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Epithelial Stem Cells and Their Possible Role in Fate of Peritrophic Membrane of *Culex pipiens* Mosquito (Diptera: Culicidae)

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Abstract: The stem cells are capable of massive proliferation and differentiation in the midgut epithelium of mosquitoes. The posterior midgut of the late 4th larval instar of *Culex (Cx.) pipiens* was dissected at 2, 6, 10 and 14h post feeding after starving for 24 h, then fixed in 10% formal saline for 24 h., dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin wax, Sectioned 2–3 µm thickness using semi-automatic rotary microtome, Dehydrated through 95% and 100% alcohol, cleared in xylene, mounted, stained with toluidine blue and examined. The morphometric measurements using the real-time microscope image was visualized using video monitor to measure the peritrophic membrane (PM) thickness mean. The morphometric analyses of the PM thicknesses were conducted to verify whether the activity of the stem cells are associated with the PM thickness occurs post feeding. By the first 2 h post feeding, the stem cells have been observed during proliferation and differentiation and the PM thickness mean was 2.1 µm. By 10 h post feeding, the maximum thickness 10.52 µm was recorded then sharply decreased to be 2.2 µm by 14 h post feeding. The increase in the PM thickness mean was not synchronized by the stem cells proliferation found post feeding. The increase in cells number of the posterior midgut was not sufficient to explain the increase in the PM thickness mean, indicating that the increase in the volume of the digestive cells may be necessary for increase of the PM thickness.

Key words: Mosquito larvae · Stem cells · Midgut epithelium · Peritrophic membrane (PM)

INTRODUCTION

The peritrophic membrane (PM) is an invertebrate-unique structure that lines the digestive tract, playing important roles in facilitating food digestion and providing protection to the gut epithelium. The PM is an extracellular envelope composed predominantly of chitin fibrils and glycoproteins and classified as two types; type I formation of a PM is thought to be the ancestral method and is found in the majority of organisms that produce a PM. In type I formation, the PM is secreted by the entire midgut and is formed simply by delamination from the surface of the midgut epithelium [1, 2].

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].

The PM can take many diverse forms; there are two types of mosquito PM (1 and 2) that have very different properties depending on the site of synthesis. Type 1 PM, which for example is produced in blood-fed adult mosquitoes, is secreted by all of the epithelia lining the midgut usually in response to the ingestion of a blood meal. This PM envelops the ingested meal in a bag like structure. In contrast, type2 PM is produced in the mosquito larvae. This type is thin tubular sleeve, lines the entire midgut and hindgut of the mosquito larvae and is constitutively secreted by a small group of cells at the foregut/ midgut junction called the cardia. The PM is continuously synthesized and is a preformed barrier in larval mosquitoes, as demonstrated by Stoltz and Summers [3]. The larval PM is colorless, transparent and semi-permeable with a certain toughness and elasticity and its main compositions are chitin, proteoglycans and proteins [4]. PM which originates from the cardia continuously lines to the end of the midgut (abdominal segments). It separates the midgut lumen into two regions, respectively the endoperitrophic space is located between PM and the food bolus and the ectoperitrophic space is located between PM and the midgut cells [5].

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In insects, chitin is not only synthesized by ectodermal cells that form chitinous cuticles, but also by endodermal cells of the midgut that secrete a chitinous PM [6]. Most of the hematophagous insects present a midgut morphologically and physiologically divided into two or more parts [7, 8]. The anterior or thoracic region is responsible for the absorption of sugar and the formation of a mucous material and the posterior or abdominal region is responsible for the absorption, synthesis and secretion of digestive enzymes and the peritrophic matrix [9-12]. The PM is involved in the protection of insects from invasion by bacteria and parasites.

In diptera, midgut changes begin in the last larval instar, the larval epithelial digestive cells degenerate, leaving the basal membrane and the regenerative cells develop into a new epithelium during the late 4th larval (L_4) instar. Epithelium renewal is followed by changes in volume and shape of the midgut [13]. The epithelium of the midgut of insects is mainly composed of digestive, endocrine and regenerative cells. The digestive cells are responsible for the production of digestive enzymes and the absorption of the digested products, while the regenerative cells restore the epithelium [14]. The regenerative cell is a cell which divides from time to time to generate a new cell population and daughter cells differentiate further in the precise way, depending on their purposes [15,16]. This study aimed to investigate the mechanism of stem cells to reconstitute the midgut epithelium and its fate role in the peritrophic membrane formation of *Culex* (*Cx*.) *pipiens* L_4 instar post feeding at different time intervals.

