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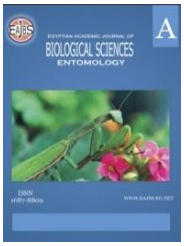

Study The Role of Intestine Stem Cells in the Midgut Epithelium Homeostasis of *Culex pipiens* post *Bacillus sphaericus* Infection

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ABSTRACT

The *Culex (Cx.) pipiens* mosquito is the main vector of Filariasis and Rift Valley Fever in Egypt. *Bacillus (B.) sphaericus* is a mosquitocidal bacterium that was recently developed as a commercial larvicide and is used to control pestiferous and vector mosquitos worldwide. Whereas B. sphaericus is highly active against larvae of Culex.The 4th larval instar of *Cx. pipiens* were dissected at 2, 10 and 14 hrs post-treatment with *B. sphaericus*, then the alimentary canal was fixed in 5% glutaraldehyde. Samples were dehydrated in an ethanol series, filtrated with epoxy resin, and stained with uranyl acetate. Semi-thin sections were stained with toluidine blue dye and then examined with 100X light microscopy (LM). The transverse sections (TS) of the mid and hindgut were examined by Transmission Electron Microscopes (TEM). The ultrastructural changes detected showed apoptosis, shrinkage, detachment from neighbours, nuclear degranulation, a number of vacuoles developed, and the bacteria began to penetrate the larval lumen. The damage to the midgut epithelium of the fourth larval instar of *Cx. pipiens* caused by *B. sphaericus* infection have an effect on the proliferation and differentiation of intestinal stem cells (ISC) in the midgut epithelium. It occurred simultaneously with a slight increase in cell division. Precursor stems cell proliferation and differentiation could replace the destroyed epithelial cells. The ISC regenerates the architecture of the midgut epithelium, thereby accelerating tissue repair. It was found that the number of stem cells and nuclei increase over time post-bacterial infection.

INTRODUCTION

The midgut of insects is the primary site of digestion and nutrition absorption, and the epithelium is in charge of producing many digestive enzymes as well as absorbing and transferring nutrients to the haemolymph (Wigglesworth,1965). The basic cellular composition of the epithelium is similar: absorptive enterocytes (ECs), which account for the majority of differentiated cells, endocrine cells (ECs), and columnar cells. Immature SCs are in charge of replacing differentiated cells lost due to damage or ageing (Jiang and Edgar 2012).

In mosquitoes, Endocrinal, columnar, and regenerative cells differentiate from midgut cells. Columnar cells play a role in both enzyme secretion and digestion product absorption (Chapman,1985). The midgut architecture of *Cx pipiens* is similar to that of other dipteran

mosquito species, such as *Cx. fatiganus, Anopheles (An.) stephensi, An. Gambiae,* and *Aedes aegypti* (Loeb *et al.,* 1999).

The midgut epithelium is the first physical barrier encountered by ingested pathogens; this single layer of epithelial cells forms a microvillar surface on the luminal side (Hecker, 1977). There has been little research into the epithelial composition and dynamics of mosquito midguts. Early histological studies identified and mapped neuropeptide production in the mosquito midgut epithelium (Broderick *et al.,* 2006), However, their physiological significance remains unknown. Brown *et al.* (1985) discovered putative "regenerative cells" in adult midgut epithelium. Maldonado *et al.* (2019) discussed DNA synthesis and/or mitoses in the midguts of several mosquito species, indicating that the mosquito midgut epithelium is regenerated.

The competence of a mosquito as a vector is determined by the successful invasion and traversal of midgut epithelial cells by orally acquired pathogens. It has previously been demonstrated that epithelial dynamics, in the form of cell sacrifice, play a role in the invasion of pathogens in mosquito midguts. The ability of the midgut's tissue homeostasis to replenish damaged cells is dependent on the presence of ISCs (Darboux *et al.,*2007).

