The digestive canal of the silkworm is composed of three parts; foregut, midgut and hindgut (Judy and Gilbert 1969). All parts are lined by a single layer of epithelium. It consists of connective tissue which surrounds and feeds the epithelium, and muscle tissue which provides contraction of the digestive tract. Midgut originates in the endoderm, while foregut and hindgut originate in the ectoderm. They have different structures and functions. Midgut provides for ingestion of food, transportion and ion balance (Levy et al. 2004 b). The foregut and hindgut have four histological layers; an envelope of connective tissues, two layers of muscle fibres, a single layer of epithelial cells and a layer of chitinous intima. The cuticle of the hindgut is thinner than the foregut. This part is responsible for some water and nutrient absorption. The hindgut has polygonal epithelial cells. There is squamous epithelium with small nuclei in the foregut. The midgut is the largest section of alimentary canal. It has four histological layers, too, but in midgut there is peritrophic membrane instead of chitinous intima. The peritrophic membrane envelops the food contents and protects the epithelium from contact with hard food particles (Mathur 1973). Three types of cell have been recognized in midgut epithelium; columnar epithelial, goblet and regenerative cells. Columnar epithelial cells have roles in the digestion and transportion of foods. Goblet cells have a role in midgut's ion balance (Billingsly and Lehane 1996). And the regenerative cells, so called 'nidus', proliferate and differentiate into new columnar and goblet cells. Regenerative cells from larvae of the Lepidoptera; *Choristoneura fumifera, Lymantria dispar, Bomnyx mori, Manduca sexta* and *Heliothis virescens* have been successfully grown in vitro as primary and secondary cultures. The cells in these cultures are mixtures of typical larval midgut epithelial cell types; columnar, goblet and regenerative cells. Regenerative cells undergo mitosis and subsequent metamorphosis to the other types in vivo (Loeb et al. 2003).

Programmed cell death (PCD) plays a key role in developmental biology. Some larval organs in *Bombyx mori* as the silk glands and larval midgut undergo programmed cell death at larval-pupal metamorphosis (Beaulaton and Lockshin 1982; Goncu and Parlak 2008; Goncu and Parlak 2011; Franzetti et al. 2012). Cells which are no longer required for organism and have fullfilled their physiological roles are eliminated by PCD. At larval-pupal metamorphosis, the digestive system of *Bombyx mori* undergo PCD in response to the steroid ecdysone. PCD generally occurs with two major mechanisms; apoptosis (type I cell death) and autophagic cell death (type II cell death) (Gavrieli et al. 1992). Apoptotic cells indicate some biochemical (DNA fragmentation, phosphatidylserine translocated to the outer of apoptotic cell membranes, the passage of cytchrome c from the inner membrane of mitochondria to the cytoplasm, proteins such as caspase 3 activation) and morphological (cell shrinkage, chromatine condensation, apoptotic bodies) changes (Tettamanti et al. 2007).

Autophagy has been related with both cell survival and cell death. It has been considered as a survival mechanism since it provides nutrient deprived cells with a means of survival. But in recent years, studies showed that autophagy seems to act together with apoptosis and has a secondary role in cell death. Under certain conditions, autophagy may kill the cell through a caspase independent, non-apoptotic type of cell death (Franzetti et al. 2011). This type of cell death is characterized by massive degradation of cellular contents, including essential organelles such as mitochondria. It occurs by means of intracellular double membrane/ vesicle reorganization and lysosomal activity (Shen and Codogno 2011; Sridharan et al. 2011). During this death double membrane bound structures are called autophagosomes. Acid phosphatase, that is one of the lyzozomal enzyme, is a marker enzyme to determine autophagic cell death.

In our study, we aimed to determine the features of digestive system degeneration in

Bombyx mori during the prepupal period. The morphological changes were indicated by using histological techniques. We determined apoptotic cells using H&E (Hematoxylen&Eosin and PAS (Periodic Acid Schiff) staining. And we also analyzed TUNEL (terminal transferase-mediated dUTP nick end-labeling) assay for apoptosis and acid phosphatase (AP) assay for autophagic cell death. We determined nuclei of apoptotic cells using TUNEL assay and we estimated AP activity in the gut tissues.

Materials and methods

Experimental animals

The eggs of *Bombyx mori* were brought from Bursa Silkworm Institute. The eggs were kept in labaratory conditions at 25±1ºC and 70-85% RH. After 10-12 days, larvae emerged from eggs and they were began to feed under 12h light; 12h dark photoperiod. The larvae were fed with fresh mulberry leaves every morning, noon and evening. The fifth larval instar lasts 10 days; 7 days for feeding, 3 days of spinning to build cocoon referred to as the prepupal period. Experiments were performed during the prepupal period.

Morphological analysis

Dissected guts were placed in Bouin fixative at 4ºC for 12h. Dehydrated guts using alcohol series were embedded in paraffin. 5 µm paraffin sections were cut and dewaxed using xylol for 20 min., rehydrated in graded series of alcohol. Haematoxylin-Eosin(H&E) and Periodic-Acid Schiff (PAS) staining was performed. The sections were examined using Leica DM3000.