MATERIALS AND METHODS

The late L_4 instar of *Cx. pipiens* mosquito larvae were starved for 24 hours and fed on tetra-mine, moved at different time intervals (2,6,10,14 h) post feeding.

Histology: The midguts were dissected in a saline solution 10% for 24 h (0.1 M NaCl, 20 mM KH_2PO4 or 20 mM Na_2HP4) and washed in tap water, dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin wax (melting point 46-54 °c). Sections of 2–3 µm thickness were prepared using semi-automatic rotary microtome (LEICA RM 2245, USA). Dehydrated through 95% and 100% alcohol for 2 times, cleared in xylene, two times for 3 minutes each and covered with resinous mounting medium, then stained with toluidine blue.

Light Microscopy: The light microscopy was used to investigate the availability of stem cells associated with formation the peritrophic membrane and restore the midgut epithelium of L_4 instar of *Cx. pipiens*.

Morphometric Analysis: The morphometric analysis is carried out on routine toluidine stained slides and examined on magnification (50X). The boundaries of the tissue was measured and adjusted to the illumination of the video monitor. The morphometric measurements represented the real-time microscope image that visualized using the video monitor. The ecto/endo- peritrophic spaces measured by drawing a line starting from the epithelial layer edge to the PM. and from the later to the food bolus using the interactive measurement software of the system on a total magnification of (200X). The results appeared automatically on the measured monitor in the form of the distant measured in (µm) with the mean, standard deviation, the minimum length and the maximum length. Morphometric analysis was performed at the Pathology Department, National Research Center using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England,).

Statistical Analysis: Simple (bivariate) and multiple linear regression analyses were performed using the REG procedure of the SAS software package. For the multiple linear regression analysis, three predictor variables were included in the initial analysis: (i) Time post feeding (ii) The peritrophic membrane thickness mean (ii) Measure of ecto- and endo- peritrophic spaces. Models employing all possible combinations of these predictor variables were generated.

RESULTS

By 2 h post feeding, the midgut columnar cells as digested and absorptive cells showed high activity through their nucleus with dense chromatin and the PM thickness mean recorded 2.1 µm. At the beginning, time intervals and the PM tightly surrounded the food particles with narrow endo-peritrophic space mean (4.7 µm). The thickness mean of the PM sharply increase from 2.1±0.14µm by the first 2 h to be 7.1 µm by 6 h post feeding. By10 h post feeding, the PM thickness mean reached its maximum mean 10.52±0.55 µm, then decreased sharply to reach 2.2±0.3 µm by14 h post feeding (Table 1, Fig. 1). The ecto-peritrophic space recorded its maximum thickness mean 34.5±4.8 µm by 10h post feeding, then decreased rapidly to reach 25.63±6.2 µm by14 h post feeding (Table 1, Fig. 1). On the other hand, the endo-peritrophic space thickness mean increased gradually during the 6 h post feeding to reach its maximum mean 12.14±1.13 µm, then decreased to be 6.45±1.4 µm by 10 h post feeding and continuously decreased to be 4.7±0.2 µm

Table 1: Statistical analysis of the peritrophic membrane thickness mean, the ecto-peritrophic space thickness mean and the endo-peritrophic space thickness mean of *Cx. pipiens* L_4 instar at different time intervals post feeding

	Statically aspect											
	PM thickness				ecto-peritrophic space				endo-peritrophic space			
Time intervals post feeding	Mean	SD ±	SE ±	(Min - Max)	Mean	SD ±	SE ±	(Min - Max)	Mean	SD ±	SE ±	(Min - Max)
2 hrs.	2.1	± 1.2	± 0.14	(0.52 - 2.31)	16.6	± 8.6	± 1.2	(4.04 - 33.44)	11.58	± 3.4	± 1	(7.08 - 16.26)
6 hrs.	7.1	± 8.8	± 1.25	(0.26 - 12.8)	10.3	± 6.35	± 1	(1.55 - 25.62)	12.14	± 7.8	±1.13	(1.29 - 27.31)
10 hrs.	10.52	± 2.1	± 0.55	(5.41 - 13.41)	34.5	± 16.46	± 4.8	(9.8 - 52.86)	6.45	± 3.7	± 1.4	(2.38 - 13.26)
14 hrs.	2.2	± 1.8	± 0.36	(0.26 - 5.67)	25.63	± 15.3	± 6.2	(5.42 - 42.34)	4.7	±0.44	± 0.2	(0.2 - 5.19)

