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Nosema bombycis (Microsporidia) suppresses apoptosis in *Bm*N cells (*Bombyx mori*)

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Abstract

Nosema bombycis (*N. bombycis*, Nb) a f g -e ated a d b gate t ace a a a te t at ca e c c eb e d ea e t e . Afte fect g t e t, e bta e e g f t ce a d e f e e a da . T b bet ee t e at ge a d t e t ce gge t t at *N. bombycis* e e t a t a d eact e ge ec e (ROS) d ct f t ce t c eate t e t a e e ta c d t f t g t a d de e e t. I t t d , d ffe e t et d e e ed t e t at *N. bombycis* e ed a t *Bm*N ce . F c t et a a e t ed t at e e ed a t f *Bm*N ce at 2 a d 5 da afte fect (*P* < 0.05). C a ed t act c D (ActD) t eat e t, a t f *Bm*N ce a a a e t ed ced afte e fect (*P* < 0.01). F t -e g t afte fect , t e ROS d ct f *Bm*N ce a d -eg ated c a ed t t at afte ActD t eat e t f 6 . F t e e, *N. bombycis* e e ted t e f at f a t e b d -eg at g t e e e f *apaf-1* a d *cytochrome C*. I add t , *N. bombycis* a -eg ated t e e e f *buffy*. We te b t a de t ated t at e dec ea ed t e e e f t c t c e C at 48 a d 98 t fect . T , e t gge ted t at *N. bombycis* b ted t e t c d a a t t c at a f t e t ce t c eate a t a e e t f t a.

Key words: *Nosema bombycis*, apoptosis, ROS, inhibition, cytochrome C

Introduction

Microsporidia are a group of intracellular pathogens that can invade a variety of hosts ranging from protists to mammals. Infection by microsporidia can be disastrous to the production of insects, livestock, fish, and shellfish [1–3], resulting in huge economic loss to relevant industries. Furthermore, microsporidia are opportunistic human pathogens that can cause long-term diarrhea in children and the elderly (especially in developing countries) and infect immunocompromised patients [4]. The general life cycle of microsporidia has three phases: the infective phase, proliferative phase, and spore-forming phase [5]. The infective phase is the only stage during which microsporidia can live outside of host cells. Currently, many cell lines can provide a suitable environment for microsporidia to grow and develop. For example, *Nosema ceranae* and *Nosema apis* can multiply in IPL-LD-65Y cells [6]. *Encephalitozoon* has been shown to infect three cell lines *in vitro* [7]. An important aspect of the pathogenesis of intracellular parasites is their ability to manipulate the activity of host cells for their own benefit. As obligate intracellular parasites, microsporidia have a skilled and sophisticated mechanism for infecting host cells without being recognized by the host defense system [8]. Microsporidia can live within host cells for several days and modulate the host cell cycle and apoptosis.

In multicellular organisms, the balance between cell death and cell growth determines homeostasis. However, the dysregulation of cell death mechanisms was involved in the pathogenesis of an increasing number of diseases. Apoptosis is an evolutionarily conserved and universal process. Lepidoptera insects have similar apoptosis signal pathways to mammalian cells. The two mechanisms of apoptosis are the intrinsic and extrinsic pathways, which both principally regulate caspase activation. The intrinsic pathway causes the activation of caspases that are regulated by the convergence of signals at the mitochondrion, such as those mediated by the Bcl-2 family of proteins. These signals lead to the release of cytochrome C from the intermembrane space of the mitochondria to the cytosol, where it interacts with the apoptosome, a large complex containing procaspase-9, apaf-1, and dATP, to activate caspase-9 [9,10]. *Bombyx mori* is a representative Lepidoptera that has important economic and scientific value. Extensive research on apoptosis in silkworm has been performed mainly focusing on two aspects: the morphological changes in tissues and cells during apoptosis induced by extrinsic factors [11

RNA extraction and quantitative real-time reverse polymerase chain reaction analysis of apoptosis genes

Total RNA was isolated from control and treated cells at 48 hpi using Trizol reagent (SANGON, Shanghai, China) according to the manufacturer's instructions. cDNA molecules were generated from the total RNA using the GoScript™ Reverse Transcription System (Promega, Madison, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the 480 fluorescence quantitative PCR Detection System (Roche, Basel, Switzerland). The PCR reaction included an inactivation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 15 s. A dissociation curve was generated at the end of each PCR cycle to verify single-product amplification. Three independent duplicate experiments were performed for each of the datasets. mRNA was quantified using the comparative threshold cycle (Ct) method [34]. The *BmRP49* gene was used as the internal control. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated as the fold change in the expression level from that of the control gene. All primers are listed in Table 1 [35].