Detection of DNA fragmentation

Apoptotic cells were identified in situ using TUNEL (TdT-mediated dUTP-biotin Nick End Labelling) technique. Tissue samples were fixed in % 10 neutral formalin for 8 h at 4 oC. After dehydration in a graded series of ethanol, the tissue was embedded in paraffin. The Biovision TUNEL kit protocol was applied. Paraffin sections were deparaffinized, rehydrated through graded ethanol washes, and rinsed with PBS. Proteinase K digestion was applied as a pretreatment for 20 min at room temperature. Incubation with the TdT reaction mix was performed for 1,5 h at 37 oC. Diaminobenzidine (DAB) solution was used for signal development for 15 min. Sections were counterstained with methyl green for 5 min.

Determination of Acid Phosphatase Activity

Lysosomal activation is important for autophagic cell death. The acid phosphatase (AP) is a suitable marker for lysosomes. Its activity was estimated in the larval gut tissues during each day of the prepupal period. It was measured using paranitrophenol phosphate (Sigma) as a substrate according to Bergmeyer et al. (1974). Guts were homogenized in 0,9 % NaCl. They were incubated for 30 min at room temperature. Then 1 ml of 0,1 N NaOH was added to stop the reaction. They were read at 405 nm using spectrophotometre (Agilent Technologies Cary 60 Uv-Vis) and AP activity was estimated in 1g tissue. Also, we used Bradford assay to determine the amount of total protein in 1 g tissue. After that we estimated the specific enzyme activity in 1 mg protein.

Results

Histologic results

The animals stopped feeding at day 7 of fifth instar. The spinning period continued 3 days and all of them became pupae at day 10. Experiments were performed during prepupal period. Morphological characterizations of the digestive system were performed using histological techniques. The alimentary canal of the silkworm consists of three parts; foregut, midgut and hindgut (Fig. 1).

In our histological study, the columnar and goblet cells in midgut epithelium have been researched. The columnar cells are numerous and long, with the apical portion showing many lengthy microvilli. The goblet cells have a large goblet-shape and they are located in between the columnar cells (Fig. 3a). The hindgut and foregut are internally lined by an epithelium covered by a chitinuous cuticular intima. The hindgut has polygonal epithelial cells with large deeply stained nuclei (Fig. 4a). In the foregut, there are squamous epithelial cells with small nuclei (Fig. 2a).

Beginning from day 7 of 5. instar, the insects stopped feeding and began spinning.

From this day, degenerative changes began in the gut cells. In the foregut and hindgut, cuticle and epithelial cells began to seperate from each other and midgut cells began to become smaller on day 7 (Figs. 2-4). On day 8, nuclear condensation and vacuoles could be seen in the midgut cells (Fig. 3). Apoptotic morphology became more obvious on day 8 and 9, especially nuclear condensation was much more obvious (Figs. 2-3-4).

