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# Chemotherapeutic effect of benzimidazole derivatives on *Nosema bombycis naegeli* in silkworm *Bombyx mori* l -an ultrastructural study

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**Abstract:** Benzimidazole derivatives, Albendazole (Zentel), Mebendazole (Mebex), Fenbendazole (Panacur) were tested *in vivo* against the parasite *Nosema bombycis* Naegeli in silkworm *Bombyx mori* L as chemotherapeutic drugs. Administration of the drugs 24hr post infection of *N bombycis* spores increased the survival rate compared to drug untreated batches. Drug treatment suppressed parasite multiplication and further development during medication period but parasite resurged during moth stage i.e. in the non medication period. Treatment after 48hr post infection was not effective and mortality due to *N. bombycis* infection was high. Ultrastructure revealed that chemical treatment caused malformation of the merogonic and sporogonic stages; reduced the cellular organelles in the development stages of *N. bombycis* which lead to vacuolation, elongation and finally collapse of the parasitic stages viz., sporont, sporoblast and early spore stage compared to drug untreated batches. However, effect of the drug on mature spore was not predominant.

**Key words: Silkworm, *Nosema bombycis*, Benzimidazole, Ultrastructure, Cure effect**

## INTRODUCTION

*Nosema bombycis* Naegeli, a parasite causing pebrine disease in silkworm *Bombyx mori* L is a threat to sericulture industry as the parasite is transmitted within the silkworm population both transovarially and *per os*. Efforts to combat the disease in insects using chemotherapeutic drugs have been reported and suggest that various benzimidazole compounds are partially effective against *N. bombycis* as a curative drug in insects and specifically in silkworm[1-5]. Carbendazim, a benzimidazole compound is reported to be effective as a chemotherapeutic drug in controlling *N. bombycis* infection in silkworm during larval stage, but resurgence occur during non feeding stages[6] and also partially effective against other microsporidians infecting silkworm[7]. It is opined that resurgence of *N. bombycis* during moth stage is due to the resistance of the mature spore to carbendazim[8]; as the structure of mature spore includes the thick spore wall[9]. In the present investigation we have assessed the *in vivo* curative effect and ultrastructural changes against *N. bombycis* after treatment with other benzimidazole drugs that are widely used in the treatment of helminthic infection like albendazole, mebendazole and fenbendazole.

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## MATERIALAND METHODS

### Preparation of inoculum

*Nosema bombycis* spore suspension maintained in our laboratory was *per os* fed to uninfected bivoltine silkworms and reared. After 10-12 days, pebrine diseased larvae were collected, homogenized and the homogenate was filtered and centrifuged at 5000 rpm for 5 minutes. The sediment was re-suspended in distilled water and the spore suspension was purified following the triangulation method[10]. The spore load was adjusted to $1x10^6$ spores/ml using Neubauer haemocytometer[11].

**Effect of benzimidazole drugs on infected silkworm**

Mulberry leaves smeared with pebrine suspension at spore load $1x10^4$ spores/larva was fed *per os* to 100 silkworms (race $CSR_2$) on 1st day of II instar. Albendazole (Zentel), Fenbendazole (Panacur) and Mebendazole (Mebex) each in three different concentrations (0.2%, 0.4% and 0.8%) were prepared in aqueous media. After 24h post infection one ml of each prepared drug solution was smeared on mulberry leaf and fed to silkworm's once daily upto spinning. After the larvae consumed drug treated leaf, normal leaf was administered. During rearing, larvae showing symptoms of non-feeding, vomiting, body shrinkage and subsequently death were considered as an indication of chemical toxicity. Dead worms were tested to confirm mortality due to toxicity or pebrine infection.

Four different treatments were maintained- Silkworms infected drug treated (IDT), uninfected drug treated (UDT), infected drug untreated (IDU), uninfected drug untreated (UDU). Mortality due to pebrine disease was confirmed by microscopic examination during larva, pupa and moth stages. Preliminary investigation revealed that larval mortality due to chemical toxicity was least in lower dosages but were not effective in controlling the disease. Based on the result, in II batch the drug was administered depending on the age of the larva (0.4% during II to III instar and 0.8% from IV instar to spinning) (Table 1).

After confirming that the chemicals are partially effective in controlling the disease when the treatment was initiated after 24hr post infection; further, analysis of the curative effect of the drug was done at different post infection period (24h, 48h, 72h, 120h) (Table 2).