Western blot analysis of cytochrome C

The control and infected *Bm*N cells were harvested by low speed centrifugation, and the pellet was washed with PBS, re-suspended in 100 μl of lysis buffer (20 mM HEPES-KOH; pH 7.5, 10 mM KCl, 1.5 mM PMSF, 1 mM DTT, and 250 mM sucrose) and homogenized in a Dounce homogenizer, and centrifuged twice at 12,000 rpm for 10 min at 4°C. The supernatant was determined using the BCA kit (SANGON), and equal amounts of protein were electrophoresed on a 12% SDS gel and then subject to western blot analysis. Mouse anti-cytochrome C monoclonal antibody (BD-Pharmingen) was used as the primary antibody, and the blot was finally developed using an ECL kit (Thermo Fisher Scientific, Waltham, USA). An antibody against β-tubulin (provided by Dr Wang Huabin from Zhejiang University) was used as the loading control.

Statistical analysis

All assays were performed in triplicate under identical conditions, and all data were presented as the mean ± SD. Comparisons between multiple groups were performed using one-way ANOVA followed by Student's unpaired *t*-test. A *P* value of <0.05 was considered statistically significant. All calculations were performed using the Statistical Package for the Social Sciences, version 13.0 (SPSS, Chicago, USA).

Results

Nosema bombycis inhibits ActD-induced *Bm*N cell death

DNA fragmentation is a characteristic of cells undergoing apoptosis [36]. To confirm that *N. bombycis* can reduce apoptosis in *Bm*N cells, cellular DNA from *Bm*N cells that had undergone various treatments was isolated and analyzed by agarose gel electrophoresis. A significant increase in DNA fragmentation was found in uninfected *Bm*N cells that had been treated with ActD, suggesting that characteristic DNA fragmentation occurred in the cells treated with ActD (**Fig. 1**, lane 2). However, the level of DNA fragmentation in the infected *Bm*N cells remained low compared with that of the uninfected and was not obviously different from that in the control cells (**Fig. 1**, lane 3).

Nosema bombycis reduced apoptotic cell ratio of ActD-induced *Bm*N cells

At various time points after inoculation with *N. bombycis*, the apoptosis ratios were detected by flow cytometry. As shown in **Fig. 2A,B**, the early apoptosis ratios of the control cells were 7.97% ± 0.58% and 24.02% ± 0.24%, respectively, at day 2 and day 5, while the early apoptosis ratios of the treated cells were 4.61% ± 0.06% and 19.32% ± 0.48%, respectively, at day 2 and day 5. The apoptosis ratio was dramatically decreased by *N. bombycis* infection ($P < 0.01$). The early apoptosis ratio of *Bm*N cells induced by ActD treatment was significantly up-regulated compared with that of the control ($P < 0.01$), and the apoptosis ratio was significantly down-regulated by infection with *N. bombycis* (**Fig. 2C**; $P < 0.01$).

Nosema bombycis reduced ROS production of ActD-induced *Bm*N cells

To determine whether ROS production was involved in ActD-mediated cell apoptosis, cellular ROS was detected using an ROS-sensitive fluorometric probe, DCFH-DA. As shown in **Fig. 3C**, a sudden increase of ROS production was observed as a bright fluorescent signal within the *Bm*N cells after 6 h of exposure to 200 ng/ml ActD. The ROS production was significantly increased compared

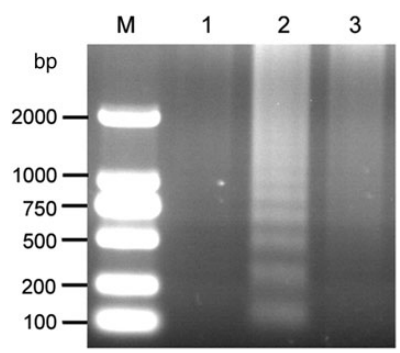

Figure 1. Inhibition of ActD-induced apoptosis of *Bm*N cells by infection with *N. bombycis* Lane M: marker; lane 1: control cells; lane 2: ActD-treated cells; lane 3: *Bm*N cells infected with *N. bombycis*.