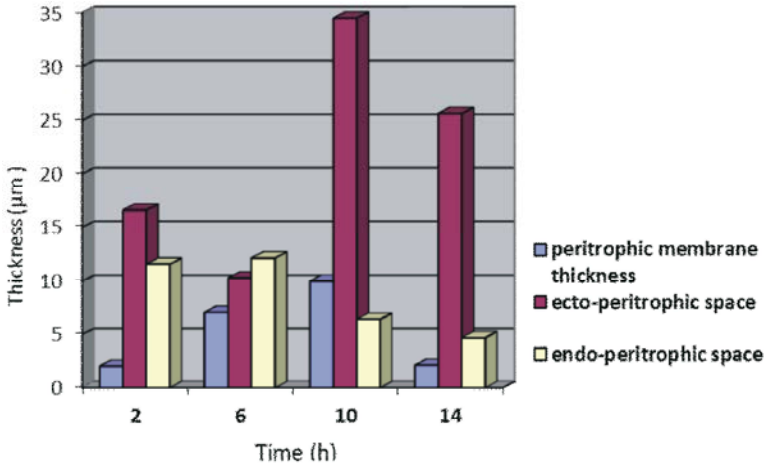

Fig. 1: Histogram of the peritrophic membrane thickness mean, the ecto-peritrophic space thickness mean and the endo-peritrophic space thickness mean at different time intervals post feeding of *Cx. pipiens* L_4 instar.

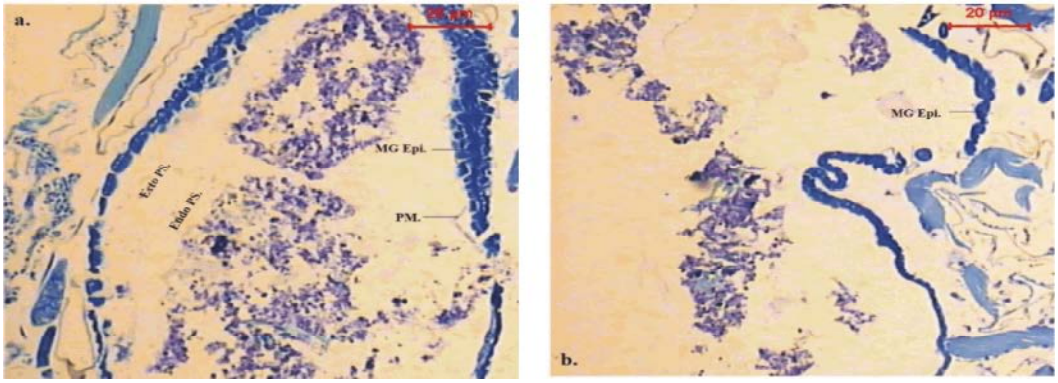

Fig. 2: Photomicrograph of Longitudinal Section of *Cx. pipiens* L_4 inster showing, the histology of midgut epithelium, PM: peritrophic membrane, MG Epi: midgut epithelial layer, Ecto-PS: ecto-peritrophic space, Endo-PS: endoperitrophic space. (a): 6 h. post feeding showing, the presence of PM: peritrophic membrane thickness, ecto- and endo-peritrophic spaces thickness and MG Epi, midgut epithelial layer. (b): control group, 6 h post starvation for 24 h showing, the absence of PM peritrophic membrane, MG Epi:midgut epithelial layer.

tightly surround the digested food by 14 h (Table 1, Fig. 1). The activity of the columnar cells decreased and associated with decreasing of the PM thickness mean (2.2 µm) by 14 h post feeding.

Close examination of the longitudinal (Fig.2 a, b) and the transverse sections of midgut revealed that it is composed of single-layer mostly digestive epithelial cells, solitary stem cells and endocrine cells.