In all the experiments, treatment and control batches comprised of 100 larvae each with 3 replications. Analysis of variance (ANOVA) was done and the treatment means were compared.

**Ultrastructural study**

Silkworms at the age of II instar 1st day were infected *per os* with pebrine spore load $1x10^4$ spores/larva. After 24 h, 72 h and 96 h of post infection treatment was given once daily through mulberry leaf for a period of 12 days. Midgut was dissected on the 12th day and fixed in Karnovsky's fixative, washed in 0.1 M Cacodylate buffer, dehydrated in an ascending series of alcohol and embedded in Epon-Araldite resin following all standard proceedures[12]. Ultra thin sections of 80 nm were cut, stained with uranyl acetate and lead citrate and observed under transmission electron microscope (TEM) (make: Jeol CXII 100) at 80 kV.

## RESULTS AND DISCUSSION

In the first experiment, effect of three different chemicals albendazole, mebendazole and fenbendazole each in 0.2%, 0.4% and 0.8% dosages against pebrine disease was studied (Table 1). Larvae showing symptoms of vomiting and body shrinkage and subsequently death were considered as an indication of chemical toxicity. Mortality due to chemical toxicity if any observed during larval stage was only upto 4-5 days of initiation of chemical feeding. There was no chemical toxicity in lower concentration, 0.2% but mortality due to pebrine was high in both larval and moth stage. In 0.4% drug administered batches low mortality due to toxicity and also pebrine infection was found during larval stage, but the moths were heavily infected with *N. bombycis*. Though 0.8% chemical concentration was toxic to silkworms it was effective in controlling *N. bombycis* development when treatment was initiated at 24h post infection. Based on the results of the 1st experiment drug dosage for the 2nd experiment was fixed depending on the age of the larva i.e. 0.4% to II and III instar and 0.8% in IV and V instar and drug treatment initiated after different post infection period (Table 2). Albendazole and mebendazole were effective in controlling the pebrine disease completely during larval stage when drug administration was initiated at 24h post infection but low (19% and 14% respectively) percentage of moths was found infected. However, the number of infected larvae and moths increased significantly when the administration of these two drugs were initiated at subsequent period of post infection (48 hr to 72hr); indicating that delayed treatment cause high infection both in the larva and moth stage and the parasite resurged during the non feeding stage of silkworm i.e. pupa and moth. The improved body weight and survival rate in chemical treated batches suggest that compared to untreated batches, chemical treatment even at low concentration (0.2%) are effective against *N. bombycis*. Among the

chemicals treated, the survival rate was high in batches treated with albendazole and mebendazole.

Complete cure of pebrine disease was not achieved probably due to the faster multiplication rate of the parasite than the cure rate of the chemical. Incomplete cure effect of *N. bombycis* in transovarially infected silkworms treated with mebendazole has been reported[1]. It is reported that after benomyl treatment small isolated centers of microsporidian stages in various tissues apparently resulted in renewed development during non feeding stages[13]. Benzimidazoles are effective in minimizing the microsporidiosis to significant extent in silkworm[7].

Ultrastructural studies revealed that under drug untreated condition the developmental stages of the parasite *N. bombycis* viz., sporont, sporoblast and spore posses abundant free ribosomes, lamellar type rough endoplasmic reticulum, diplokaryotic nuclei and neat and parallely arranged polar filament coils around the posterior vacuole (Fig.1-3). However, the drug treatment caused severe damage to the merogonic and sporogonic stages of *N. bombycis* in the midgut (Fig. 4-9). All the chemical treated batches caused depletion of the ribosomes, endoplasmic reticulum and other cell organelles of the parasite and deformation of the polar filament leading to vacuolation, elongation and finally collapse of sporont, sporoblast and early spore stages, however changes in the mature spore structure was not predominant except the pitted spore coat.

The only infective stage of parasite is the environmentally resistant spore, which has the protein rich spore wall that supposedly contributes to their infection and resistance[14]. This indicates that the early developmental stages are more vulnerable to the chemical treatment compared to mature spores, probably due to the protective spore coat of the spore that comprises of proteinaceous exospore and chitinous endospore that may become impermissible to the drugs[9]. Similarly it is showed that prevention of microtubule assembly will inhibit the intranuclear spindle formation in *Glugea anamola* by benzimidazole derivatives[15] and polar filament deformation in *N. apis* due to itraconazole[16].

