

Proliferation and Differentiation of Stem Cells in the Midgut Epithelium of *Culex pipiens* (Diptera: Culicidae) Mosquito Larvae Post Feeding

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Abstract: The late fourth instar (L_4) of *Culex (Cx.) pipiens* mosquito larvae were starved for 48 hours, then fed on tetra-mine, moved at 2,6,10 and 14 h intervals post feeding. The posterior midgut was dissected, fixed and observed using the transmission electron microscopy (TEM). The posterior midgut epithelium of the 4th instar larvae of *Cx. pipiens* was found to be composed of single-layered digestive cells, endocrine cells and regenerative cells. The digestive cells have microvilli, produce digestive enzymes and absorb digestion products. These cells have regular geometrical shapes (usually pentagonal or hexagonal) and spherical nuclei. The regenerative cells are positioned basally in the epithelium as single cells, small and cone-shaped and of darker appearance than the digestive cells. Well-developed peritrophic membrane was observed in the gut lumen to protect the midgut cells from possible damage by abrasive food particles. Numerous cell organelles were observed throughout the cell. Regenerative cells of the midgut epithelium fulfil the role of stem cells. They are proliferate intensively and differentiate into distinct cell types with high rate through the first 2 h post feeding and decrease slowly as the time intervals increase.

Key words: *Culex pipiens* • Mosquitoes • Stem cells • Posterior Midgut • Transmission Electron Microscopy

INTRODUCTION

Insects present a reproductive and adaptive capacity that allows them to occupy several different environments. Part of this adaptive success is due to a great structural variety and the physiology of their gastrointestinal tract, which allows them to use a wide variety of substrates as a source of nutrients essential for their survival [1]. In holometabolous insects, during metamorphosis, the gastrointestinal tract is completely renovated. Many new cells are formed, while others die and some are just modified [2, 3]. These modifications are linked to changes in diets, which are, in general, different for larvae and adults [4, 5]. Interfering with the normal development of the midgut might reduce the larva's ability to absorb, or store, nutrients and, as a consequence, reduce adult fecundity. Thus detailed knowledge of regulators of midgut development could identify targets that may be modulated to affect female fecundity and control mosquito populations [6].

Type II formation of a PM is considered to be a derived technique, and is found only in some families of the diptera, dermaptera, embioptera and Lepidoptera orders of insects [1]. In type II formation, the PM is produced by a specialized group of cells present on the proventriculus of the anterior midgut [2]. Type II formation is a continuous process which is carried out regardless of the presence or absence of a food bolus and increase with feeding process.

In general, the midgut is the location where digestion and absorption of almost all nutrients ingested by insects occur [7]. Its anterior limit is the cardiac valve and the posterior limit is the pyloric valve. It is usually internally coated by the peritrophic membrane instead of the cuticle observed in other regions [8]. It is surrounded by fine muscle fibers arranged in two layers. The mid gut epithelium is composed of three cell types: digestive or principal cells, regenerative cells and endocrine cells [9-12]. The digestive cells are cylindrical, tall and present microvilli in their apical portion; the basolateral region

presents numerous folds and invaginations. These cells are responsible for the production of digestive enzymes and the absorption of digested products [7]. They vary along the length of the midgut, according to its physiological moment or the role it plays in a certain region [13].

Among the digestive cells of most insects, small regenerative cells are single isolated or in pairs or forming small groups called cell nests, which are fundamental for the restoration of the digestive epithelium [13,14]. These cells are called stem cells in *Manduca sexta* [15, 16]. Regenerative cells of the insect midgut epithelium fulfill the role of stem cells. They are proliferating intensively and differentiate into distinct cell types [13, 17-23]. They may transform into columnar cells of epithelial character; either into endocrine or goblet cells, if present in the midgut epithelium [15, 21, 24, 25]. The new regenerative cells can repair or renewal the degenerative cell in a cyclic or continuous way [1,19,23,24,26-28]. This work aimed to investigate the stem cells proliferation and differentiation in the midgut epithelium of *Cx. pipiens* mosquito larvae. It may be able to understand the relation among the feeding, proliferation, differentiation of stem cells, midgut epithelium regeneration and potential mitoses.