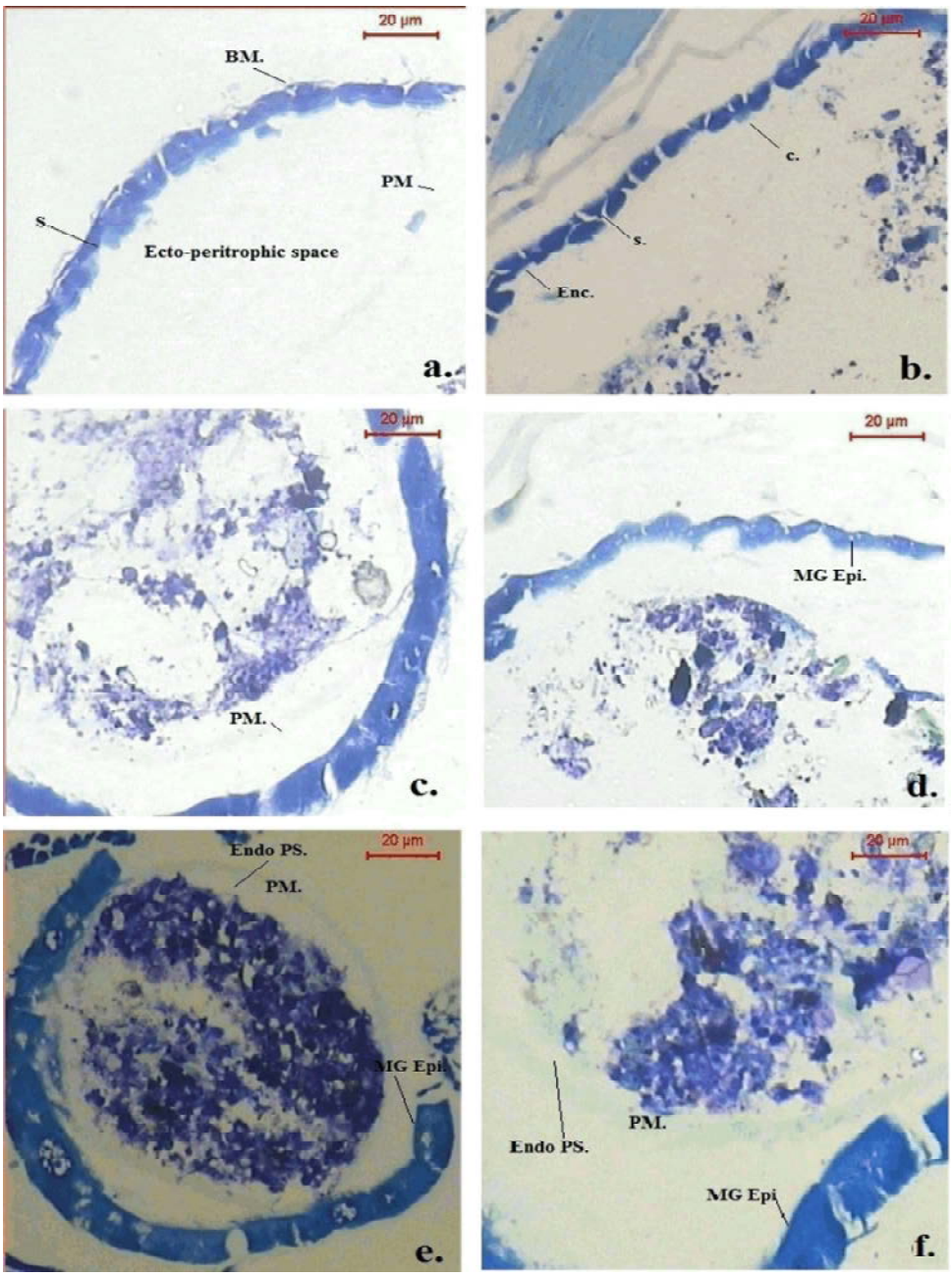

Fig. 3: Photomicrograph of Transverse Section of *Cx. pipiens* L_4 instar showing, the histology of midgut epithelium. PM: peritrophic membrane, EnC: endocrine cell, C: columnar cell, S: stem cell, BM: basement membrane, DS: divided stem cell, Int G: intercellular gabs, mv:microvilli. (a): 2 h post feeding, showing the presence of PM thickness, ecto- and endo-peritrophic spaces thickness, S: stem cells, BM: basement membrane. (b): control group 2 hrs. post starvation for 24 h, showing the absence of PM, C:columnar cells with its basal nuclus and dense microvilli towards the gut lumen, EnC: endocrine cells with central nucleus and microvilli, S: stem cells. (c): 6 h post feeding, showing the presence of PM thickness, ecto- and endo-peritrophic spaces thicknesses. (d) control group by 6 h post starvation for 24 h, showing the absence