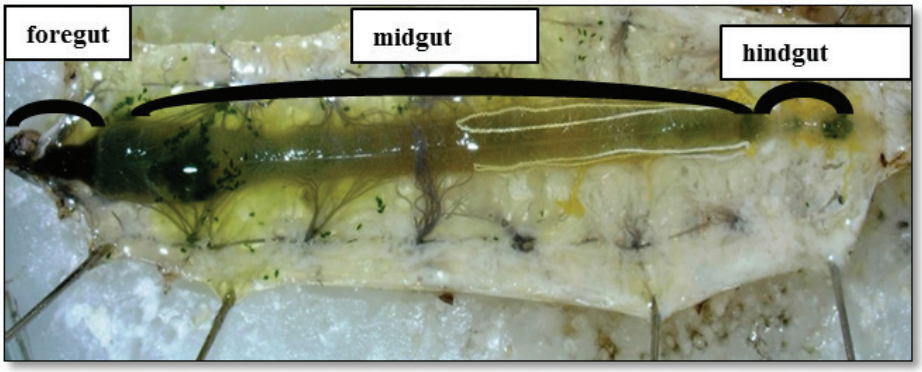

Figure 1. Digestive system of *Bombyx mori*

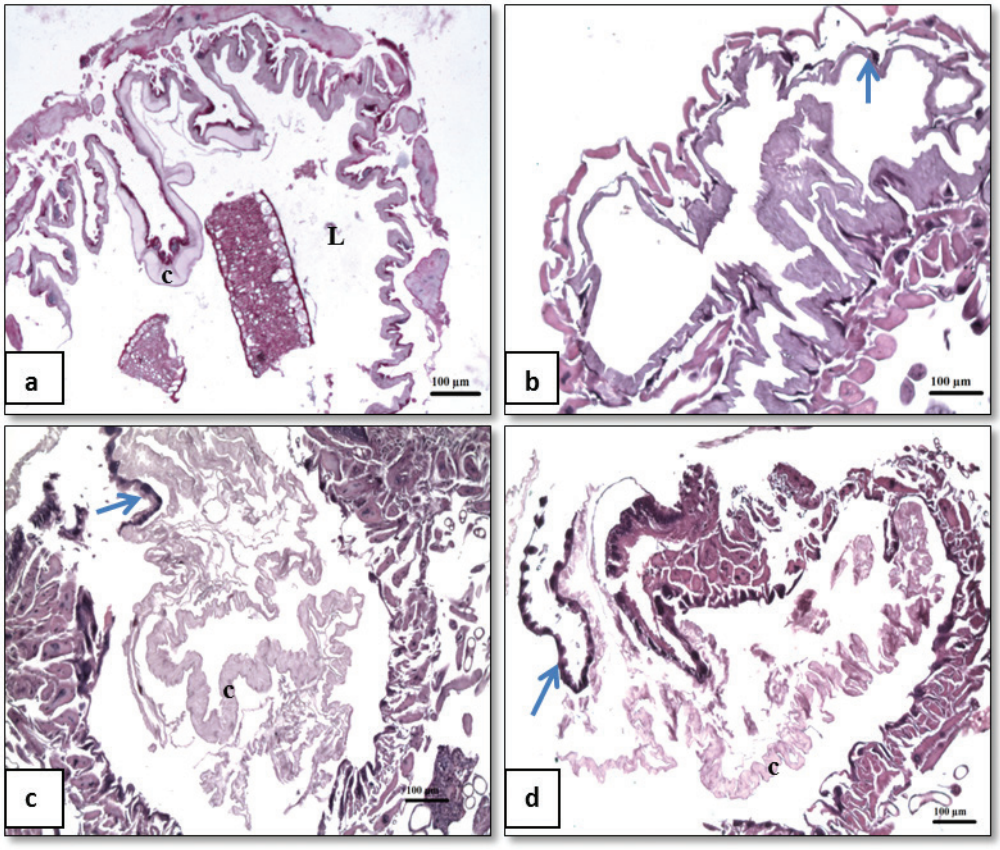

Figure 2. Larval foregut (crop region) during prepupal period of *Bombyx mori*. There were not seen any degeneration on day 6(a). Degeneration in the foregut begins slowly on day 7(b). Degeneration proceeded and the cuticle and foregut epithelial cells began to separate from each other on day 8(b). On day 9(d), the epithelial cells and cuticle completely separated from each other. C,Cuticle; Arrows,foregut squamous epithelial cells; L,Lumen; f,food. (PAS staining)

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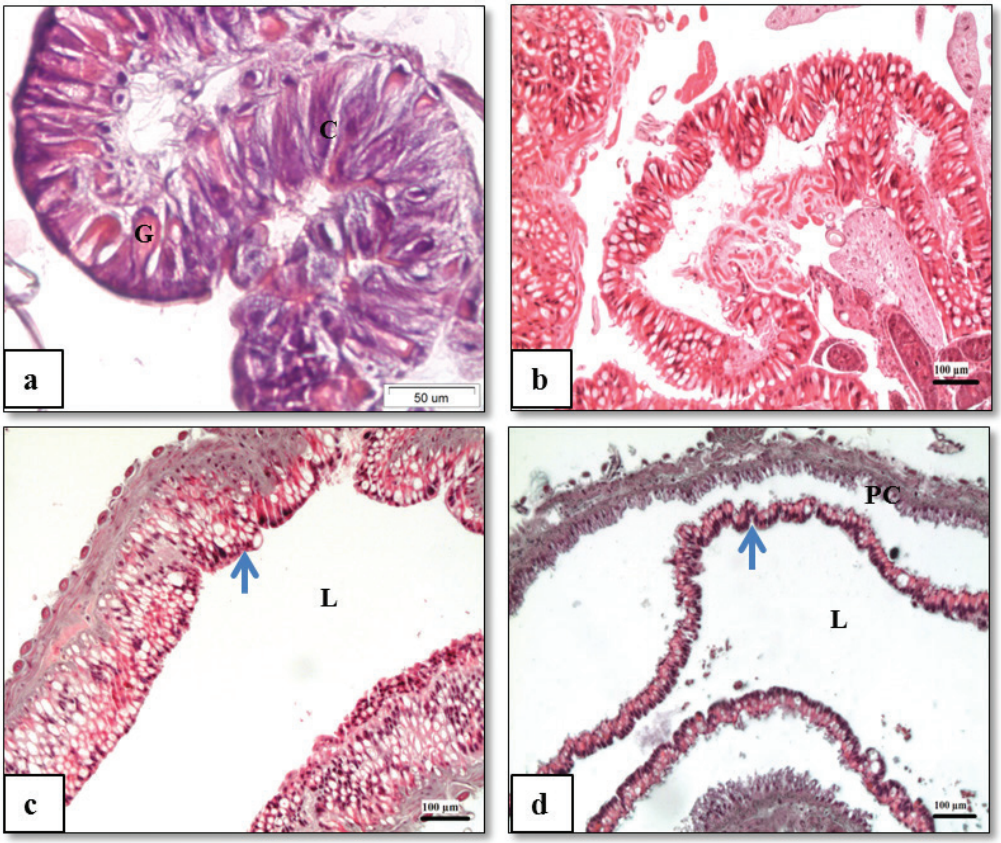

