

Original Article

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

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Abstract

Nosema bombycis (*N. bombycis*, Nb) is a fungus-related and obligate intracellular parasite that causes chronic pebrine disease in the silkworm. After infecting the host, spores obtain energy from host cells and survive for several days. This symbiosis between the pathogen and the host cell suggests that *N. bombycis* prevents apoptosis and reactive oxygen species (ROS) production of host cells to create the optimal environmental conditions for its growth and development. In this study, different methods were used to prove that *N. bombycis* suppressed apoptosis in *BmN* cells. Flow cytometry analysis results showed that spores suppressed apoptosis of *BmN* cells at 2 and 5 days after infection ($P < 0.05$). Compared with actinomycin D (ActD) treatment, apoptosis of *BmN* cells was apparently reduced after spore infection ($P < 0.01$). Forty-eight hours after infection, the ROS production of *BmN* cells was down-regulated compared with that after ActD treatment for 6 h. Furthermore, *N. bombycis* prevented the formation of apoptosomes by down-regulating the expression of *apaf-1* and *cytochrome C*. In addition, *N. bombycis* also up-regulated the expression of *buffy*. Western blot analysis demonstrated that spores decreased the level of host cytochrome C at 48 and 98 h post infection. Thus, our results suggested that *N. bombycis* inhibited the mitochondrial apoptotic pathway of the host cells to create an optimal environment for its own survival.

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 [11–14] (such as ecdysone and hemolymph), intrinsic factors [15–17] [such as actinomycin D (ActD), ultraviolet light, and viruses], and apoptosis-related genes and proteins, which have been cloned and identified [18–20]. Cytochrome C is released into the cytoplasm in stress-induced apoptotic *Sf-9* and *BmE* cells, indicating that the mitochondrial apoptotic pathway functions in Lepidoptera [21].

Apoptosis has been recognized as an important defense mechanism against viral, bacterial, and parasitic pathogens [22,23]. However, pathogens have developed various strategies to manipulate the activity of host cells involved in normal cell proliferation, cell development, and cell death. Pathogens can up-regulate or down-regulate host cell apoptosis [24,25]. Microsporidia appear to modify the host cell cycle by inhibiting host cell apoptosis. During evolution, these organisms developed simplified organelle structures with no typical mitochondria or Golgi. And they decreased the size of their genomes and reduced the number of biochemical pathways encoded in their genomes. All these features resulted in dependence on the host cell's physiology for the parasite to develop [26]. *Nosema algerae* can grow and develop within the host cell for several days without inducing apoptosis [27]. *Encephalitozoon* spp. is able to multiply in the host cell without activating the p53 apoptotic pathway in infected cultured cells [28]. Thus, by attenuating host cell death, parasites can optimize their environment and extend the period during which they can grow and differentiate within the host cells [27]. *Nosema bombycis* causes silkworm pebrine and significant economic losses for the silk industry due to horizontal transmission and vertical transmission. During the developmental stages, *N. bombycis* has direct contact with the host cytoplasm for several days, and the infected cells maintain normal architecture and appearance during this period. This tolerance of the parasite by the host cells indicates that the parasite may inhibit the host's apoptosis pathways to allow its own proliferation. In the present study, the effects of *N. bombycis* infection on the death of host cells were investigated. The apoptosis of *BmN* cells infected with spores was detected and compared with that of uninfected controls. Fragmented DNA was detected, and the early apoptosis ratio, the reactive oxygen species (ROS) production, and the expression levels of apoptosis genes and proteins were measured. Our results suggested that *N. bombycis* suppresses apoptosis in *BmN* cells.

Materials and Methods

Cell culture and apoptosis induction

BmN cells, which were originally derived from *Bombyx mori* ovaries, were cultured in 35 × 10 culture dishes (Greiner Bio-one, Frickenhausen, Germany) in TC-100 insect medium (Sigma, St Louis, USA)

supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, USA) at 27°C [29]. Cells were treated with 200 ng/ml ActD (1 mg/ml stock solution prepared in water; Sigma) to induce apoptosis.