MATERIALS AND METHODS

The late fourth L₄ instar of mosquito larvae of *Cx. pipiens* were starved for 48 hours, then fed on tetra-mine, moved at 2, 6, 10, 14 h intervals post feeding. Individual midgut were dissected and fixed in 2.5% glutaraldehyde for 3-4 h and washed with phosphate buffer 0.1 M/L for 10 min. Kept overnight in a new phosphate buffer and post fixed in 2% osmium tetra oxide (OsO₄) for 1.5 h at room temperature. Specimens dehydrated in a graded series of alcohol (30%, 50%, 70%, 90%, 95% and 100% for 15 min each), followed by acetone (15 min). The material was embedded first in propylene oxide two times for 15 min each, then a mixture of epoxy resin (ER) and propylene oxide (PO) 1:2 (ER: PO) for 1 h in rotator; 1:1 (ER: PO) overnight, 2:1 (ER: PO) for 1 h then move to 100% resin only overnight at 60 Celsius. Semi-thin and ultra-thin sections were cut using UCT25 Ultramicrotome (Leica, Reichert Ultracut and Leica Ultracut UCT). Semi-thin sections were stained with toluidine blue dye then observed with Olympus BX60 light microscopy (Correct Tokyo Seiwa Optical).

Ultra-thin sections placed on copper grids and stained with uranyl acetate and lead citrate for TEM (Jeol JEM 100S) examination at the National Cancer Institute, Cairo, Egypt.

RESULTS

The examined posterior midgut of *Cx. pipiens* mosquito found to be composed of a single layered epithelium surrounded by a network of circular and longitudinal muscle bundles. The digestive cells have morphological characteristics of enterocytes cells and basolateral membrane. The free surface of the enterocytes cells has a regular array of microvilli. These cells have regular geometrical shapes (usually pentagonal or hexagonal) and spherical nuclei (Figure 1). The absorptive cells are the major cellular component of the posterior midgut epithelium. The cell membranes close to the basal lamina are extremely in folded. Other cell types found in the midgut epithelium are stem cells and endocrine cells. Both stem cells and endocrine cells scattered among the enterocytes in the basal region (Figure 1). The stem (Regenerative) cells are found as solitary cells, have little cytoplasm and the nuclei have condensed chromatin. The cytoplasm has profile suggesting low metabolic activity in these cells.

The TEM shows stem cells with predominantly euchromatic nucleus at the base of epithelium. The stem cells initiate the differentiation process by forming a continuous layer of irregular and highly electron dense cells under the old cells. At this stage, the new digestive cells present poorly developed (Figure 2). By 2h post feeding, the activity of stem cells increased, the old stem cells mitotically divided into two new stem cells with two nuclei, which morphologically similar but gradually differed in size, one of them increased in size and became large and extending further into the apical region of the midgut epithelium. The second stem cell keep in stem cell phase, this cell remain close to the basal membrane (Figure 2), the stem cells undergo differentiation, involving cell growth, gradual development of the nucleolus and the basal labyrinth and the development of microvilli contained in an apical compartment. Differentiating cells are observed at distinct heights of the epithelium demonstrating their asynchronous but progressive growth toward the lumen (Figure 2). By 6, 10 and 14h post feeding, the testing groups did not show any divided cells.