Due to the lack of specific markers for progenitor cells for mosquitoes. Taracena *et al.* (2018) used morphological and physiological parameters to define the presence of ISCs in the adult females of *A. aegypti*. Progenitor cells are well characterized for their basal positioning. ISC hallmark capacity is to undergo mitosis. This allowed us to successfully identify the presence of ISC in the epithelium, and to quantify the number of cells dividing in the different conditions. Evaluated when the midgut epithelium was exposed to pathogenic bacteria ingested with the food meal.

Because of the presence of SCs in treated midguts, growth factors that promote stem cell proliferation and/or differentiation are produced and secreted. Furthermore, the presence of stem cells in control midguts is predicted by the fact that mature cells treated with toxin secrete proteins that induce stem cell-based epithelial regeneration (Castagnola and Jurat-Fuentes, 2009). Pathogenic challenge (Houtz *et al.,* 2017), reactive oxygen species (Hochmuth *et al.,* 2011), and ageing (Biteau et al., 2008) all change the kinetics of division, differentiation, and endocycling to reshape the microbial community.

The objective of this study is to explore the ultrastructure alterations in the midgut epithelium of the 4th larval instar of *Cx. pipiens* post *B. sphaericus* infection and the role of ISC in the midgut epithelium homeostasis (Taracena *et al.,*2018).

MATERIALS AND METHODS

Mosquito Rearing:

Mosquitoes were originally captured from Nahia, Giza Governorate, Egypt. Mosquitoes were colonized in an insectary at the Research and Training Centre on Vectors of Diseases at Ain Shams University. Mosquitoes maintained in the insectary at 28 °C and 70-80 % humidity using a 12:12 light: dark photocycle, under temperature (27 $\pm$ 2 °C). The late 3rd instar and early 4th larval instar were continuously supplied with cups containing clean tap water and fed on fish pellet food.

Treatment with *B. sphaericus*:

For bioassay, late 3rd and 4th instar larvae were placed in 5 mL of dH_2O and maintained in a humidified room at 28°C. Mortality was assessed at 12, 24 and 48 hrs post addition of *B. sphaericus stran 2362*. LC_{80} detected 0.174 ppm using Soliman et al method (2000). Larvae were evaluated at intervals 2-, 10-, and 14 hours post bacterial infection. Abbott Laboratory, North Chicago, IL, USA, provided us with *B. sphaericus* strain 2362.

Light Microscope (LM):

An individual's larval midgut was dissected and prepared for light and TEM examination. The morphometric analysis was carried out on routine toluidine stain slides and examined on magnification (100X) of Micro Blue - Euromex microscope (Holland) at Zoology Department, Faculty of Science, Suez University.

Transmission Electron Microscope (TEM):

At least five larvae were fixed for 2 hrs at room temperature in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0), rinsed in the same buffer, and fixed in 1% osmium tetroxide for 2 hrs at room temperature. Samples were dehydrated in an ethanol series ranging from 10% to 90% for 15 minutes in each alcohol dilution, followed by 30 minutes in absolute ethanol. In a graded series, samples were infiltrated with epoxy resin and acetone, culminating in_pure resin. Formvar-coated copper grids were used to collect ultrathin sections. Sections were stained twice with uranyl acetate and lead citrate. The stained sections were examined using a TEM (JEOL JEM 1010) according to Martins et al (2011). ETM examination has been done at the Regional Centre for Biotechnology and Mycology (RCMB), Al-Azhar University.

RESULTS

The LM Examination:

The midgut epithelium of control *Cx. pipiens* mosquito larvae showed clear 4 nuclei and stem cells (Fig.1 A, B). Two hrs post-treatment, TS of midguts showed 6 nuclei with stem cells mitotically active; divided into two new stem cells (Fig. 1 C, D). Post 10 hrs of treatment, 9 nuclei with stem cells appeared (Fig. 1 E, F). Fourteen hours post-treatment showed clearly 11 nuclei with stem cells.