Figure 3. Larval midgut during prepupal period of *Bombyx mori*. There was not any degeneration on day 6 (a). On day 7, it was seen that the midgut cells began to shrink (b). On day 8, Apoptotic morphology was seen clearly; midgut cells became smaller and nuclear condensation was seen (c). On day 9, the pupal and the larval midgut cells were separated from each other, pupal cells could be clearly distinguished surrounding the condensed larval midgut cells(d). G, goblet cells; C, columnar epithelial cells; L, lumen; PC, pupal midgut cells; Arrows, nuclear condensation of degenerative larval midgut cells. (H&E staining).

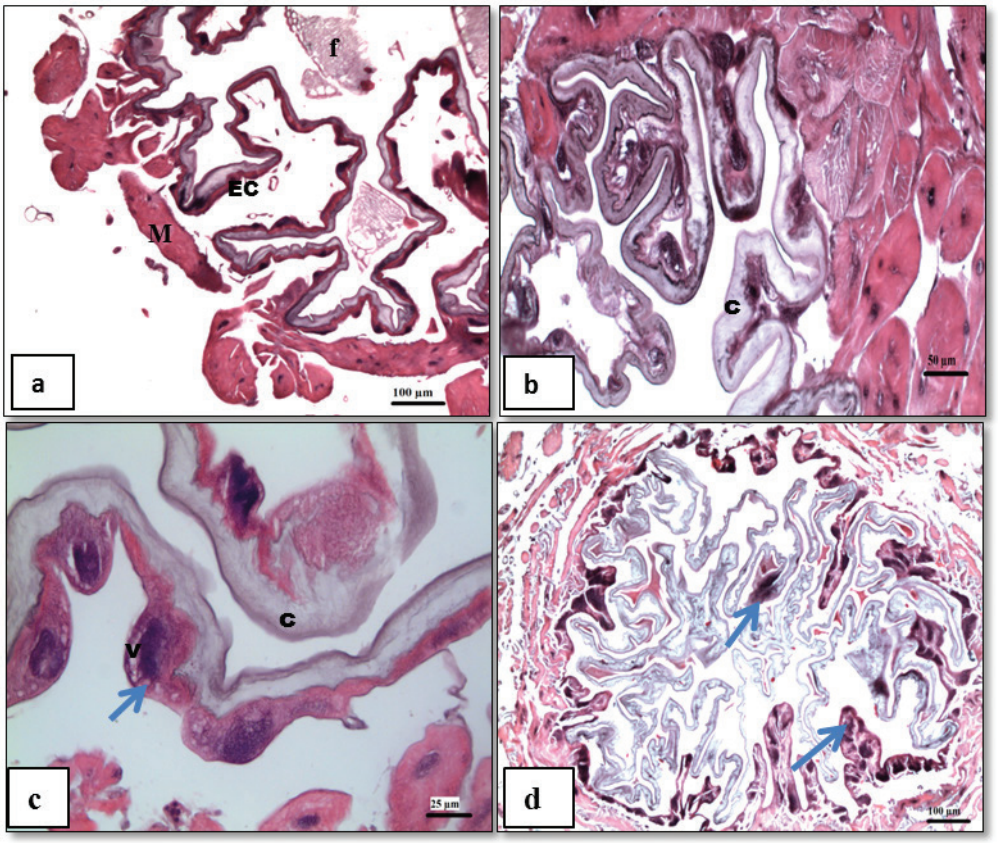

Figure 4. Larval hindgut during prepupal period of *Bombyx mori*. There were not seen any degeneration on day 6(a). Degeneration in cells began to appear on day 7(b). Nuclear condensation and vacuoles were seen in cells on day 8(c). Cuticle and degenerative epithelial cells seperated from each other on day 9 (d). f,food; c,cuticle; v,vacuoles; EC, epithelial cells; M, muscles; Arrows, nuclear condensation of degenerative cells. (PAS staining)

TUNEL assay

The TUNEL assay is one of the most accepted techniques in the examination of apoptotic cells. Sections from staged prepupa were stained using Biovision TUNEL Kit in order to determine whether larval gut is accompanied by DNA fragmentation. In apoptotic cells tunel positive nuclei stained brown color with DAB, on the contrary the cells which are not apoptotic stained green color with methyl green that was used for counter staining.

The first morphological signs of apoptosis occurs on day 7 of fifth instar but TUNEL positive nuclei strongly stained on day 8. Especially in midgut; nuclear fragmentation was easily identified by days 8 and 9; we observed strong TUNEL staining on these days. The newly formed pupal midgut cells could be clearly distinguished on day 9, surrounding the condensed TUNEL positive larval cells (Fig. 6). Also we observed a TUNEL positive staining in foregut and hindgut on days 8 and 9 (Figs. 5-7).