Nosema bombycis spore preparation and infection of cells

Nosema bombycis was originally isolated from infected silkworms in Laboratory of Invertebrate Pathology, College of Animal Sciences, Zhejiang University (Hangzhou, China) [30]. Spores were propagated and purified as previously described [31]. To rule out bacterial contamination, purified spores were added to the normal culture medium. If no bacterial contamination was found at 48 h postinoculation, the purified spores were considered suitable for infection [32]. Spores were stored in sterilizing saline at 4°C before use. Approximately 3×10^7 spores were treated with 0.2 M KOH at room temperature for 40 min. Spores were concentrated at 2500 g for 1 min and re-suspended in 100 µl of culture medium. Then, the spores were added to $\sim 3 \times 10^6$ *BmN* cells. At 48 (proliferative phase) and 96 (spore-forming phase) hours post infection (hpi), the control and infected cells were treated with ActD for 6 h. The cells were then used for the subsequent experiments.

Extraction of fragmented DNA

DNA was isolated by the method of Chejanovsky and Gershburt [33]. Infected and uninfected *BmN* cells were harvested by centrifugation, and the cell pellets were suspended in TES [10 mM Tris, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), pH 8] with proteinase K at a final concentration of 70 µg/ml. After 2 h of incubation at 55°C, the DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). Then, DNA was precipitated with 1/10 volume of 3 M sodium acetate and two volumes of ethanol. The DNA pellet was washed twice with 70% ethanol, re-suspended in Tris-EDTA buffer solution with RNAase (20–50 µg/ml), and then incubated at 37°C for 2 h. For each DNA sample, 0.1 µg DNA of each samples was analyzed by 2% agarose gel electrophoresis with ethidium bromide staining.

Flow cytometry analysis of apoptotic cells

Apoptosis was evaluated using annexin V-FITC/propidium iodide (PI) staining followed by flow cytometry. Briefly, after being treated with ActD for 6 h, control and infected *BmN* cells were collected and centrifuged at 200 g for 10 min. The cells were washed twice with cold phosphate buffer saline (PBS) to remove excessive culture medium. Then, the cells were re-suspended at 1×10^6 cells/ml in binding buffer and incubated with PI and annexin V-FITC (BD Biosciences Pharmingen, San Diego, USA) at room temperature for 15 min in the dark. A total of at least 6000 cells were collected and analyzed by flow cytometry (FACSCalibur, BD Biosciences Pharmingen).

Microscopic observation of ROS production

ROS was detected using an ROS-sensitive fluorometric probe DCFH-DA. Primary cultured *BmN* cells were incubated at 27°C for 12 h prior to the experiment. Then, the infected and uninfected *BmN* cells were challenged with ActD (200 ng/ml) for 6 h followed by incubation with 1 µM DCFH-DA (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) at room temperature. *BmN* cells were observed and photographed under an ECLIPSE Ti fluorescence microscope (Nikon, Japan).

RNA extraction and quantitative real-time 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 ($2^{-\Delta\Delta C_t}$) 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 *BmN* 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 *BmN* cell death
DNA fragmentation is a characteristic of cells undergoing apoptosis [36]. To confirm that *N. bombycis* can reduce apoptosis in *BmN*

cells, cellular DNA from *BmN* cells that had undergone various treatments was isolated and analyzed by agarose gel electrophoresis. A significant increase in DNA fragmentation was found in uninfected *BmN* 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 *BmN* 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 *BmN* 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 *BmN* 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 *BmN* 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 *BmN* cells after 6 h of exposure to 200 ng/ml ActD. The ROS production was significantly increased compared

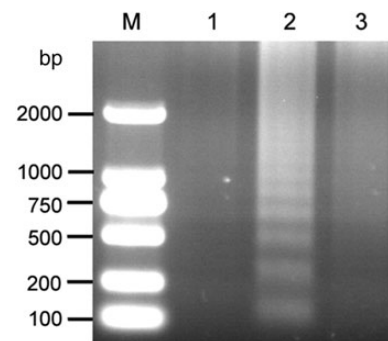


Figure 1. Inhibition of ActD-induced apoptosis of *BmN* cells by infection with *N. bombycis*. Lane M: marker; lane 1: control cells; lane 2: ActD-treated cells; lane 3: *BmN* cells infected with *N. bombycis*.