TEM Examination:

The midgut epithelium of control *Cx. pipiens* mosquito larvae have a single layer surrounded by a network of circular and longitudinal muscle bundles. The cells possessed the morphology of epithelial cells and a basolateral membrane. Epithelial cells have a consistent array of microvilli on their free surface (Fig.2B, C). Cells have spherical nuclei and a well-organized appearance with an intact membrane (Fig.2 A).

The structure of the midgut epithelium of the 4th larval instar of *Cx. pipiens* showed modifications at different time intervals post *B. shaericus* treatment. Two hrs post-treatment, the epithelial cells layer showed few vacuoles, and cells began to expand with elongated nuclei (Fig.2 G, I). Clear and distinct bacteria toxins crystals appeared as dark granules located between the epithelial cells and the microvilli without entering the midgut. Stem cells were mitotically active and divided into two new stem cells with two nuclei that were morphologically similar but gradually differed in size (Fig. 2 D). Differentiating cells are seen at various epithelial heights, demonstrating their asynchronous but progressive growth toward the lumen.

Bacillus sphaericus spores were found in the lumen post 10 hrs of treatment, The number of vacuoles in the epithelial layer increased. Some positions of microvilli were lost. Bacteria were visible in the lumen and started to invade the epithelial layer (Fig. 2 L). The midgut epithelium showed numerous cyto-pathologies affecting mitochondria. More deterioration cells (Fig. 2 O) and some stem cells near the membrane (Fig. 2 K, M and N). Fourteen hrs post-treatment, Epithelial cells scarified and harmed microvilli. More vacuoles, nuclei and deterioration cells appeared (Fig. 2 Q and R).

