Light and Electron Microscopic Study on the Development of *Nosema Bombycis* Naegeli in the Midgut of Silkworm *Bombyx Mori* L.

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Infection effect of *Nosema bombycis* on the midgut of silkworm *Bombyx mori* and subsequent appearance of spores and the performance of larvae was studied. Autopsy of larvae showed white pustules on the surface of midgut at 5 days of post infection (pi). At later stage, important organs like midgut, silk gland and gonads reduced in size and all these organs showed white pustules. Light microscope observation of pustules revealed enormous spores. Spore multiplication was at a faster rate in young larvae. Infection of the adult larvae resulted in pebrinized pupa and moths. Larval weight, cocoon weight and cocoon shell ratio reduced as the post infection period increased. Transverse sections of midgut showed *N. bombycis* infection limited to a few columnar cells at 3-5 days of pi. At 7 days pi, cell volume increased, cells were swollen and elongated. Heavily infected cells looked like sacks filled with parasite and the apical region of certain cells were bulging into the gut lumen. Later at 8-9 days of pi, spores or its developing stages leaked into the lumen either freely or enclosed within the globules of host cytoplasm. Besides columnar cells, development of *N. bombycis* was observed in the regenerative cells and rarely in goblet cells. Development of *N. bombycis* was also observed in both longitudinal and circular muscles at the late pi period. The histopathological changes, deformities and spore production time in the host were all influenced by the spore dosage and age of the host.

Key words: *Nosema bombycis*, *Bombyx mori*, Midgut, Light and electron microscope

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Introduction

The microsporidium, *Nosema bombycis* Naegeli is a serious intracellular parasite and causes a deadly disease, 'Pebrine' in silkworm, *Bombyx mori* L. The horizontal spread of the disease occurs when the silkworm ingests mulberry leaves contaminated with spores. Ingested spores germinate in the midgut due to the alkaline gut juice and initially infects the epithelial cells of the midgut, then invades and multiplies in almost all organs of the body (Thomson, 1960).

Development of the parasite is reported to have a profound effect on the tissues of the host. Damage of epithelial cells of the midgut leading to loss of functions of the digestive and excretory system due to *Nosema apis* in honeybee, *Apis mellifera* (Shabanov, 1976). Enlargement and disarrangement of cells of midgut in silkworm due to infection of *N. bombycis* has been observed under light microscope (Wafa and Kotby, 1971). Though, ultra structure of uninfected midgut is well documented in silkworm (Akai, 1970; Mathavan *et al.*, 1989), no detailed studies are reported on the infection of *N. bombycis* to the midgut and its effect on the development of silkworm and cocoon parameters. Hence the histopathological study on the midgut which is the primary site of infection will be a basis for the further studies on other microsporidians infecting silkworms.

Materials and Methods

Collection of Inoculum

Nosema bombycis infected moths were collected and homogenized in a single cup pestle and mortar. The homogenate was filtered and purified by triangulation method (Cole, 1970). The spore load was counted using Neubauer hemocytometer (Cantwell, 1974) and adjusted to 1×10^8 spores/ml.

Experimental infection

Silkworms (race CSR2) in 1st day of II, IV and V instar were per orally infected with spore load, 1×10^6 spores/larva by smearing the spore suspension on mulberry leaf and feeding to each larva. After consumption of infective feed, larvae were given normal leaf and further reared following standard rearing methods (Krishnaswamy, 1990). Dead and unequal larvae were subjected to microscopic examination and number of pebrine infected larvae were recorded. Silkworms that reached spinning stage were allowed to spin cocoons. Cocoon weight, shell weight were recorded and cocoon shell ratio was calculated.