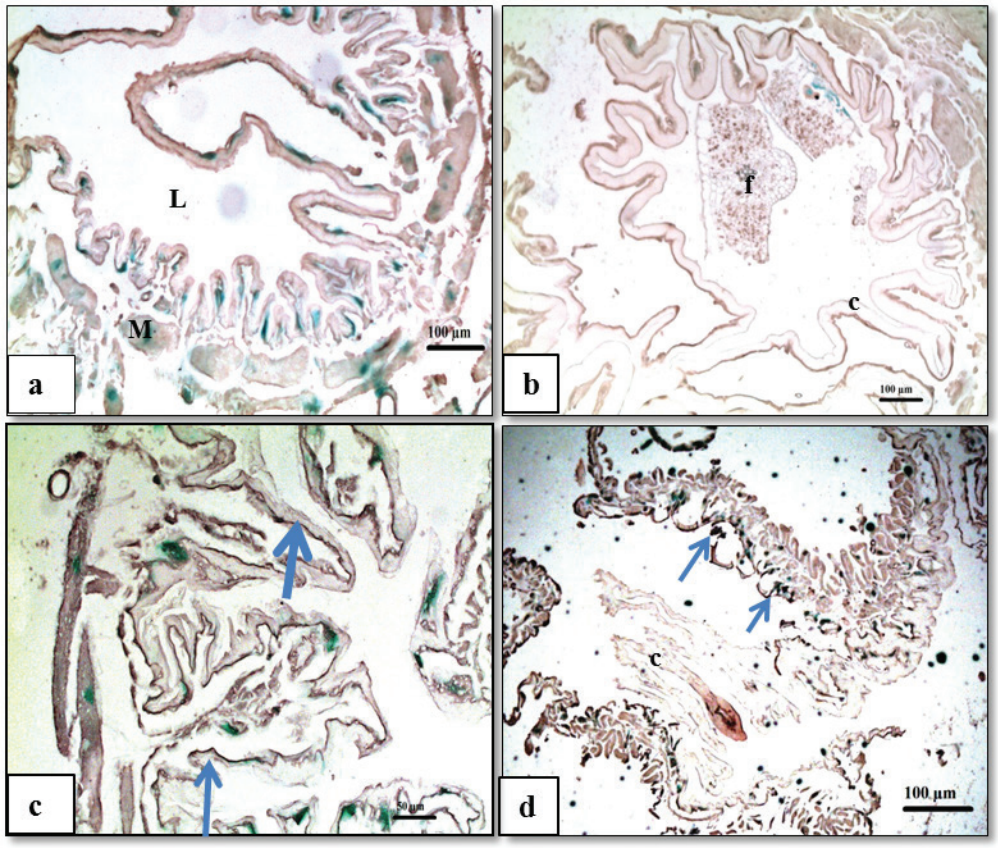

Figure 5. Foregut sections of staged prepupae were analyzed using TUNEL assay to detect DNA fragmentation. Strongly Brown(Tunel-positive) nuclear staining indicates the presence of apoptotic cells. Green(Tunel-negative) staining indiates nuclei of non-apoptotic cells. Foregut epithelial nuclei were stained by the methyl green and there were not seen TUNEL positive(brown) staining on day 6(a). On day 7, some of the nuclei were slightly stained but there were not strongly brown nuclear staining (b). Great numbers of TUNEL positive(brown staining) nuclei were clearly indicated on day 8(c). On day 9, the foregut began to disappear. Cuticle and epithelial cells were seperated from each other and apoptotic nuclei could be observed(d). C,Cuticle; Arrows, apoptotic foregut epithelial cells; f, food; L,lumen; M, muscles.