Table 1. Primers used in qRT-PCR

Gene	GenBank number	Sequence
<i>apaf-1</i>	NM_001200008	F: 5'-TATGCTGCGTCCCCTG-3' R: 5'-GTGCCATTATCTCGTTTTG-3'
<i>buffy</i>	NW_004582072	F: 5'-GCTATGTGCGGCGTTGGAG-3' R: 5'-CCCTTGTGACCCGTCTTGC-3'
<i>cytochrome C</i>	NW_004582024	F: 5'-TCATACTCCGATGCCAATA-3' R: 5'-TAGGCAATAAGGTCAGCAC-3'
<i>BmRP49</i>	NW_004624556.1	F: 5'-CAGGCGGTTCAAGGGTCAATAC-3' R: 5'-TGCTGGGCTCTTCCACGA-3'

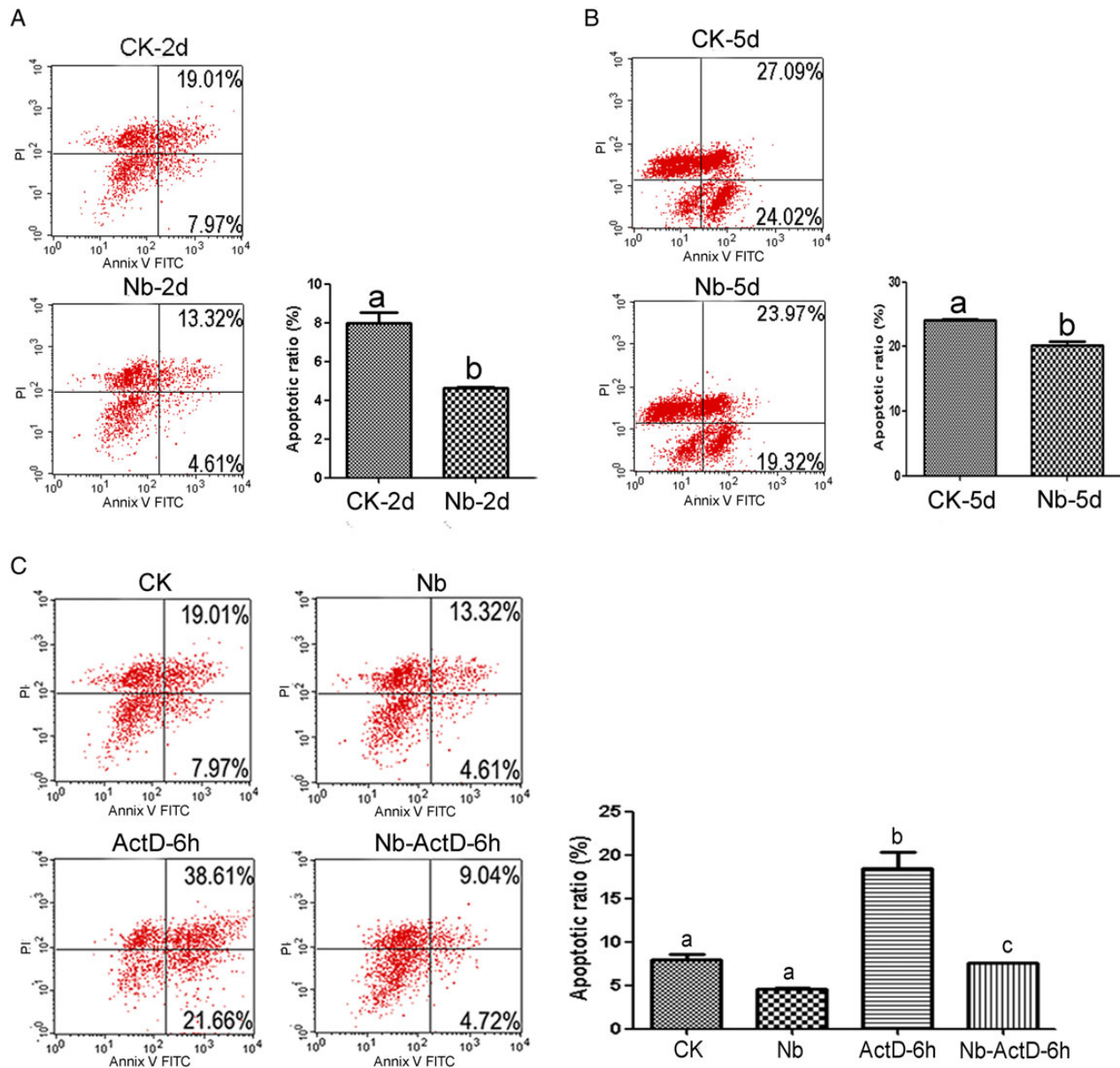


Figure 2. Apoptosis of *BmN* cells determined by flow cytometry (A) Apoptosis ratio of control *BmN* cells and *N. bombycis*-infected *BmN* cells at 2 days after infection. (B) Apoptosis ratio of control *BmN* cells and *N. bombycis*-infected *BmN* cells at 5 days after infection. (C) Apoptosis ratio of control *BmN* cells, *BmN* cells infected with *N. bombycis* for 2 days, *BmN* cells treated with ActD for 6 h and *BmN* cells infected with *N. bombycis* for 2 days and then treated with ActD for 6 h. All samples were measured using flow cytometry with double staining of Annexin V-FITC and PI. Different letters were significantly changed, $P < 0.05$.

with the control group (Fig. 3A; $P < 0.05$). At 48 hpi with *N. bombycis*, the ROS production of *BmN* 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 *BmN* cells challenged with 200 ng/ml ActD (Fig. 3D). The number of *BmN* cells which increased ROS production was calculated (Fig. 3E).

Nosema bombycis adjusted mRNA expression of apoptosis-related genes in *BmN* cells

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 reduced cytochrome C releasing by western blot analysis