The present study indicates that mebendazole and albendazole treatment is effective compared to fenbendazole and has a potential for destruction of intracellular developmental stages of *N. bombycis*. However, these drugs only partially contribute to the cure

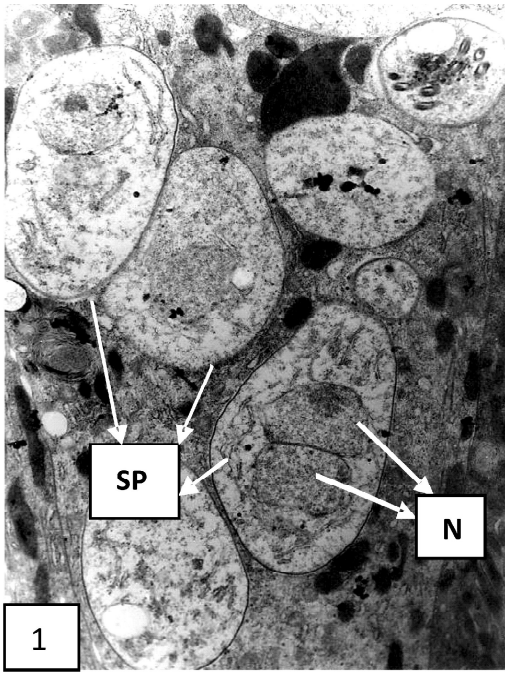


Fig.1:Stages of *N. bombycis* under chemical untreated condition. 1 Sporont stage with diplokaryotic nuclei. (Mag. X13,000)

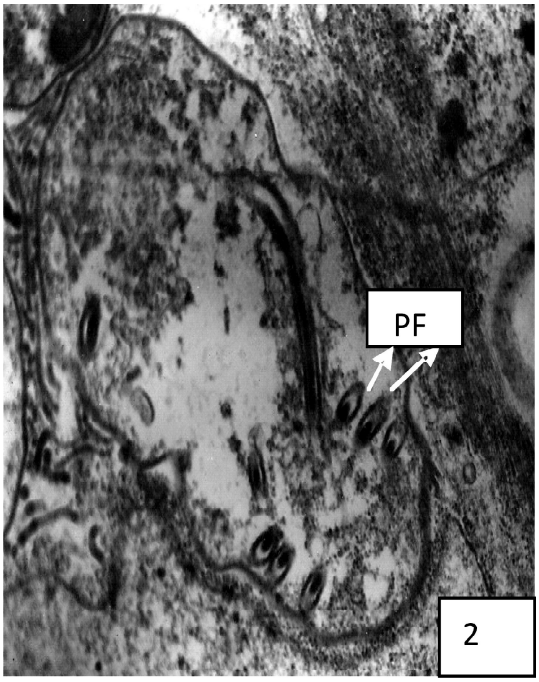


Fig.2: Stages of *N. bombycis* under chemical untreated condition. Early sporoblast with polar filament coils. (Mag. X24,000)

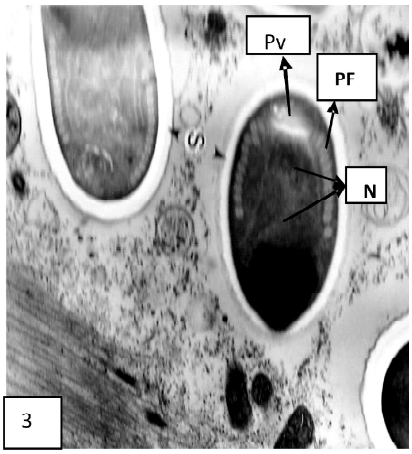


Fig.3:Stages of *N. bombycis* under chemical untreated condition.Mature binucleated spore with clearly visible polar filament coils (Mag. X22,000).

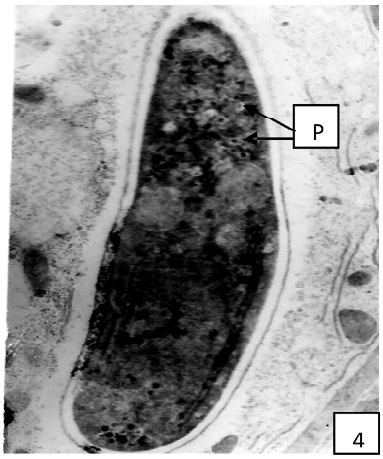


Fig.4:Note the vacuolation, elongation and collapse of the parasitic stages due to albendazole treatment. Elongated and pitted sporont (Mag. X22,000)