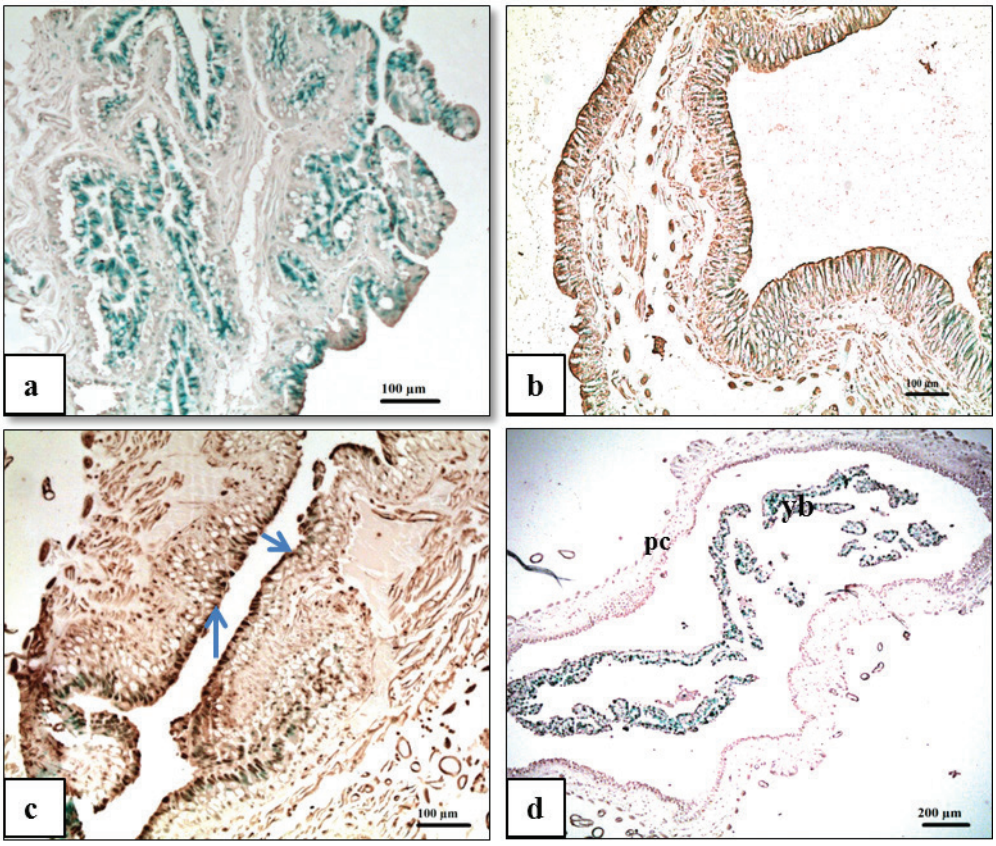

Figure 6. Midgut sections of staged prepupae were analyzed using TUNEL assay to detect DNA fragmentation. Strongly Brown(Tunel-positive) nuclear staining indicates the presence of apoptotic cells. Green(Tunel-negative) staining indicates nuclei of non-apoptotic cells. On day 6, midgut nuclei were stained with green; Tunel-positive(Brown) staining was not observed(a). On day 7, some nuclei were stained Tunel-positive(Brown) but, some of them were stained by the methyl green(b). Great numbers of TUNEL positive nuclei were clearly indicated on day 8(c). On day 9, larval midgut epithelium completely detached from basal lamina and was called as yellow body and moved into the lumen. In the yellow body, apoptotic nuclei could see stained with Brown(tunel-positive)(d). PC, pupal midgut cells; yb, yellow body; Arrows,apoptotic nuclei.

