**Toxoplasma gondii** Induces Apoptosis via Endoplasmic Reticulum Stress-Derived Mitochondrial Pathway in Human Small Intestinal Epithelial Cell-Line

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**Abstract:** *Toxoplasma gondii*, an intracellular protozoan parasite that infects one-third of the world’s population, has been reported to hijack host cell apoptotic machinery and promote either an anti- or proapoptotic program depending on the parasite virulence and load and the host cell type. However, little is known about the regulation of human FHs 74 small intestinal epithelial cell viability in response to *T. gondii* infection. Here we show that *T. gondii* RH strain tachyzoite infection or ESP treatment of FHs 74 Int cells induced apoptosis, mitochondrial dysfunction and ER stress in host cells. Pretreatment with 4-PBA inhibited the expression or activation of key molecules involved in ER stress. In addition, both *T. gondii* and ESP challenge-induced mitochondrial dysfunction and cell death were dramatically suppressed in 4-PBA pretreated cells. Our study indicates that *T. gondii* infection induced ER stress in FHs 74 Int cells, which induced mitochondrial dysfunction followed by apoptosis. This may constitute a potential molecular mechanism responsible for the foodborne parasitic disease caused by *T. gondii*.

**Key words:** *Toxoplasma gondii*, ER stress, mitochondrial apoptosis, FHs 74 Int cell

**INTRODUCTION**

*Toxoplasma gondii* (*T. gondii*) is one of the world’s most successful parasites, in part because of its ability to infect and persist in most warm-blooded animals [1]. Natural infection by *T. gondii* occurs via oral ingestion of cyst-containing meat or oocyst-contaminated water and produce. As a result, the first encounter between parasite and host occurs in the small intestinal epithelium [2]. Tissue cysts that rupture in the small intestine and release zoites infect locally before disseminating throughout the host, where they form cysts that persist for the lifetime of the infected individual. This can have serious consequences to human health: reactivation of cysts in people whose immune systems are compromised can result in severe encephalitis and death [3]. Furthermore, spontaneous abortion, stillbirth, and severe birth defects can occur if the infection occurs during pregnancy and is transmitted to the fetus [4].

Excretory/secretory proteins (ESP) from *T. gondii* plays an important role in generating suitable conditions for parasite invasion into host cells [5]. One of the mechanisms by which the parasite escapes host immune responses is antigen shedding, a mechanism in which parasite ESP is released into the host body and evokes the immune system [6]. Apoptosis is a form of programmed and controlled cell death that accounts for the majority of cellular death in bioprocesses [7]. Mitochondria is important in the regulation and transmission of apoptotic signals, which are regulated by maintaining a balance between the levels of Bcl-2-family proteins [8]. *T. gondii* can modulate the host cell response, and apoptosis is one of the mechanisms targeted by this parasite [9]. A recent study revealed that *T. gondii* triggers trophoblast apoptosis through oxidative stress and mitochondrial dysfunction in mice [10]. Infection of murine astrocytes by the highly virulent RH strain led to decreased expression of the antiapoptotic protein survivin, p53 upregulated modulator of apoptosis (PUMA), and Bcl-2 and increased expression of the proapoptotic protein Noxa in the early stage of infection [11]. Conversely, *T. gondii* and ESP have the ability to inhibit apoptosis in several murine...
and human host cells treated with a broad spectrum of pro-apoptotic stimuli [12-14]. Presumably, *T. gondii* interferes with different processes to inhibit apoptosis and maintain chronic infection.

Bcl-2 plays an important role in the maintenance of mitochondrial integrity by preventing apoptosis-induced cytochrome c release, and cytochrome c is involved in activating downstream caspases that trigger apoptosis [15]. Several in vitro studies have reported that *T. gondii* infection regulates mitochondrial apoptosis through modulation of Bcl-2 family protein expression [10,13,14]. The endoplasmic reticulum (ER) functions in protein folding and assembly, lipid biosynthesis, vesicular trafficking, and cellular calcium storage. Its function can be disturbed by various factors, such as inhibition of protein glycosylation, calcium depletion, changes in redox status, and the expression of misfolded proteins. These dysfunctions cause proteotoxicity in the ER, collectively termed ER stress, which leads to activation of the unfolded protein response (UPR) [16,17]. *T. gondii* induces apoptosis of neural stem cells via the endoplasmic reticulum stress pathway [18]. However, little is known about the molecular mechanism of apoptosis in human small intestinal epithelial cells induced by *T. gondii* infection.

Here, we aimed to investigate the possible implication of endoplasmic reticulum stress-mediated mitochondrial apoptosis and its mechanisms in *T. gondii*-infected and ESP-treated FHs 74 Int cells. FHs 74 Int cells were infected with *T. gondii* or treated with ESP alone or in combination with the ER stress inhibitor 4-phenylbutyric acid (4-PBA), and then, cellular cytotoxicity, apoptosis, Bcl-2 family members and ER stress signaling pathways were evaluated.