Table 1. Primers used in qRT-PCR

Gene	GenBank number	Sequence
apaf-1	NM_001200008	F: 5′-TATGCTGCGTCCCCTG-3′ R: 5′-GTGCCATTATCTCGTTTTG-3′
buffy	NW_004582072	F: 5′-GCTATGTGCGGCGTTGGAG-3′ R: 5′-CCCTTGTGACCCGTCTTGC-3′
cytochrome C	NW_004582024	F: 5′-TCATACTCCGATGCCAATA-3′ R: 5′-TAGGCAATAAGGTCAGCAC-3′
BmRP49	NW_004624556.1	F: 5′-CAGGCGGTTCAAGGGTCAATAC-3′ R: 5′-TGCTGGGCTCTTTCCACGA-3′

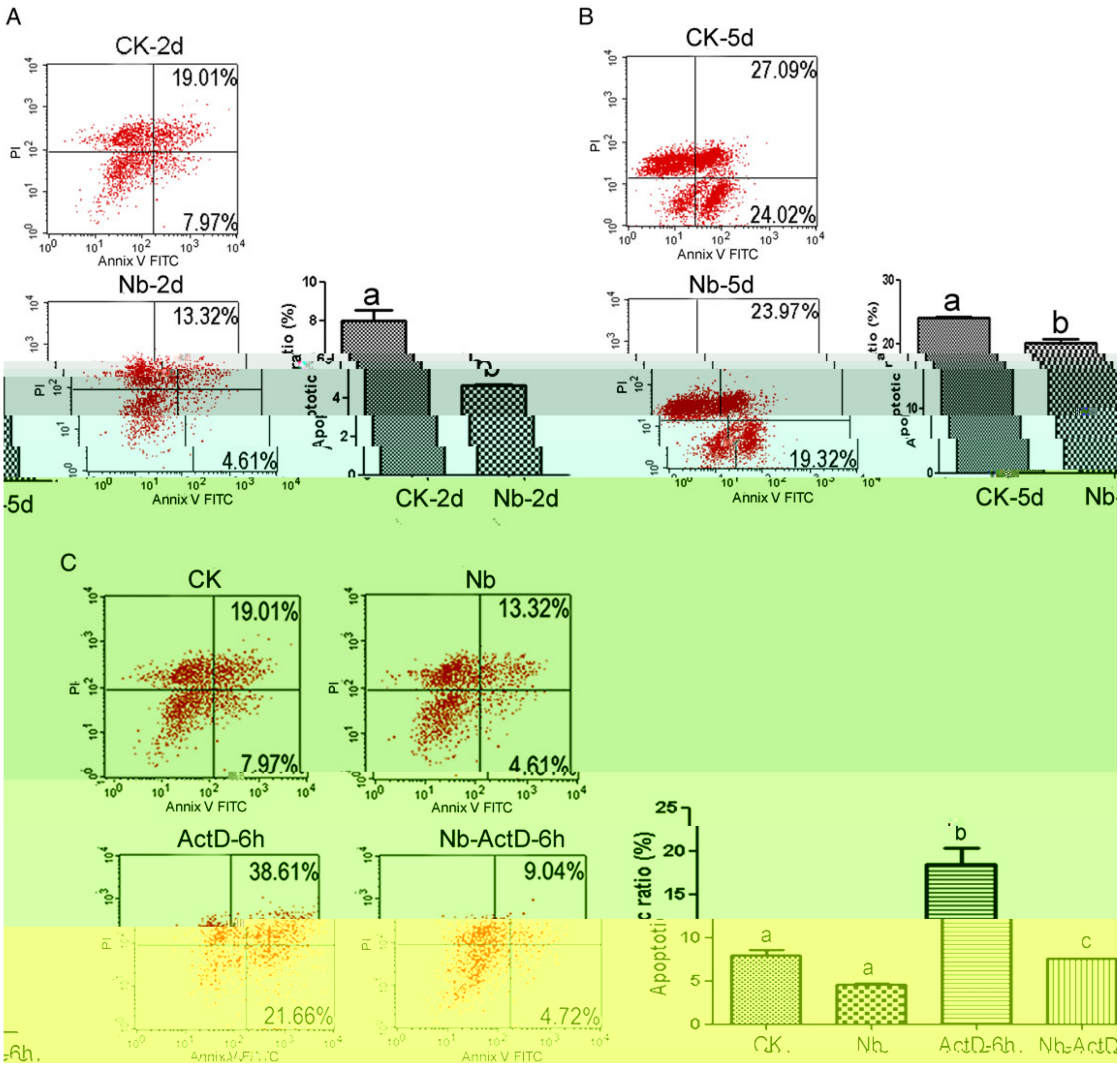

Figure 2. Apoptosis of *Bm*N cells determined by flow cytometry (A) Ap pt   at   f c t *Bm*N ce   a d *N. bombycis*- fected *Bm*N ce   at 2 da   afte   fect . (B) Ap pt   at   f c t *Bm*N ce   a d *N. bombycis*- fected *Bm*N ce   at 5 da   afte   fect . (C) Ap pt   at   f c t *Bm*N ce , *Bm*N ce   fected   t *N. bombycis* f 2 da , *Bm*N ce   treated   t ActD f 6   a d *Bm*N ce   fected   t *N. bombycis* f 2 da   a d t e treated   t ActD f 6 . A   a p e   e e ea ed   g fl   c t   et   t d   be ta   g f A e V-FITC a d PI. D ffe e t ette   e e g fica t   c a ged, $P < 0.05$.

with the control group (**Fig. 3A**; $P < 0.05$). At 48 hpi with *N. bombycis*, the ROS production of *Bm*N cells drastically decreased to 38.5% of that of the control group (**Fig. 3B**; $P < 0.05$). The fluorescence microscopy results suggested that *N. bombycis* decreased ROS production in *Bm*N cells challenged with 200 ng/ml ActD (**Fig. 3D**). The number of *Bm*N cells which increased ROS production was calculated (**Fig. 3E**).

Nosema bombycis ad   ted   RNA e p e   f ap pt   -e ated ge e   *Bm*N ce

The mRNA expression of the *B. mori* adaptor protein apaf-1 which belongs to the WD40 superfamily was examined. It contains caspase recruitment domain and nucleotide-binding adaptor domains, which shares high similarity with *Drosophila* Dark, a key component of the *Drosophila* apoptosis machinery [37]. *Buffy* is a Bcl-2 family homolog gene that participates in a crucial point of anti-apoptotic pathways and has at least one of four BH domains (BH1, BH2, BH3, or BH4) [20]. As determined by qRT-PCR analysis, the mRNA levels of the apoptosis genes (*apaf-1* and *cytochrome* C) were down-regulated and the anti-apoptosis gene (*buffy*) was up-regulated after infection with *N. bombycis* compared with those after ActD treatment (**Fig. 4**). In other words, *N. bombycis* reversed the expression pattern of these genes and changed the apoptosis of the host cells.

Nosema bombycis red ced c t c   e C e ea   g b   e te   b t a a

In the intrinsic pathway of apoptosis, mitochondria sense catastrophic cellular changes and irreversibly commit cells to apoptosis by releasing death factors such as cytochrome C. To elucidate the mechanism of