of PM peritrophic membrane, MG Epi:midgut epithelial layer (f): 10 h post feeding, showing the maximum thickness of the PM membrane and the ectoperitrophic space (e): 14 h post feeding, showing the decrease in thickness of the PM thickness and the ectoperitrophic space

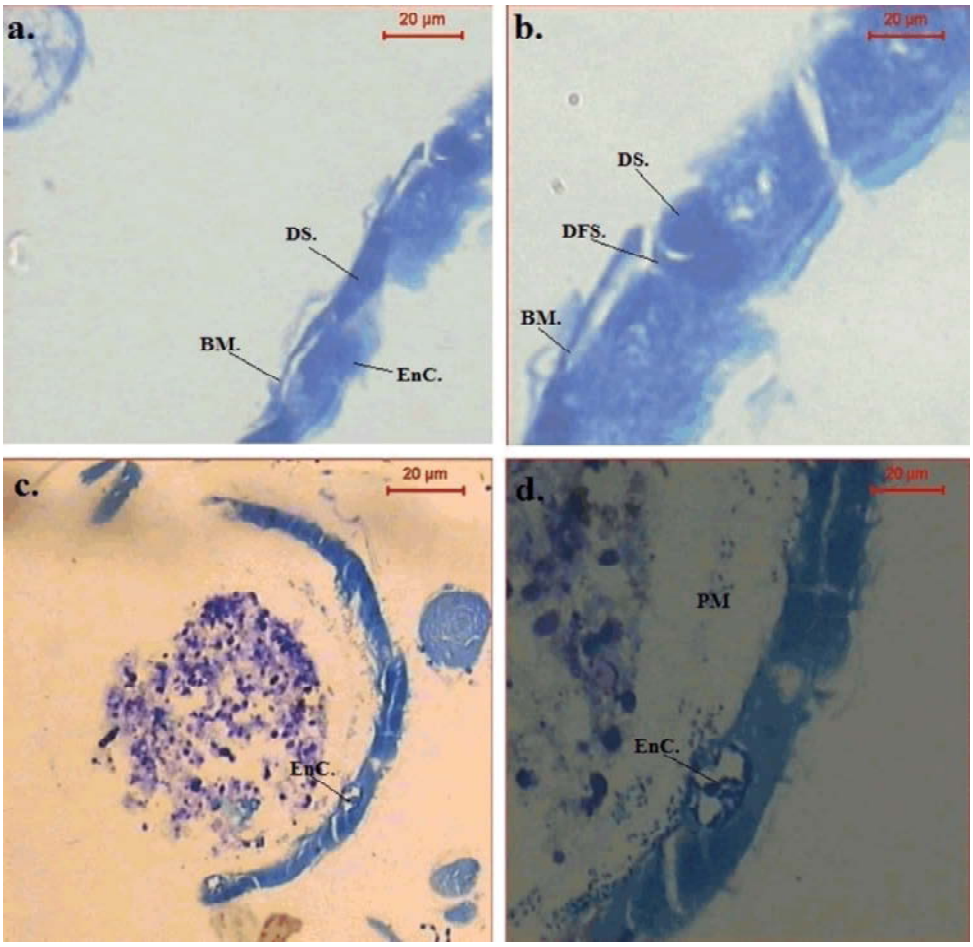

Fig. 4: Photomicrograph of Transverse Section of *Cx. pipiens* L_4 instar 2 h. post feeding showing, the histology of midgut epithelium. EnC, endocrine cell, C:columnar cell, DS: divided stem cell, DFS: differentiated stem cell, BM: basement membrane, DS: divided stem cell, Int G: intercellular gabs, mv: microvilli (Scale bar: 20 µm, Toluidine blue stain) showing the activity of endocrine cells. (a):The dividing stem cell, (b):the differentiated stem cell (c,d): showing the activity of EnC: endocrine cells, PM:peritrophic membrane, Ecto- and Endo-peritrophic spaces.