Transmission electron microscopy

For transmission electron microscopy, silkworms were drawn at every 24 h up to 12-15 days of post infection (pi), starved for 3 h to minimize leaf content in the midgut and facilitate fixing and processing of tissues. Larvae were dissected in cold insect Ringer solution; midgut and silk gland smear was observed under light microscope and stages of the parasite were recorded. Simultaneously, dissected midgut was immediately fixed in Karnovsky's fixative for 2 h at 4°C (Karnovsky, 1965), washed in 0.1 M cacodylate buffer with two changes and stored overnight in the same buffer at 4°C. The tissue was post-fixed in 2% osmium tetroxide prepared in 0.1 M cacodylate buffer (1:1) and finally rinsed with distilled water with two to three changes at an interval of 10 min each. The specimen was dehydrated in an ascending series of alcohol and transferred to propylene-oxide and then embedded in Epon-Araldite resin mixture. Semi-thin

sections of 0.80 µm were cut using glass knife in Reichert Jung Ultra microtome (Germany). Sections were picked on to clear glass slides, stained with 1% aqueous crystal violet and photographed. Ultra thin sections of 70-80 nm were cut in the same ultra microtome using diamond/glass knife. Sections were picked on to formvar-carbon coated copper grids and stained with 3% alcoholic uranyl acetate for 45 min and Reynolds lead citrate (Reynold, 1963) for 30 min. Stained sections were observed in JEM CX-100 (Jeol, Japan) at 80 Kv and electron micrographs were taken.

Results

Gross pathology

Infected larvae did not show any external disease symptoms up to 3 days post infection (pi) but after 5 days pi, larvae showed symptoms of sluggishness, poor food intake, irregular growth, delayed moulting and gradual reduction in larval weight. Autopsy of these larvae revealed white pustules in patches on the surface of the midgut. After 8-10 days pi, the organs like silk gland, Malpighian tubules and gonads also showed white pustules. Light microscope observation of the pustules revealed enormous sporoblasts and spores.

Larvae infected in II instar showed sporoblast stage in the midgut at 3 days pi while, it took 5-7 days for the sporoblasts to appear when larvae were infected in IV and V instar. However, larvae infected in II instar and IV instar showed sporoblast stages in the silk gland after 5 and 7 days

Table 1. Effect of *N. bombycis* infection on the rearing performance of silkworm *B. mori*

Infection age of silkworm		Spore multiplication (pi days)			Larval wt. pi days (g/larva)			Mortality due to pebrine (%)			Cocoon wt. (g)	CSR (%)
		3	5	7	3	5	7	Larva	Pupa	moth		
II instar 1 st day	IC	MG	Sb ⁺⁺	Sb ⁺⁺ S ⁺⁺	0.02	0.10	0.46	100	-	-	-	-
		SG	-	Sb ⁺ S ⁺⁺								
	UC	-	-	-	0.03	0.13	0.66	0.0	0.0	0.0	1.92	18.22
IV instar 1 st day	IC	MG	-	Sb ⁺ S ⁺⁺	0.63	1.20	2.27	86	11	3	1.27	10.23
		SG	-	- Sb ⁺⁺								
	UC	-	-	-	0.66	1.35	2.80	0.0	0.0	0.0	1.92	18.22
V instar 1 st day	IC	MG	-	- Sb ⁺⁺	2.10	3.23	3.40	0.0	23	77	1.30	13.07
		SG	-	-								
	UC	-	-	-	2.11	3.50	4.00	0.0	0.0	0.0	1.92	18.22

MG-Midgut, SG-Silk gland, Sb-Sporoblast, S-Spore, IC-Infected control, UC-Uninfected control, CSR- Cocoon shell ratio, Pi-Post infection, - not observed +++ heavy, ++ medium, + low.

pi respectively and these stages were rarely seen in the silk gland when infected in the V instar (Table 1). Spore multiplication was at a faster rate if infected in the early stages of the host and larvae are more susceptible to *N. bombycis* infection. Infection at the late instars caused reduced rate of spore production during larval stage but mortality increased at the pupa and moth stages. Increase in infection period

has a direct effect on the growth and performance of silkworm hence reduction in larval weight and cocoon weight (Table 1).