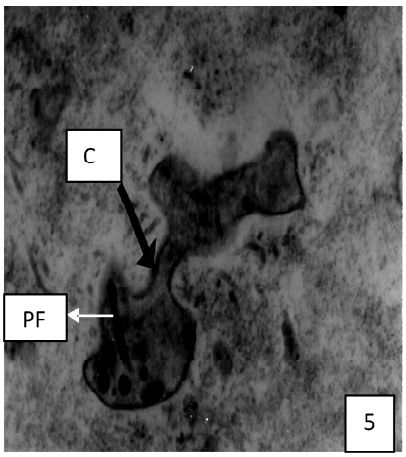


Fig.5:Note the vacuolation, elongation and collapse of the parasitic stages due to albendazole treatment.

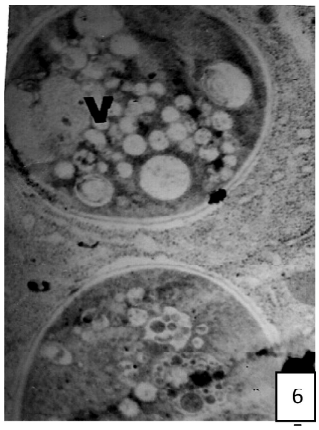


Fig.6:Note the vacuolation, elongation of the sporont and whorls within the parasite due to mebendazole treatment. Fig. 6 Vacuolated sporont (Mag. X20,000)

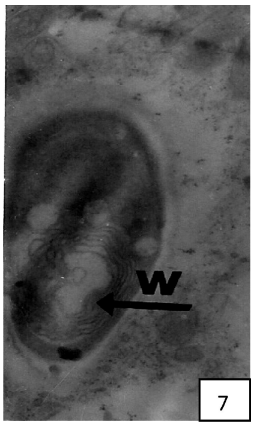


**Fig.6:**Note the vacuolation, elongation of the sporont and whorls within the parasite due to mebendazole treatment. Vacuolated sporont with whorls (Mag. X35,000)

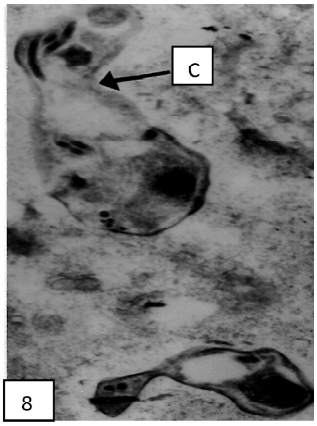


**Fig.8:**Note the vacuolation, collapse and distortion of the parasite shape due to fenbendazole treatment. Disrupted polar filament coils, vacuolation and shrinking of the sporoblast. (Mag. X7,000).

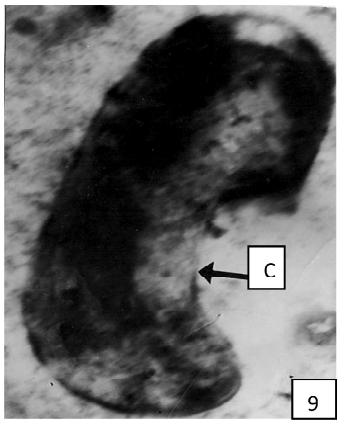


**Fig.9:**Note the vacuolation, collapse and distortion of the parasite shape due to fenbendazole treatment. Collapse of the early spore (Mag.X11,000).

of the disease in silkworm as the chemically unaffected mature spores multiply in the moth stage i.e. the non medication period and hence low infection appear. A drug can be considered effective only if complete cure of the disease is achieved.