The free surface of the digestive cells has a regular array of "brush border" microvilli. The cell membrane was close to the basal lamina, extremely folded (Fig. 3 a,b) The control group post starvation for 24 h showed normal activity and distribution of both stem cell and endocrine cells, the later was normal and had no vacuoles or cavities (Fig.3b). The activity of stem cells proliferation and differentiation observed during the first 2 h post feeding, the mother stem cells mitotically divided into two daughter stem cells with two nuclei, which morphologically similar but gradually differed in size, the first daughter stem cell increased in size and became large and extending further into the apical region of the midgut epithelium. The second daughter stem cell did not pass the same cycle and keep in stem cell phase, this cell remain close to the basal membrane (Fig. 4 b). The morphological observations of the sequential divisions of the stem cells proved proliferation and differentiation into normal columnar midgut epithelial cells and endocrine cells.

The activity of the endocrine cells as a secretory cells increased and formed vacuoles containing refractory fibrotic material may be PM feeding illustrated (Fig.3c,e). Consequently, the endocrine cells basally located nuclei, possessed a distinct apical cavity. The size of the nucleus varied, but was typically of comparable size to the nuclei of normal midgut epithelial cells. These cells became close to the apex surface of the midgut epithelium to release its apocrine secretion (Fig.3), throughout the midgut restructuring process, a cavity

form was observed at the digestive cells' apex (Fig.4 c, d). The active endocrine cells open at the surface of the midgut epithelium. The activity of these endocrine cells as a secretory cells increase, enlarge and forming vacuoles released a granular material by the first 2h post feeding (Fig.4 d). There is a definite space between the epithelium and the PM. This space is filled with scattered granular material released from the apex of the digestive endocrine cells via apocrine secretion toward the intestinal lumen (Fig. 4 d) and adjacent to the microvilli. The numbers of such cells vary throughout the different intervals post feeding in posterior midgut.

DISCUSSION

The insect midgut is the chief site of both digestion and absorption of nutrition and the epithelium is responsible for the production of many digestive enzymes or the uptake and transfer of nutrients to the haemolymph [17]. The columnar cells are concerned with enzyme secretion and with absorption of the product of digestion [18]. The histology of the midgut architecture of *Cx. pipiens* is similar to those of other dipteran mosquito species *Cx. pipiens fatiganus*, *An. stephensi*, and *An. gambiae* [19], *Ae. aegypti* [20-24,26]. The midgut of 4th larval instar of *Cx. pipiens* is functionally the most important part of the digestive system, responsible for digestion and absorption of nutrients as in other insects [25].

In this investigation, the activity of the stem cells to proliferate has been observed in 4th larval instar of *Cx. pipiens* midgut epithelium during the first 2 h post feeding only. The regulation of stem cells proliferation in insects may be a basic biological process controlled by many regulatory factors derived from haemolymph and fat bodies [26]. *In vitro* four midgut differentiation factors affecting and inducing proliferation and differentiation of lepidopteran midgut stem cells to be mature midgut cell types (MDFs 1-4). These factors were isolated and characterized from conditioned media of *Heliothis virescens* and *Lymantria dispar* haemolymph [27]. The stem cells in larval *Drosophila* midgut are stimulated via an epidermal growth factor receptor (EGFR) signaling pathway. In early larvae, surrounding visceral muscles produce Vein, a weak EGFR ligand that stimulates a low level of stem cells proliferation. Later on in the larval stage, two other ligands are produced by the stem cells that provide signals that augment the first signal and lead to a higher level of proliferation [27, 28].