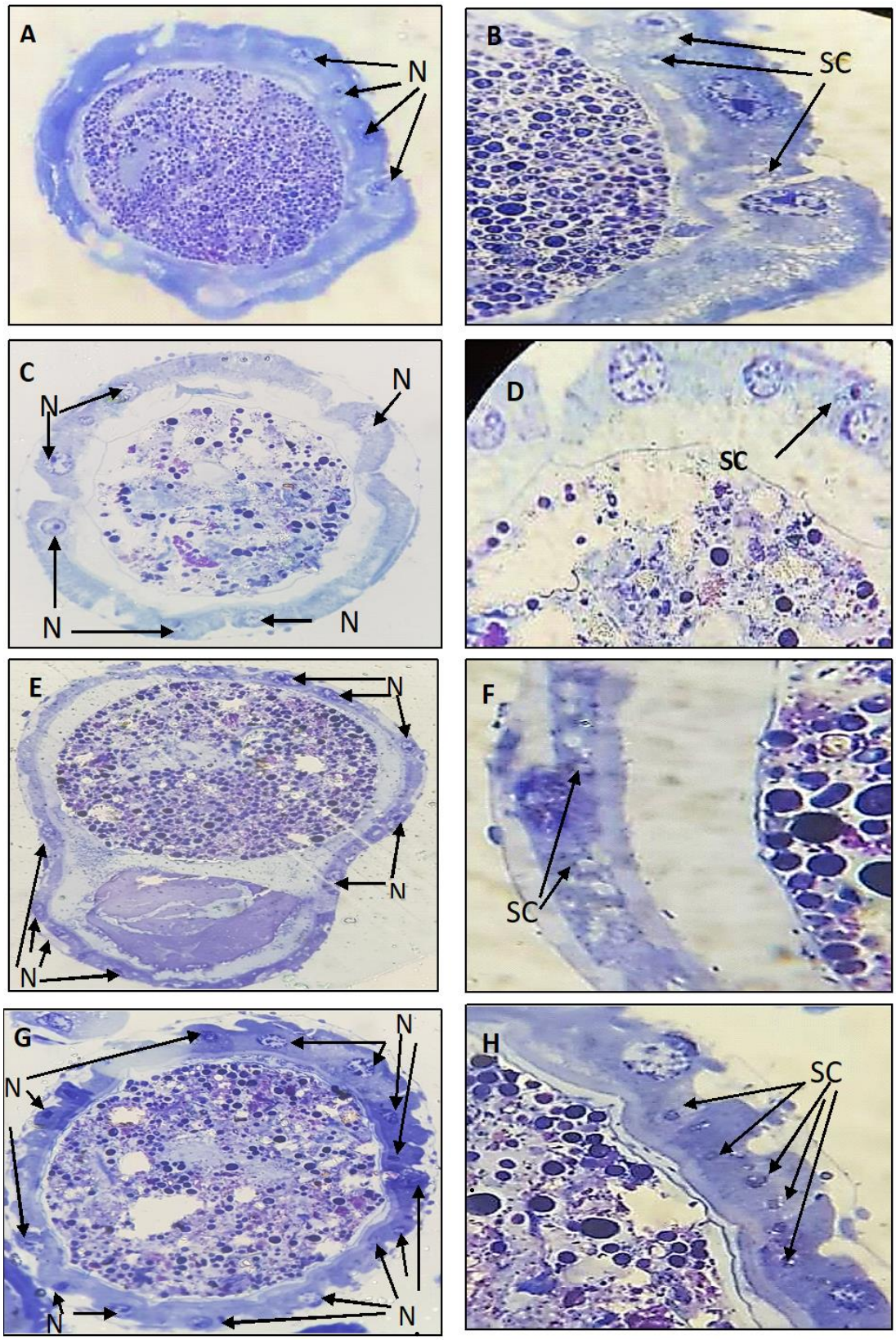
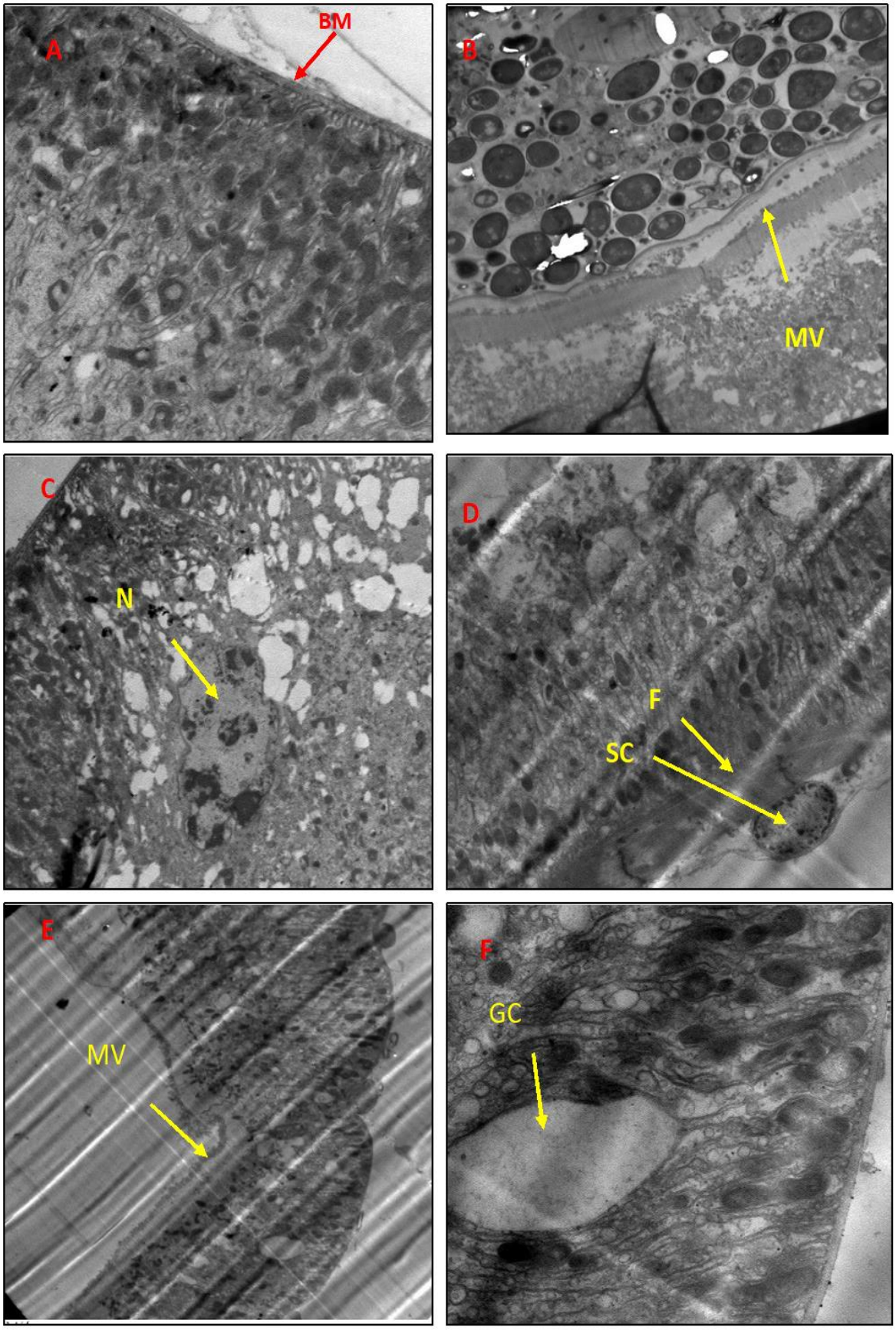