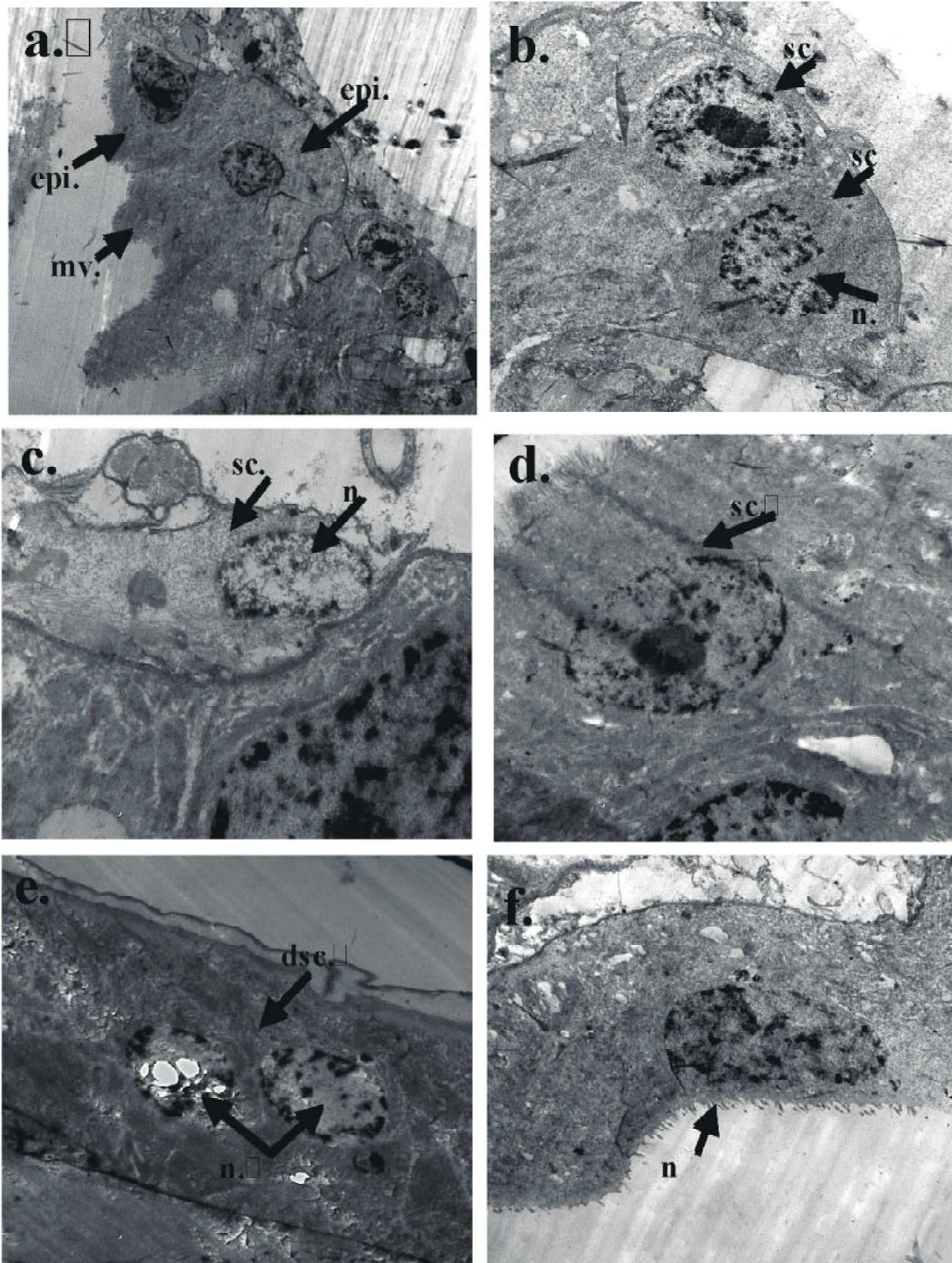


Fig. 1: Transverse section of the ultrastructure of a midgut *L4* instar of *Cx. pipiens* showing. [a] The epithelial cells with stained nuclei. [b] undifferentiated and differentiated stem cells. [c] Stem cell at the proliferation phase. [d] The semifinal stage of stem cell differentiation. [e] Recently divided stem cells. [f] The final stage of stem cell differentiation