## REFERENCES


1. **Chandra A K & Sahakundu A K. 1983**. The effect of drug on pebrine infection in *Bombyx mori* L. *Indian J Seric*. 21& 22, 67-69.
2. **Ditrich O, Kucerova Z & Koudela B. 1994.** *In vitro* sensitivity of *Encephalitozoon Cuniculi* and *E. hellen* to albendazole. *J. Eukaryotic Microbiol* 41, 109. Workshop on Opportunistic Protists.
3. **Haque A M D, Hollister W S, Willcox A & Canning E U. 1993**. Antimicrosporidial activity of albendazole. *J Invertebr Pathol.* 62, 171-177.
4. **Hsiao T H & Hisao C. 1973**. Benolmyl a novel drug for controlling a microsporidian disease of the alfa alfa weevil. *J Invertebr Pathol*. 22 , 303-304.
5. **Sahay, A, Sahay, D. N., Roy, D. K., Singh, G. P., Roy, G.C., Suryanarayana, N. 2008.** Efficacy of evolved drug formulation and Metronidazole against pebrine disease in tasar silkworm *Antharaea mylitta* D; in *Proceedings of the National Workshop on Recent Advances in Tasar culture*, pp115-117, Warangal, India,.
6. **Liu S X. 1987.** The effect of chemotherapy on pebrine disease of silkworm, *Bombyx mori*. *Sericologia*, 27, 405-410.
7. **Bhat, S. A., Nataraju &, Ifat Bashir, 2012**. Efficacy of different benzimidazole derivatives on microsporidiosis of Lamerin breed of the silkworm, *Bombyx mori* L. *J Entomol and Nematol*. 4, 12-14.
8. **Jyothi, N.B., Patil, C. S. & Dass, C.M.S. 2005.** Action of carbendazim on the development of *Nosema bombycis* Naegeli in silkworm *Bombyx mori* L, *J Applied Entomol.* 129, 205-211.
9. **Vavra J & Larsson J. 1999.** Structure of the microsporidia; in *The microsporidian and miscrosporidiosis*. Whittner M, Weiss L.M. (eds.). ASM Press Washington. D.C. 7-84.
10. **Cole R J. 1970.** The application of the triangulation method to the purification of *Nosema* spores from insect tissues. *J Invertebr Pathol.*15, 193-195.
11. **Cantwell G E. 1974.** Honeybee diseases, parasite and pests; in *Insect diseases*. G E Cantwell (ed.), pp 501-547, Marcel Dekker Vol. II, New York.
12. **Jyothi N B. 2003**. Studies on pebrine disease caused by *Nosema bombycis* Naegeli and its effect on the development of the host *Bombyx mori* L. Ph.D. thesis, Bangalore University, Bangalore.
13. **Brooks, W.M., Cranford, T.D. & Pearce, C.W. 1978**. Benomyl: Effectiveness against the microsporidian *Nosema heliothidis* in the corn earworm *Heliothis zea. J Invertebr Pathol*. 31, 239-245.
14. **Tu, Z., Liu, Q. & Wan, Y. 2011**. Reduced infectivity and environmental resistance of microsporidian *N. bombycis* after spore wall proteins- removal treatment, *Am J Microbiol Res.* 5, 5319-5324.
15. **Schmahl, G. & Benini, J. 1998.** Treatment of fish parasites. 11. Effects of different benzimidazole derivatives (albendazole, mebendazole, fenbendazole) on *Glugea anomala*, Moniez, 1887 (Microsporidia): ultrastructural aspects and efficacy studies. *Parasitol Res.* 84, 41-49.
16. **Liu, T.P. & Myrick, J.R. 1989.** Deformities in the spore of *Nosema apis* as induced by itraconazole treatment, *Parasitol Res*. 75, 498-502.


## LEGEND FOR FIGURES

**Fig.1 to 3:** Stages of *N. bombycis* under chemical untreated condition. Fig. 1 Sporont stage with diplokaryotic nuclei. (Mag. X13,000). Fig. 2 Early sporoblast with polar filament coils. (Mag. X24,000). Fig. 3 Mature binucleated spore with clearly visible polar filament coils (Mag. X22,000).

**Fig. 4 to 5:** Note the vacuolation, elongation and collapse of the parasitic stages due to albendazole treatment. Fig.4 Elongated and pitted sporont (Mag. X22,000), Fig. 5 Collapsed sporoblast with disrupted polar filament (Mag. X8,000, )

**Fig. 6 to 7:** Note the vacuolation, elongation of the sporont and whorls within the parasite due to mebendazole treatment. Fig. 6 Vacuolated sporont (Mag. X20,000). Fig. 7 Vacuolated sporont with whorls (Mag. X35,000).

**Fig.8 to 9:** Note the vacuolation, collapse and distortion of the parasite shape due to fenbendazole treatment. Fig. 8 Disrupted polar filament coils, vacuolation and shrinking of the sporoblast. (Mag. X7,000). Fig. 9 Collapse of the early spore (Mag.X11,000).

V-Vacuolation, C-Collapse**,** W-Whorls, PF-Polar filament coils, N- Nucleus, P-Pitted, Sp-Sporont, S-Spore, Pv-Posterior vacuole.

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