Fig.1: TS showed the histology of the midgut epithelium of the 4th larval instar of *Cx. pipiens* mosquito[A] Control showed 4 nuclei(N), [B] Control showed stem cells (SC), [C] Treated after 2hrs showed 6 nuclei(N), [D] Treated after 2hrs showed stem cells(SC), [E]Treated after 10 hrs showed 9 nuclei(N), [F] Treated after 10 hrs showed stem cells(SC) ,[G] Treated after 14 hrs showed 11 nuclei(N), [H] Treated after 14 h showed stem cells(SC).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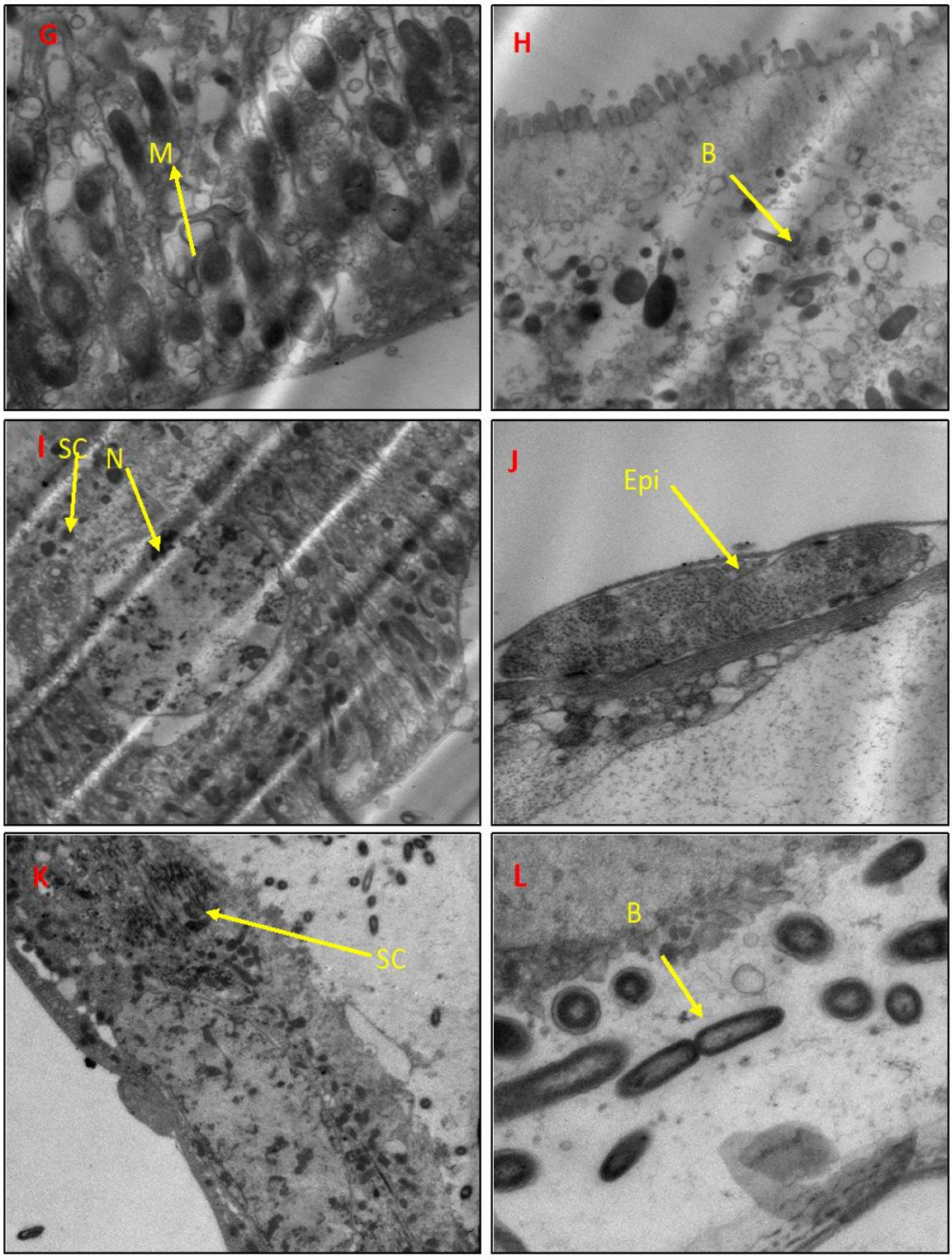
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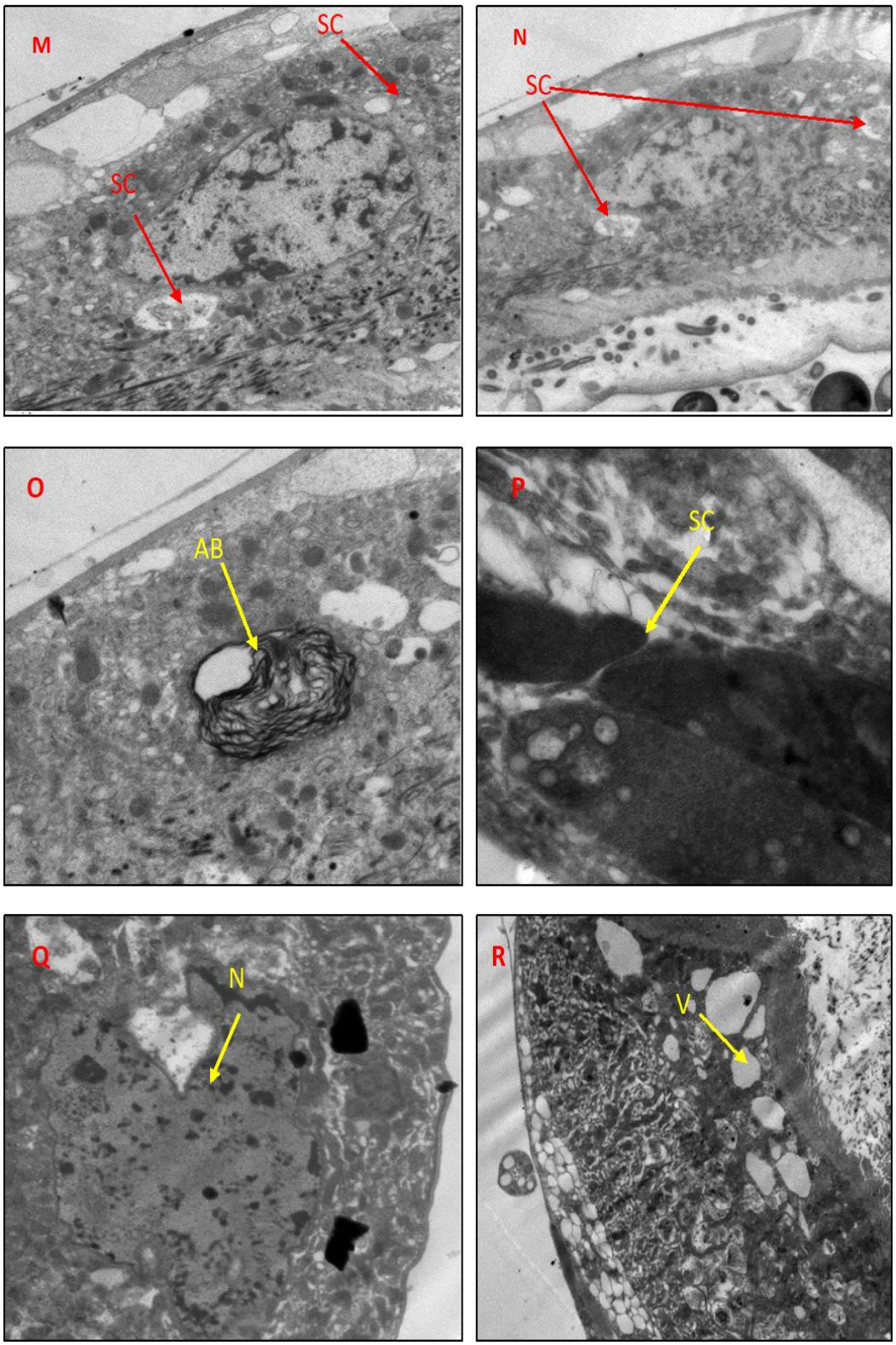