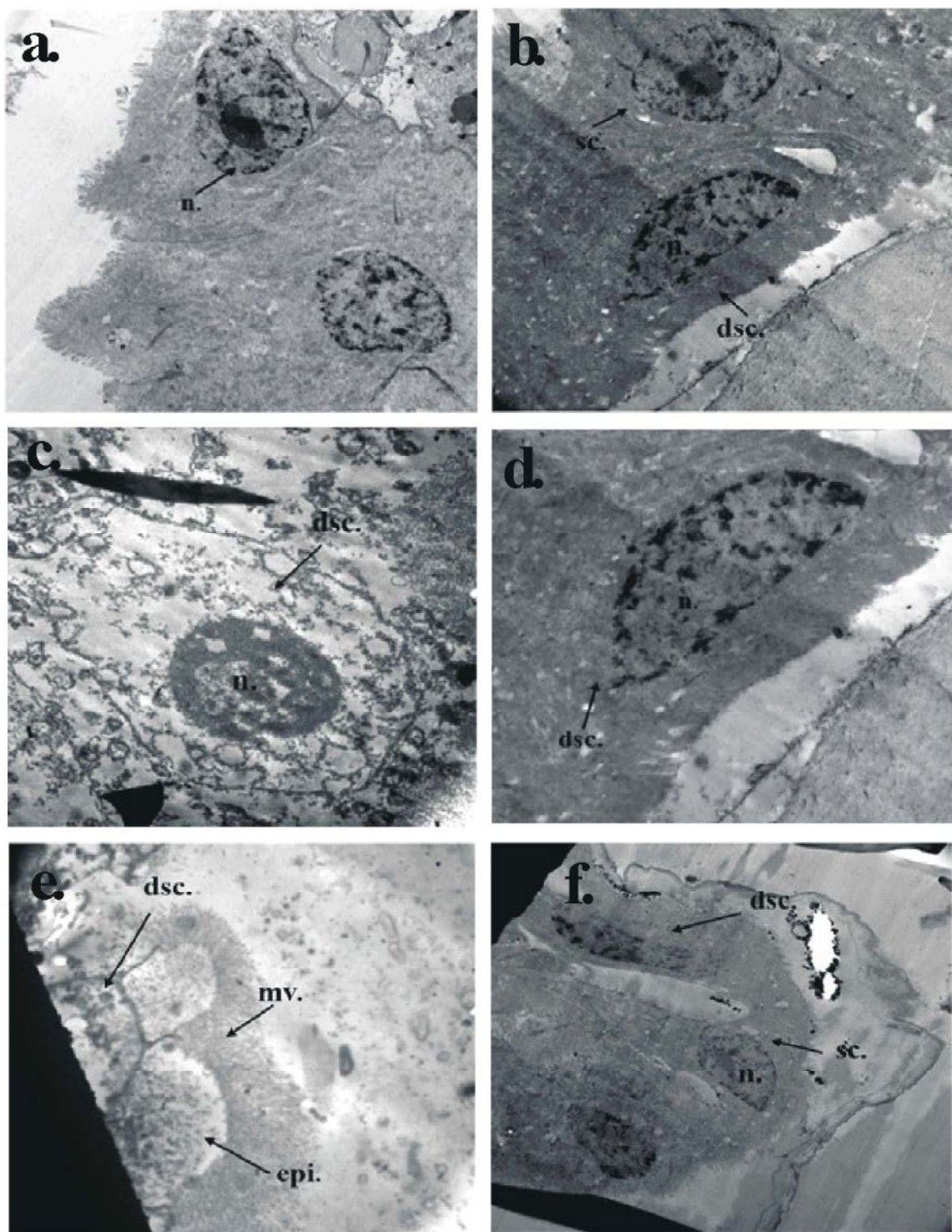


Fig. 2: Transverse section of the ultrastructure of a midgut L₄ instar of *Cx. pipiens* showing . [a] The well –developed epithelial cells with microvilli on the apical side of the epithelium. [b] The stem cells undergo differentiation, involving cell growth.[c] gradual development of the nucleolus leaving the basal labyrinth [d] The final phase of stem cells differentiation.[e] Differentiating cells at distinct heights of the epitheliumand progressive growth toward the lumen. [f] The nucleus is similar to that of the mature stem cell

DISCUSSION

The midgut epithelium morphology in the L_4 of *Cx. pipiens* is similar to those of other mosquito species [19, 29-31]. The finding of this study showed that the digestive cells, have morphological characteristics of enterocytes cells and basolateral membrane. The free surface of the enterocytes cells has a regular array of microvilli. These cells have regular geometrical shapes and spherical nuclei. It is known that midgut epithelial cells degenerate during digestion and new cells originate from regenerative cells due to their mitotic activity [32, 33]. The timing of cell division differs between the anterior and the posterior midgut *Cx. pipiens* and *Aedes. aegypti* [34, 35].