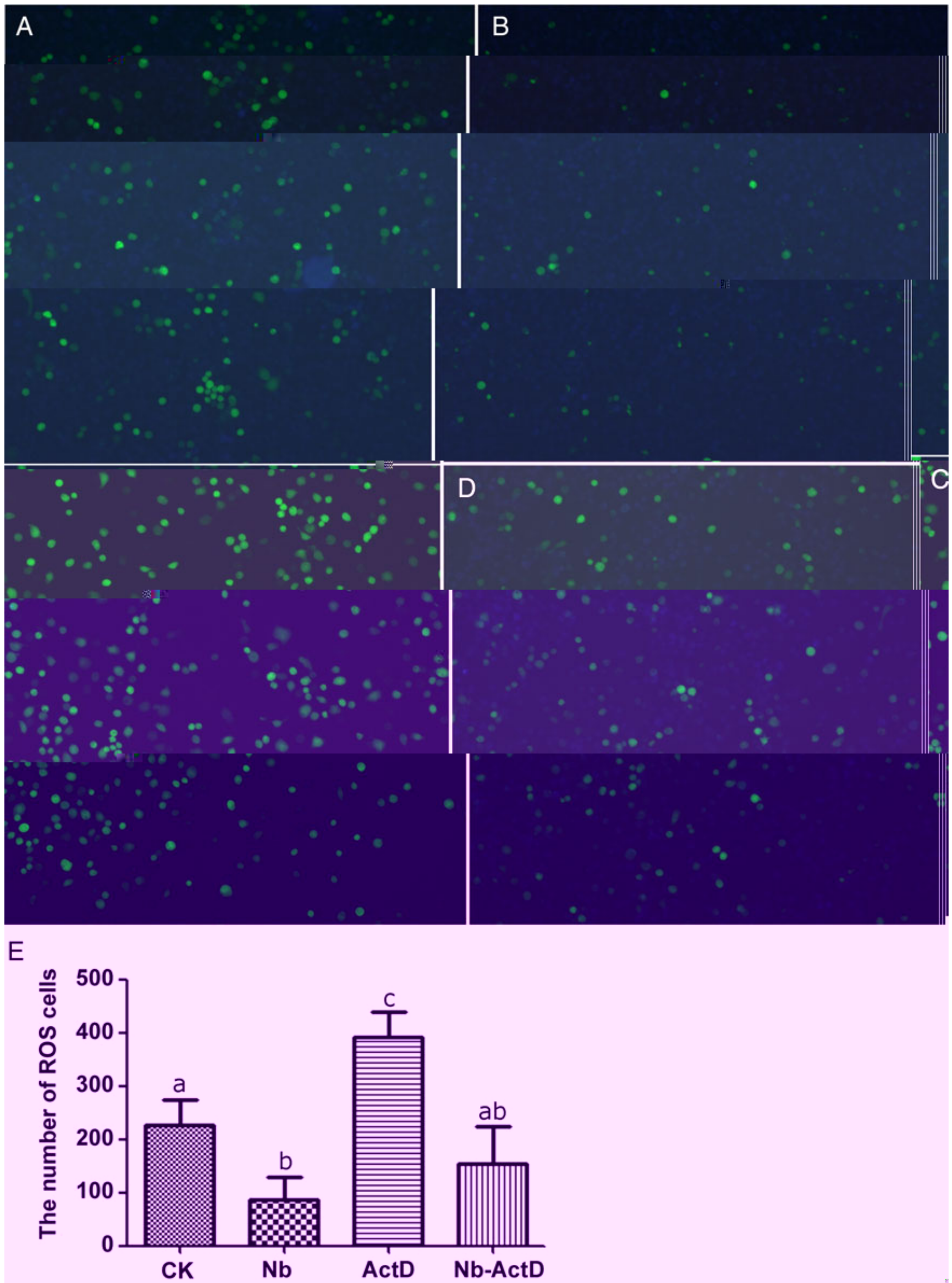

Figure 3. ActD-induced increase of ROS production in *Bm*N cells *Bm*N ce e e . c bated t 200. g/ ActD a d e a . ed f ROS d ct. . b DCFH-DA.

action of *N. bombycis*, the content of cytochrome C, an important factor in the apoptosis pathway, was detected by western blot analysis. Cytochrome C was down-regulated at 48 and 96 hpi, particularly at 48 hpi, after infection with different doses of spores (**Fig.** 5). These results verified that *N. bombycis* suppressed host cell apoptosis by adjusting the level of cytochrome C to intervene with the formation of the apoptosome.

Discussion

Intracellular parasites (viruses, bacteria, fungi, and protozoa) invade cells to exploit host's resources and reproduce, which usually causes death of the host cell in the process. Apoptosis has been recognized as an important defense mechanism against pathogen invasion [22]. Conversely, some intracellular parasites have developed a variety of strategies to evade this host defense mechanism and manipulate the host for their own benefit [28,38–42]. Inhibiting apoptosis appears to be a common mechanism used by microsporidia [27,28].

As an obligate intracellular pathogen, *N. bombycis* depends on its hosts for replication. Thus, *N. bombycis* needs to manipulate host cells and prevent host cell apoptosis. In this study, our results demonstrated that *N. bombycis* inhibits apoptosis by preventing the activation of the lepidopteran mitochondrial signaling pathway. Like other types of