Fig. 2: T.S. of the midgut epithelium of 4th larval instar of *Cx. pipiens* mosquito**.** [A, B & C] Control showed basement membrane (BM), regular Microvilli (MV) and nucleus (N), [D to I] Treated post 2hrs showed Fibres (F)and Stem cell(SC), Microvilli (MV), Goblet Cell (GC), Mitochondria (M), Bacteria (B) & nucleus (N) and Stem cells(SC), [J & K]Treated post 10 hrs showed Epithelial cells (Epi) and Stem cells (SC), [L] Bacteria spore in the lumen, [M&N] Stem cells (SC), [O] Apoptotic body(AB)**,** Post 14h [P] Two Stem cells(SC), [Q] Two nucleus (N), [R] Vacuoles(V).

DISCUSSION

Ultrastructural modifications have been reported in epithelial cells of *Cx. pipiens* within a few hours post-treatment with soluble and activated *B. sphaericus*. Large vacuoles appear, swelling of mitochondrial cristae and endoplasmic reticula, expanding of vacuoles and condensation of the mitochondrial matrix. Physiologically, the oxygen uptake by mitochondrial and the choline acetyltransferase might be inhibited in *B. sphaericus*-treated larvae cells. A generally occurring symptom is mitochondrial swelling in *Cx. pipiens* when it is treated with a very high dose of spore/crystal complex (Davidson and Titus; 1987). The midgut cells, especially those of the posterior stomach and the gastric caeca, are the cells most severely damaged by toxins, and delayed damage in neural tissue and in skeletal muscles has been also observed (Cokmus *et al.,* 1997).

After 2 hr of *B. sphaericus* treatment, the epithelial cells began to develop a large number of vacuoles (Fig. 2b) with nuclei. The microvilli were not harmed (Fig. 2c). The presence of vacuoles in the lumen cytoplasm was discovered during the examination. The rapid digestion of *B. sphaericus* cells in the midgut could explain the early appearance of cytological disturbances after bacterial treatment (Labib and Mohamad 2003).