The present study showed that the cell proliferation begins at the posterior midgut of L_4 instar and this may be associated with the onset of midgut histolysis. Stem cell division is initiated in the L_4 instar and completed at the early two hours and no mitotic division observed after that time with unknown evidence of a relationship between proliferation of the stem cell and digestive larval cell death. These results came together with Nishiura *et al.* [35]. Conversely, this and temporal separation of cell division reported in the midgut of dipterans has not been reported for other species like Collembola, Lepidoptera and Coleoptera [36-39].

The kind of food is a key factor in polyploidia/politenization, since the largest nuclei were found in the nurse workers of bees [14], including the older, which fed on pollen. In *Locusta migratoria* (Orthoptera) the variations in the size of the nuclei of the midgut digestive and regenerative cells were related to a differentiation process of the digestive cells. This process occurs through cycles of endopolyploidia or politenization, involving the genes necessary for the adaptation of the digestive cells to a given diet [40]. Three hypotheses for the differentiation the midgut regenerative cells in *Cx. pipiens* and *Ae. aegypti* mosquito larvae have been suggested; First the regenerative diploid cells may replicate during early instars and later gave rise mitotic diploid cells as well as endoreplicating cells. Second, 1st instars may have population of diploid cells that were committed to the endoreplication cycle but only divided during the 4th instar. Third, the new endoreplicating cells may migrate into midgut during larval development [36].

Regional differences were observed in the epithelium of the midgut of bees [12] and other insects [39, 41]. The variations in the size and the amount of digestive and regenerative cells among the regions of the midgut of the same individual and among the castes demonstrate

possible physiological differences among segments of this organ in the digestive process [14]. Those characteristics among the midgut regions are also reflected in the larval development of *Cx. pipiens* significantly contribute the size of the midgut in which the growth of the midgut anterior region occurs by increasing the size of the cells, while there is an increase in the number of cells in the posterior region [36].

The present study verified that the stem cells have little cytoplasm and the nuclei have condensed chromatin. The cytoplasm profiles suggesting low metabolic activity in these cells. These undifferentiated cells are supported by the basal lamina and their surfaces do not reach the light. The massive nucleus presents dispersed chromatin and prominent nucleolus, the cytoplasm is poor in organelles [5, 11, 42]. Degeneration and regeneration of the midgut epithelium may proceed cyclically or continuously. Cyclic cellular renewal in an insect midgut appears at each molt (Metamorphosis), when the digestive tract is growing, the larval midgut epithelium is ejected and its reorganization occurs from regenerative cells. During the regenerative differentiation cells elongate in direction to the midgut lumen acquiring microvilli, followed by an increase in the nuclear and cytoplasmic volume [11, 27, 43-46]. These results confirmed by Cruz-Landim *et al.* [12], Hecker [29], Chiang *et al.* [43] and Cruz-Landim [47] who verified that stem cells has its own extracellular environment, forming the stem cell niche. The niche is created by neighboring differentiated cells which allow stem cells to present the unique ability of self-renewal. The signal to the cells must come from the stem cell niche, which induces proliferation and differentiation, or ends these processes [27, 44, 48-50].

CONCLUSION

In *Cx. pipiens* L_4 instar stem cells proliferation and differentiation proceed intensively post feeding and decrease with time. The cells enlarged their content as they departed from the stem cells phase to proliferate and differentiate. The replication of genetic material is related to the need for an increase in the synthesis of specific proteins for digestion and initiates the cell cycle and cell division to produce new epithelial cells.

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