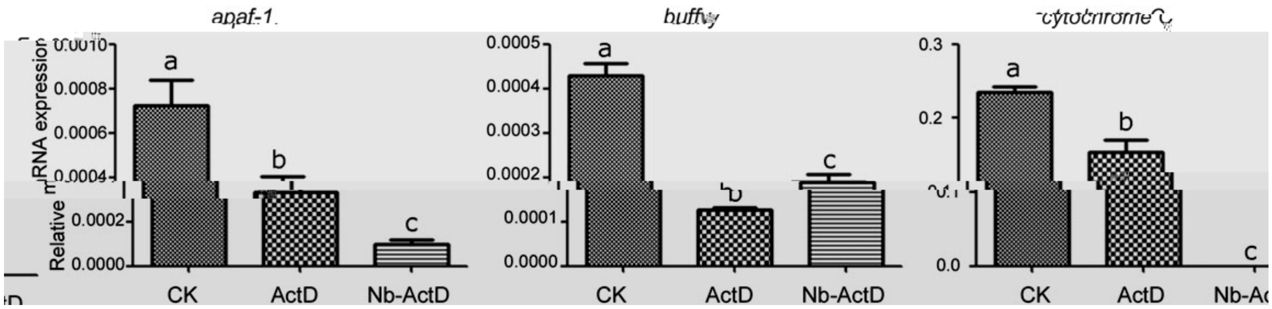

Figure 4. The relative expression of apoptotic genes and anti-apoptosis gene determined by qRT-PCR A t t c ge e e e f ce de ActD t eat e t a d *N. bombycis* fect (CK: c t ; ActD: t eated t ActD ; Nb-ActD: t eated t ActD afte fect Nb 2 da) e e detected b RT-PCR. T ee e cate e e e t e e c d cted. D ffe e t ette e e g fica t c a ged, $P < 0.05$.

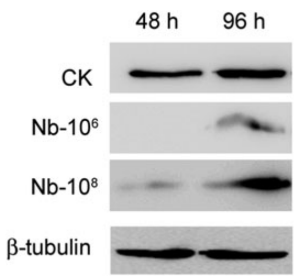

Figure 5. Western blot analysis of cytochrome C Ce t eated t d ffe e t d e f *N. bombycis* ed dec ea ed e e f c t c e C at 48 a d 96 . β-T b a ed a a te a c t . NB-10^6 a d NB-10^8 e e t e be f *N. bombycis*.

lepidopteran insect cells [43,44], after incubation with ActD, uninfected *Bm*N cells detach from the substrate, become blebby (data not shown) and undergo DNA fragmentation. According to the results of flow cytometry, the early apoptotic ratio of infected cells was decreased compared with that of uninfected *Bm*N cells. Moreover, *N. bombycis* distinctly down-regulated apoptosis after ActD treatment. Meanwhile, the low levels of DNA fragmentation in infected cells indicated that infection with *N. bombycis* does not induce host apoptosis. Similarly, *N. algerae* was previously shown to decrease the susceptibility of human lung fibroblasts (HLFs) to apoptosis [27].

Furthermore, the expression of the anti-apoptotic gene *buffy* was up-regulated by inoculation with *N. bombycis*. It has been reported that *N. algerae* infection adjusts the Bcl-2/Bax expression ratio in HLFs [27]. Both *N. bombycis* and *N. algerae* have diplokaryotic nuclei and have direct contact with cytoplasmic components. For the two parasites, the mechanism of inhibiting apoptosis might both rely on Bcl-2.

Apoptosis is initiated by a variety of stimuli, including binding of receptors to death ligands, irradiation, cellular stress, etc. [20,45]. Transduction of these pro-apoptotic stimuli via different signaling pathways results in the activation of a family of cysteine proteases, called caspases that are the central component of the apoptotic machinery [46]. Cytochrome C plays an essential role in lepidopteran cell apoptosis [47]. Our results demonstrated that in *N. bombycis*-infected *Bm*N cells, the protein levels of cytochrome C and the gene levels of *apaf-1* and *cytochrome C* were significantly inhibited during the infection. Some of these mechanisms have been previously described for *N. ceranae*, such as inhibition of the expressions of immune-peptides and immune-related genes [48], reduced re-epithelization of infected ventriculi [49,50] and induction of increased energetic stress [51,52]. Modulation of p-53-mediated apoptosis by *Encephalitozoon* spp. has been described in infected cell cultures [28].

It has been reported that excessive ROS induces apoptosis in inflammatory cells and other types of cell [53–55]. Our previous work demonstrated that the expressions of proteins involved in oxidative stress were up-regulated during the infection phase [56]. Induction of apoptosis of *Bm*N cells by ActD treatment involves up-regulation of ROS production, which is decreased in cells infected with *N. bombycis*. Thus, the production of ROS and its associated apoptosis potential must be tightly regulated by this pathogen. The adaptation of the parasitic strategy has led to a number of profound changes that result in a seemingly paradoxical mixture of parasitic characteristics [26]. The results of this study suggested that *N. bombycis* adjusts the apoptotic machinery of host cells by increasing the expression of anti-apoptotic genes and proteins and decreasing the expression of pro-apoptotic genes and proteins of the host cells. Further studies will be necessary to determine whether microsporidia share common genetic pathways to inhibit apoptosis.

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