Histopathology

Histology of uninfected larvae showed epithelium con-

Fig. 1. Transverse section of the uninfected midgut with compactly arranged cells. Mag.160x.

Fig. 2. Transverse section of the midgut at 5 days of post infection. Mag.160x

Fig. 3. Section of the midgut showing cells heavily loaded with different stages of *N. bombycis* at 7 days of post infection. Mag.400x.

Fig. 4. Enlarged portion of a ruptured columnar cell with spore and other stages being released into the gut lumen. Mag.1000x.

Fig. 5. Uninfected goblet cells in between the infected columnar cells. Regenerative cells with sporoblast stage of the parasite and towards the basal end infected longitudinal and circular muscles are found. Mag.1000x.

Fig. 6. An enlarged portion of the uninfected midgut towards the apical end showing microvilli. Mag.6000x.

taining columnar cells with a distinct brush border of microvilli along apical or lumen margin (Fig. 1 and 6) with numerous mitochondria, vesicular or lamellar type endoplasmic reticulum, evenly distributed free ribosomes and granules. Nucleus is centrally located with large and compact chromatin blocks (Fig. 7). The goblet cells are distributed between the columnar cells and have the apical border deeply invaginated into the cell forming a large cavity connecting the gut lumen by a narrow canal. The goblet cells have very little cytoplasm and the cavity is lined by microvilli with mitochondria (Fig. 5). The entire

Fig. 7. Uninfected midgut columnar cell cytoplasm showing ribosomes, vesicular endoplasmic reticulum, mitochondria and nucleus in the midgut. Mag.13000x.

Fig. 8. Enlarged portion of the columnar cell with *N. bombyci* in early stages of development at 5 days of pi. Mag.4000x.

epithelium rests on the thick basement membrane and is distinguishable from the cell cytoplasm. The circular muscles lie adjacent to the epithelium and longitudinal muscles are present towards the hemocoel (Fig. 5).

Fig. 9. Midgut columnar cell heavily loaded with mature stages of *N. bombycis* and depleted cell organelles. Mag.13000x.

Fig. 10. Basal end of the goblet cell with goblet cell microvilli, nucleus and spore. Mag.10000x.

Transverse section of the infected midgut showed parasite infection in only few cells at 3 days pi. Infected cell number increased at 5 days pi but, there was no change in cellular structure or in cell shape (Fig. 2), while at 7 days pi, the cell volume increased, cells were elongated and swollen. As infection progressed these cells looked like parasite filled sacks and the apical region of some cells bulging into the gut lumen was observed (Fig. 3). Further, the cellular damage increased with rupture of cells at the apical region and spore/its developing stages leaked into

Fig. 11. Enlarged portion of the infected circular muscles. Mag.10000x.

Fig. 12. Enlarged portion of the infected longitudinal muscle. Mag.15000x. Regenerative cells-RC, Columnar cell-CC, Goblet cell-GC, Goblet cell cavity- ↑ Ribosomes-R, Vesicular endoplasmic reticulum-VER, Mitochondria-M, Nucleolus-N, Nuclear membrane-Nm, Microvilli-Mv, Sporont -Sp, Sporoblast-Sb, Spore-S, Nuclear membrane- ▽.

the gut lumen, either free or globules of host cytoplasm filled with spores (Fig. 4). There was no remarkable change in the host cell cytoplasm at the early stages of *N. bombycis* development (Fig. 8) but later host cell organelles like ribosomes, mitochondria, both rough and smooth endoplasmic reticulum depleted due to the enormous multiplication of the parasite (Fig. 9). However, parasite development was not observed in any of the cell nucleus. Development of *N. bombycis* was mainly observed in the columnar and regenerative cells (Fig. 5) and rarely in the goblet cells (Fig. 10). Though parasitic stages were observed in the basal end of goblet cells, spore production rate was very low compared to columnar and regenerative cells. This is the first report on the development of *N. bombycis* in the goblet cells. Multiplication

rate and development of *N. bombycis* was enormous in both circular and longitudinal muscles (Fig. 11 and 12) and both revealed the lyses of the organelles at late pi period. The rate of infection reached about 90% or more by 8-9 days pi and infection spread to all organs in the silkworm. Damage to some of the infected cells was more severe and apparently healthy cells were also found in the immediate vicinity of those severely affected.