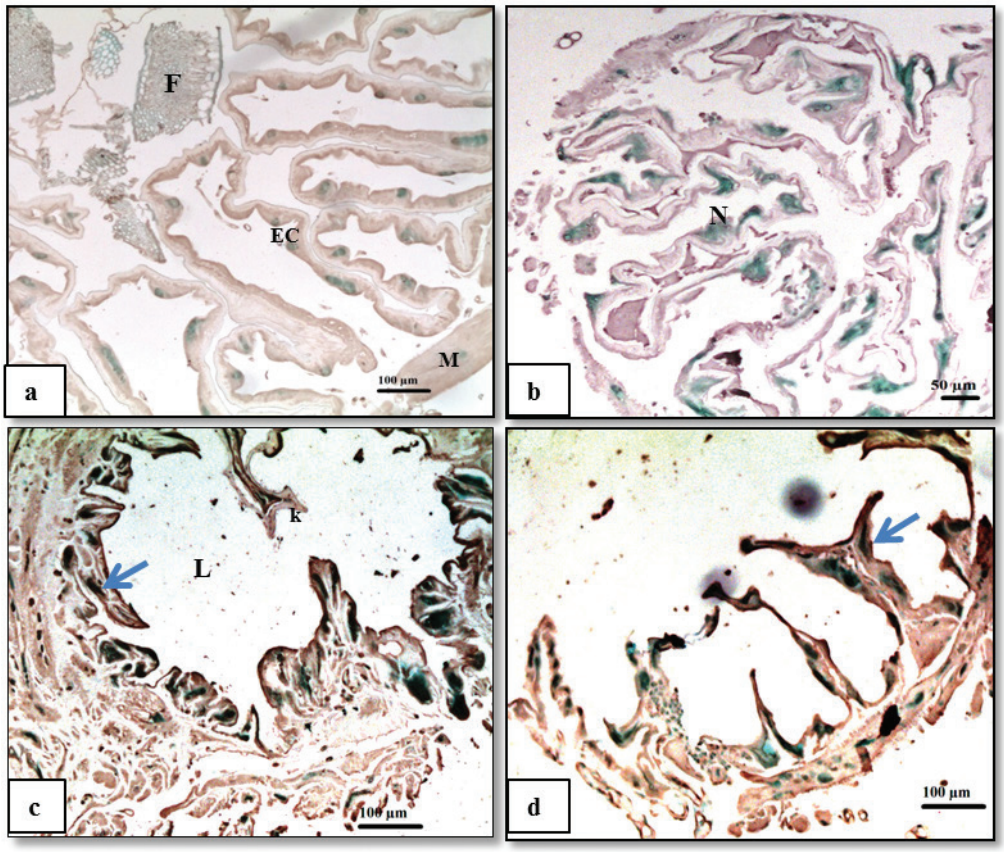

Figure 7. Hindgut sections of staged prepupae were analyzed using TUNEL assay to detect DNA fragmentation. Strongly Brown(Tunel-positive) staining indicates the presence of apoptotic cells. Green(Tunel-negative) staining indicates nuclei of non-apoptotic cells. Hindgut epithelial nuclei were stained by the methyl green and there were not seen Brown(TUNEL-positive) staining on day 6(a) and on day 7(b). Great numbers of TUNEL-positive nuclei were clearly indicated on day 8(c). On day 9, hindgut cells began to disappear; cuticle and the epithelial cells separated from each other and apoptotic nuclei could be observed(d). C, Cuticle; EC, Epithelial cells; Arrows, apoptotic cells; L,lumen; M, muscles.

Acid phosphatase activity

We determined acid phosphatase (AP) activity during prepupal period as an indicator of autophagic cell death. We estimated specific AP activity in the larval gut tissues for each day of the prepupal period.

AP was found low on day 6, but after interruption of feeding especially on day 8 and 9, specific AP activity increased slightly. The higher activity of the enzyme was observed on day 9 in fore, mid and hindgut (Fig. 8). And these results suggest that autophagic activity begun at the end of the feeding.

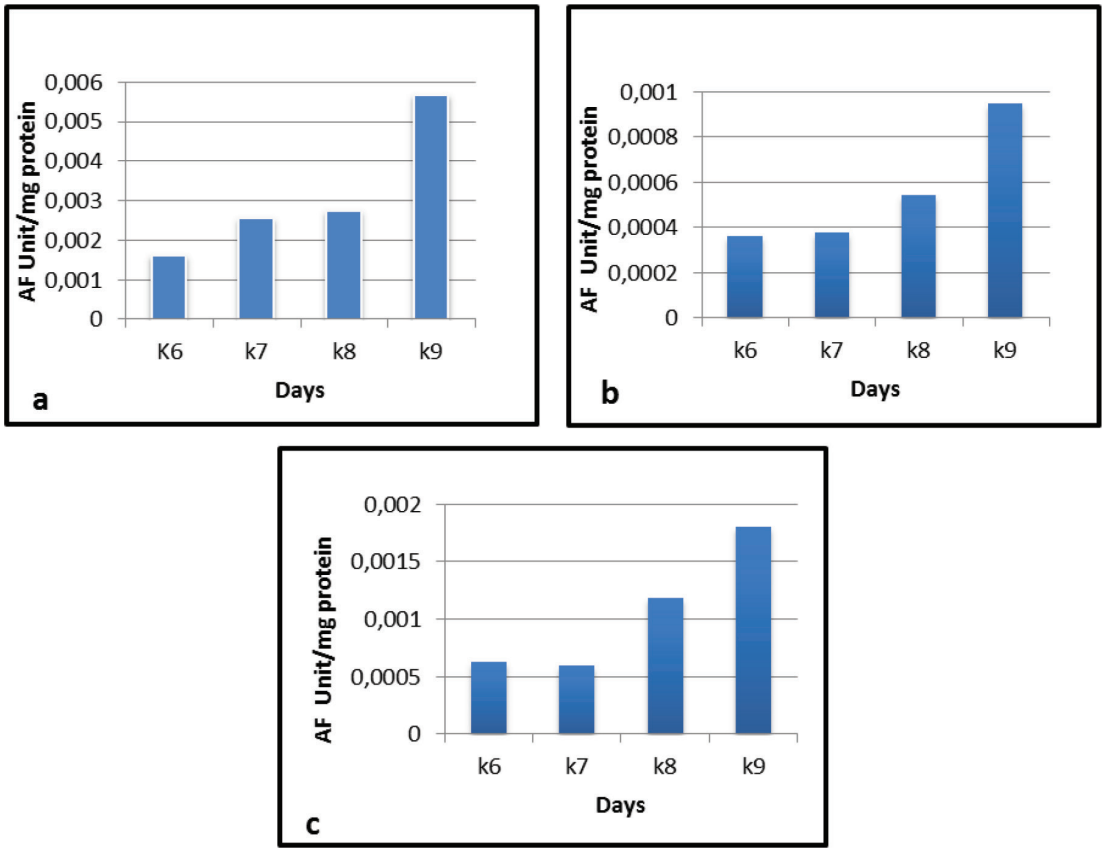

Figure 8. Acid phosphatase activity in the foregut (a), midgut (b) and hindgut (c) homogenates during prepupal period.

Discussion

In the silkworm *Bombyx mori* undergoing complete metamorphosis, the digestive canal degenerates during the larval-pupal metamorphosis through programmed cell death. Programmed cell death is a physiologically and evolutionarily conserved process and plays an important role in many vital events such as embryological development and tissue health especially in multicellular organisms.