The expression profile of a putative basement membrane protein gene *hmg176* in the midgut of *He. armigera* larvae was increasing during both molting and feeding of this larvae [29,30]. Also, The thickness of the basement membrane in diptera is due to the good nutrition during the larval stage which facilitates the transport of products between the intestine and the haemolymph [31, 32]. Cytological evidence was obtained suggesting that, in both the anterior and posterior *Ae. aegypti* larval midgut, diploid regenerative cells give rise to new endoreplicating cells that significantly contribute to the growth and metabolism of the midgut. This hypothesis was supported by BrdU incorporation studies showing that diploid cells, as well as large and small endoreplicating cells, synthesize DNA during the 2nd, 3rd and 4th instars. Cytological studies of the *Cx. pipiens* larval midgut suggested that anterior midgut growth in this species was primarily by cell enlargement and increased in number in the posterior midgut [33].

The results of this study indicated that the release of digestive enzymes from midgut cells of *Cx. pipiens* is merocrine secretion where a high density secretory granules and a maximum amount of associated membrane has been observed by 10 h post feeding. The release of the secretory granules from the insect midgut occurs largely by classic exocytosis. The release of digestive enzymes from midgut cells in oriental fruit fly is merocrine due to high concentration of secretory granules in the apical part of the cells [34,35]. The presence of numerous secretory granules and numerous vesicles of rough endoplasmic reticulum in *Chrysomya megacephala* near the apical part of the cell may account for the production of PM [36]. It is surrounded the freshly food bolus and isolated it from the surface of the midgut epithelium, this observation confirmed the previous study, in the anterior part of the cardia between inner and outer surface of the epithelium a continuous layer with refreshment structure lead to the formation of the PM at the outer surface of the microvilli of the midgut epithelium [37]. By the first 2 h post feeding, divided stem cells were observed, by 6, 10 and 14 h post feeding, only differentiate cells were observed. The morphological observations described above are presumed to represent the sequential stages of division and differentiation of regenerative cells into normal columnar and endocrine midgut epithelial cells.

The PM is secreted as an unbroken, concentric, “sleeve like” structure. Although the PM is secreted continually, the presence of a food bolus significantly increases the rate of production. In addition, the presence of a food bolus stimulates the production of multiple

matrices which surround the bolus. Following the secretion of a primary PM, subsequent matrices are secreted underneath the first matrix to create a layered peritrophic envelope [1]. Consequently, the statistical analysis of the PM thickness through different time intervals post feeding could be estimated. By 6 h post feeding, the boundary margin seemed to have an increase in the PM thickness mean to be 7.1 µm with continuous increase to reach the maximum thickness mean 10.52 µm by 10 h tightly enveloped the digested food, then sharply decreased to reach 2.2 µm by 14 h post feeding. Our results came together with Filimonova [36] Lee *et al*. [37] who stated that the PM completely surrounds the food bolus, separating it from the midgut epithelium and acts as a molecular sieve that mediates the traffic of molecules.

The stem cell is a cell which divides from time to time to generate a new cell population and daughter cells differentiate further in fate way to renew the midgut epithelium [38, 39]. Stem cells play a role also in forming the meconium. The meconium is composed of degenerating larval midgut epithelial cells which begin to slough into the lumen late in the L_4 instar of mosquitoes and complete this process early in the pupal stage. The regenerative cells remnant from midgut metamorphosis are only located at the end of this organ's posterior region at reduced numbers [33]. Interestingly, such mitotic cells are concentrated in specific midgut regions during mosquito development, where in proliferation is initiated in the anterior L4 region. Such cell proliferation advances along the midgut and switches to the posterior region in the pupal stage. The timing of cell division differs between the anterior and the posterior midgut in *Cx. pipiens* [40-42]. The sloughed larval midgut cells are replaced basal regenerative cells which proliferate and form the new adult posterior midgut epithelium.

CONCLUSION

It can be concluded that the digestive cells in the posterior midgut epithelium of *Cx. pipiens* L_4 instar are responsible for the production of digestive enzymes and the absorption of the digested products in presence of PM. The feeding process may be accelerating the stem cells proliferation and differentiation in the midgut epithelium of the L_4 instar of *Cx. pipiens* mosquito. The stem cells are able to restore the epithelium architecture including the digestive and endocrine cells as well as the PM as a result of accelerating some genes expression which responsible of digestive enzyme secretion. The proliferation and differentiation of the stem cells synchronized with a slightly increase of the morphometric measurements of the PM. The increase in the cells number of the posterior midgut was not sufficient to explain the increase of the PM thickness mean, indicating that the increase in the volume of the digestive cells of the posterior midgut may be necessary for increase of the PM thickness.

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