One of the major cytotoxic responses of Culex mosquitos to Bin intoxication was reported to be cytoplasmic vacuolization. The binary toxin (Bin) induced vacuolization in *B. sphaericus* infection. Vacuolization is a transient phenomenon that affected autolysosomes and is associated with the induction of autophagy in intoxicated cells (Opota *et al.,* 2008). Large vacuoles appeared early in the midgut cells of *Cx. pipiens*, and rough endoplasmic reticula ruptured into small vesicles. The number of vacuoles in the epithelial layer increased 10 hrs post-treatment (Fig. 3a, b). The most dramatic feature of Bin intoxication was the appearance of abnormal, clear vacuoles, indicating important cellular stress (Silva-Filha *et al.*, 1997).

Microvilli were damaged and lost in some places. Explosion in the basement membrane, which could be explained by the fact that the Cx. pipiens larval midgut was the primary target of (Bin) present in *B. sphaericus* parasporal inclusions (Darboux *et al.* 2007). Spores of Bacillus sphaericus were discovered in the lumen 10 hours after treatment (Fig. 3a). Microvilli could still be found. The bacterium began to invade the microvilli and became abundant in the lumen (Fig.3 b). Bacteria began to infiltrate the epithelial layer. The microvilli, mitochondria, and rough endoplasmic reticulum were found in the midgut epithelial cells of Bin-intoxicated mosquito larvae, but Bin toxin-induced apoptosis in vivo via an intrinsic or mitochondrial pathway with more deterioration of cells, potentially contributing to larval death (Tangsongcharoen *et al.,* 2015).

Bacteria were abundant in the lumen 14 hrs post bacterial treatment (Fig. 4a), confirming the findings of Labib and Mohamad (2003), who discovered that the number of viable spores peaked between 12 and 24 hr. Epithelial cells were killed. Microvilli were also harmed (Fig. 4d). The first step in delta-endotoxin action was thought to be binding to specific receptors on the apical microvilli membrane (Ravoahangimalala *et al.,* 1993).

The midgut infection with *B. sphaericus* has been associated with an increase in the number of nuclei and ISC in *Cx. pipiens*. According to Janeh *et al.* (2017) after bacterial damage, cell cycle activation has been observed in the midgut of *Aedes albopictus*. These stimuli increased the number of regenerative cells, allowing the midgut to restables homeostasis. Evidence suggests that delayed or immediate activation of ISC to division influences midgut infection. This activation is thought to be a tissue repair strategy.

The present study verified that the stem cells have little cytoplasm and the nuclei have condensed chromatin. The cytoplasm profiles indicate that these cells have low metabolic activity. The basal lamina supports these undifferentiated cells, and their surfaces are not

exposed to light. The massive nucleus has dispersed chromatin and a prominent nucleolus, whereas the cytoplasm lacks organelles (Janeh *et al.*, 2017). During regenerative differentiation, cells elongate towards the midgut lumen, acquiring microvilli and increasing nuclear and cytoplasmic volume. These findings were corroborated by Cruz-Landim and Vanger (2003). The dynamics of midgut epithelial cells are important at all stages of the insect lifecycle, including the end. Microbiota presence accelerates ageing by increasing the rate of gut epithelium turnover. Epithelial dynamics, manifested as cell sacrifice, have previously been shown to contribute to the bottlenecking of invading pathogens in mosquito midguts (Hixson et al., 2021). There is evidence that after infections or immune challenges, cell cycle dynamics in specific tissues, including the midgut, change, triggering regeneration and/or immune functions in order to achieve homeostasis (Maldonado *et al.*, 2019).

Conclusion:

It can be concluded that the damage of the midgut epithelium of the 4th larval instar of *Cx. pipiens* post-infection with *B. sphaericus* may have the proliferation and differentiation of stem cells in the midgut epithelium. The proliferation and differentiation of stem cells coincided with a slight increase in cell division, which could replace the destroyed epithelial cells. The ISC regenerates the architecture of the midgut epithelium and accelerates the tissue repair strategy.

Abbreviations: *B.: Bacillus; Cx.: Culex*; MV: microvilli; SC: stem cell; M: mitochondria; BM: basement membrane; N: nucleus; F: Fibres; GC: Goblet Cell; B: Bacteria; Epi: Epithelial cells; AB: Apoptotic body; V: Vacuoles.

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