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

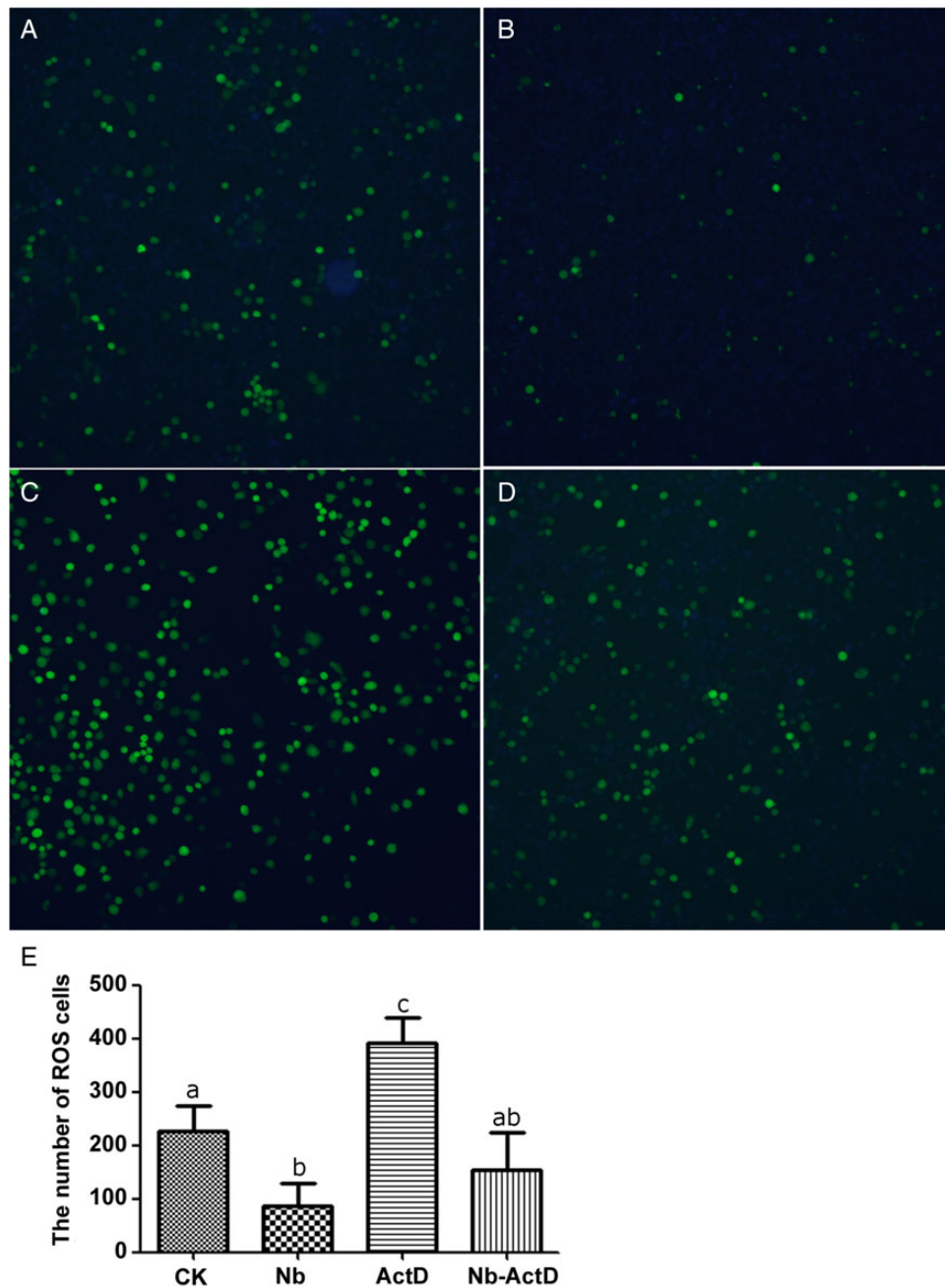


Figure 3. ActD-induced increase of ROS production in *BmN* cells *BmN* cells were incubated with 200 ng/ml ActD and examined for ROS production by DCFH-DA. Representative images of *BmN* cells were obtained by fluorescence microscopy. (A) Control cells. (B) *BmN* cells infected with *N. bombycis* and not treated with ActD. (C) *BmN* cells treated with ActD. (D) *BmN* cells infected with *N. bombycis* and treated with ActD. (E) The number of *BmN* cells which increased ROS production. Different letters were significantly changed, $P < 0.05$.

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

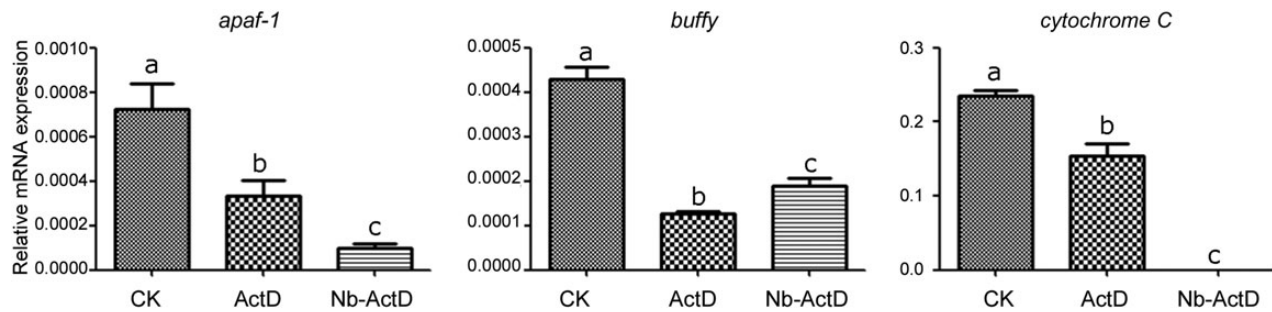


Figure 4. The relative expression of apoptotic genes and anti-apoptosis gene determined by qRT-PCR. Apoptotic gene expressions of cells under ActD treatment and *N. bombycis* infection (CK: control; ActD: treated with ActD only; Nb-ActD: treated with ActD after infection Nb 2 days) were detected by qRT-PCR. Three replicate experiments were conducted. Different letters were significantly changed, $P < 0.05$.

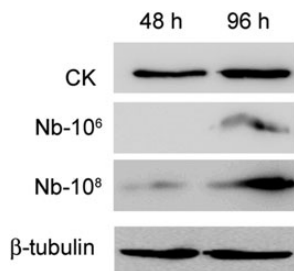


Figure 5. Western blot analysis of cytochrome C. Cells treated with different doses of *N. bombycis* showed decreased levels of cytochrome C at 48 and 96 hpi. β-Tubulin was used as an internal control. NB-10⁶ and NB-10⁸ were the number of *N. bombycis*.

lepidopteran insect cells [43,44], after incubation with ActD, uninfected *BmN* 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 *BmN* 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 *BmN* 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-epithelialization 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 *BmN* 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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