Several studies conducted in recent years have investigated programmed cell death by separating it into different types of death. Of these, apoptotic and autophagic cell death occurring during the development of animals are the most prominent two forms of cell death (Kerr et al. 1972; Schweichel and Merker 1973; Clarke 1990; Lockshin and Zakeri 2004).

Lepidopteran larval organs, like gut, are an ideal in vivo model system for studying cell death. In fact, during metamorphosis, the larval gut epithelium degenerates and the cells die via programmed cell death.

In our study, the three parts of the digestive system (the foregut, midgut and hindgut) were examined separately during the prepupal period, and the resulting degeneration was revealed. First, all the three parts were separately studied with histological staining methods, such as H&E and PAS, and what degeneration processes they underwent was observed.

The foregut responsible for the transmission of nutrients to the midgut and the hindgut responsible for the absorption of nutrients begin to degenerate during the prepupal process, lose this function during the pupal period and become blunted. In our histological examinations of this degeneration process, cell shrinkage and cuticle-epithelial detachment which can be detected at the light microscope level were observed. The general morphological structure of foregut and hindgut in insects

was elucidated by many studies (Levy et al. 2004b; Nardi et al. 2009; Gaikwad et al. 2011) ; however, the number of studies conducted on the degeneration occurring during larval-pupal metamorphosis is not adequate.

The midgut which constitutes the largest part of the digestive system is the most important part where the digestion takes place in the larvae. Degeneration signs like cell shrinkage, vacuole formation and apoptotic bodies observed in our histological findings obtained through H&E and PAS staining of the midgut composed of different cell types such as goblet and columnar epithelial are important findings. Similar results were detected in the larval midgut of *Anticarsia gemmatalis* (Levy et al. 2004a).

In our study, more specific methods were used to find out what type of cell death led to the degeneration occurring in the foregut, midgut and hindgut. The presence of apoptosis was revealed by the TUNEL method, the presence of autophagic cell death was revealed with acid phosphatase enzyme activity.

Apoptosis, is the main form of regulated cell death in multicellular organisms. Previous studies have described the occurrence of apoptotic cell death in some larval organs of Lepidoptera during metamorphosis (Gavrieli et al. 1992; Terramanti et al. 2007; Goncu and Parlak 2008; Goncu and Parlak 2011), but the role of autophagy and its relationship with apoptosis are still subject of debate (Malagoli et al. 2010; Tettamanti et al. 2011).

As in our study; apoptosis was observed in the silkworm digestive system remodeling previous studies (Parthasarathy and Palli 2007; Tettamanti et al. 2007). 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 (Lockshin and Zakeri 2004). We found similar signs in our histologic and TUNEL findings.

Although autophagy is well known for survival-promoting role in response to nutrient deprivation and other stressors, providing both nutrients and energy for the starved cells, it has a secondary role in cell death (Guillon-Munos et al. 2006; Eisenberg-Lerner et al. 2009; Shen and Codogno 2011; Franzetti et al. 2011). This type of cell death is characterized by massive degradation of cellular contents, including essential organelles such as mitochondria, by means of intracellular double membrane/ vesicle reorganization and lysosomal activity (Tettamanti et al. 2011). Autophagy is activated from the wandering stage and it reaches a high level of activity during the prepupal stage, as demonstrated by specific autophagic markers. During the prepupal period in *Bombyx mori* digestive system, we demonstrated high AP levels in fore, mid and hindgut. As in our research; high AP values were observed in the midgut during the post embryonic stage in Cavalcante and Cruz Landim's (2004) study of *Apis mellifera*.

Tettamanti et al. (2007) observed midgut development during the fifth larval instar in the tobacca budworm *Heliothis virescens*. They indicated that this remodeling of the alimentary canal involves the destruction of the old cells by programmed cell death mechanisms (autophagy and apoptosis). As in our study, TUNEL-positive signals and high acid phosphatase levels were determined in the digestive system of *Bombyx mori*.

Franzetti et al. (2011) observed that larval midgut of silkworm degradation was due to the concerted action of the two mechanisms of PCD, which occured at different times and had different functions. They also demonstrated that autophagy reached a high level of activity during the prepupal stage similar to our observations. In our study, we researched the whole digestive system (foregut, midgut and hindgut) of *Bombyx mori* and unlike their work, we showed the two mechanisms of PCD in foregut and hindgut, too.

In conclusion, this study provides cellular and biochemical evidence of the involvement of programmed cell death in the gut remodeling process during the prepupal period in *Bombyx mori*. Our observations indicated that a co-occurrence of apoptosis and autophagy ocurred during the cell death of the digestive canal. The larval digestive canal degenerate via apoptotic and autophagic cell death and this combined intervention of two different PCD

processes in the gut epithelium of Lepidoptera during the prepupal period provides a powerful experimental model system to determine apoptosis and autophagy.

Acknowledgements

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