Discussion

The digestive system of silkworm is divided into foregut, midgut and hindgut. The cell organelles of uninfected silkworm has abundant free ribosomes, vesicular and lamellar type of RER, uniformly distributed mitochondria, microtubules etc. as described by earlier workers (Akai, 1970; Mathavan *et al.*, 1989). Midgut is the largest part of the alimentary canal and cells carry out the dual function of secretion of digestive enzyme and absorption. Nutrients are absorbed through the brush border of columnar cells where absorption includes transport across the mucosal border mixing in the cytoplasmic pool and exit from the cell across the basal membrane into the hemolymph (Sachi and Wolfersberger, 1996). Protease is an enzyme involved in digestion of leaf protein in the midgut and 96% of ingested protein in the form of nitrogen is used for silk synthesis (Fukuda, 1960). Larval weight is positively correlated with silk gland weight and this in turn is significantly correlated with shell weight (Singh and Vineet kumar, 1996).

Midgut of silkworm is also conducive for the parasite, *N. bombycis* multiplication because of the alkaline pH of the gut juice and optimum temperature for the spore germination (Wafa and Kotby, 1971). Parasite multiplies in the cytoplasm of midgut epithelial cells and produce large number of spores, which finally lead to the mechanical distention of the cells and extending into the gut lumen. Food intake in an infected silkworm gradually decreases due to damage of midgut epithelium resulting in disruption of cell structure, metabolic disturbance and hence shrinkage of larval body size, retarded growth and finally leading to death. Development of the pathogen in the midgut also has an adverse effect on the secretion of digestive enzymes like proteases and absorption of the digested food (Pathak and Sharma, 1996). Damaged epithelial cells have affected the overall growth of the larva as could be seen from the reduced larval weight and this has an effect on the cocoon weight and shell ratio. Sinha *et al.* (1991) found low amount of amino acids in the haemolymph of pebrine infected larvae of *Antheraea mylitta* compared to healthy ones and have attributed this to the utilization by

the pathogen. Pebrine disease adversely affects some of the important cocoon characters (Noamani *et al.*, 1971).

It can be inferred from the results of the present study that, production of enormous spores in the cell has probably created pressure within and as a result the cell might have ruptured at the apical end releasing spores into the gut lumen. The release of spores or spore filled globules into the lumen can be interpreted as a defense reaction of the host midgut epithelium to get rid of the parasite from its body but from the epizootiological standpoint this phenomenon would help in the fast spread of the parasite through the dispersal of contaminated faecal matter in the silkworm habitat. There is a direct correlation in the appearance of spores in the midgut and their elimination along with faecal matter (Patil *et al.*, 2001).

The present findings indicate that increase in number of parasites and their accumulation in the host cell causes a distension/increase in cell size leading to the formation of white pustules commonly and rarely hypertrophy of the cells. Hypertrophy of infected cells is reported in insects infected with *Nosema sp.* infection but not tumors (Canning and Hulls, 1970).

Further observation of healthy cells adjacent to heavily infected and damaged cells suggests that the parasite exponentially multiplies in some host cells deriving nourishment but not affecting normal functioning of the host cell up to a certain extent. This indicates that infection effect on the host by *N. bombycis* is primarily mechanical disruption of infected cell organelles to draw nourishment from the host cell but there is no sudden death of the host associated with the invasion and development of the parasite. Thus the *N. bombycis* infected host survives for a longer period and this ensures the parasite for the perpetual spread in the environment. The present study forms a basis for the future understanding of other microsporidians infecting silkworm.

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