**MATERIALS AND METHODS**

Reagents and antibodies

A CytoTox 96® Non-Radioactive Cytotoxicity Assay was obtained from Promega (Madison, Wisconsin, USA). A JC-1 Mitochondrion detection kit was obtained from Dojindo (Kumamoto, Kumamoto, Japan). The ER stress inhibitor 4-phenylbutyric acid (4-PBA) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). 4-Phenylbutyrate (4-PBA) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The ER stress inhibitor 4-phenylbutyric acid (4-PBA) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The ER stress inhibitor 4-phenylbutyric acid (4-PBA) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The ER stress inhibitor 4-phenylbutyric acid (4-PBA) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The ER stress inhibitor 4-phenylbutyric acid (4-PBA) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The ER stress inhibitor 4-phenylbutyric acid (4-PBA) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). 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tion was measured using a Bradford assay (Bio-Rad Laboratories, Hercules, California, USA), and samples were stored at −70°C until use.

Experimental designs
FHs74 Int cells were seeded on 96-well plates (for LDH and MTS assays), 12-well coverslips (for immunofluorescence), and 100-mm culture dishes (for western blotting) at various densities and grown to confluence at 37°C in 5% CO₂.

FHs74 Int cells were stimulated with tachyzoites of the T. gondii RH strain at MOIs of 10 or with 1 µg/ml ESP for 0, 2, 8, and 24 hr. Then, cell cytotoxicity, apoptosis, apoptotic features, induction of ER stress and its mechanism of action, and Bcl-2 family-related protein expression were evaluated using a LDH assay, immunocytochemistry and western blotting. Untreated FHs74 Int cells were used as controls. Each experiment was performed at least 3 times in triplicate.

LDH Assay
The LDH assays were performed for cytotoxicity quantification using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit according to the manufacturer’s protocol. Briefly, 1 × 10⁴ FHs74 Int cells were seeded in 96-well plates and infected with T. gondii at an MOI of 10 or treated with 1 µg/ml ESP for the indicated times (0, 2, 8, and 24 hr) in an incubator (5% CO₂, 90% relative humidity, 37°C). Then, 50 µl aliquots from all infected and control wells were transferred into a new 96-well plate, and 50 µl of CytoTox 96® reagent was added to each sample aliquot. The plate was covered with foil to protect the plate, and 50 µl of CytoTox 96® reagent was added to each sample aliquot. The plate was covered with foil to protect the samples from light and incubated for 30 min at room temperature. After addition of 50 µl of stop solution to each well, the absorbance of the solution was measured immediately at 490 nm using a microplate reader (TECAN, Crailsheim, Germany). LDH release levels in the medium were quantified and compared to control values according to the kit instructions.

MMP Assay
MMP was measured using a JC-1 MitoMP detection kit (Dojindo, Kumamoto, Japan). Briefly, FHs74 Int cells were seeded onto coverslips in 12-well plates at a density of 1 × 10⁴ cells/well and infected with T. gondii at an MOI of 10 or treated with 1 µg/ml ESP under various conditions with or without an ER stress inhibitor. The cells were then incubated with 4 µm JC-1 fluorescent dye at 37°C for 30 min in the dark and rinsed 3 times with HBSS. The stained cells were mounted onto microscope slides in VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories), and images were collected using a laser confocal microscope (Leica, TCS SP8, Wetzlar, Germany). The intensities of green (excitation/emission wavelength = 485/538 nm) and red (excitation/emission wavelength = 485/590 nm) fluorescence were analyzed in ≥6 microscopic fields in each sample. The intensities of red and green fluorescence were calculated using Image J software (NIH, Bethesda, Maryland, USA).

Western blotting analysis
SDS-PAGE and western blotting analysis were performed to determine the expression of numerous proteins. FHs74 Int cells were cultured in 100-mm dishes and underwent serum deprivation for 4 hr to remove stimulation from serum factors. Then, the cells were stimulated with T. gondii or ESP as indicated. After washing with PBS, proteins were extracted using PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Korea). The extract was incubated with complete protease inhibitor cocktail (Roche, Basel, Switzerland) for 30 min on ice followed by boiling for 10 min and then centrifuged at 14,000 g for 15 min at 4°C. The supernatant was collected, and equal amounts of protein from each sample were separated via SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). For blocking, the membranes were immersed for 1 hr at room temperature in 5% skim milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBST). After one wash in TBST, the membranes were incubated overnight at 4°C with primary antibodies diluted in TBST supplemented with 5% BSA. Following 3 consecutive washes in TBST, membranes were incubated for 90 min with HRP-conjugated anti-mouse or anti-rabbit secondary antibody diluted 1:5,000 with 5% skim milk, as described above. The membrane was soaked with Immobilon Western Chemiluminescent HRP Substrate (Jackson ImmunoResearch Laboratories), and chemiluminescence was detected using a Fusion Solo System (Vilber Lourmat, Collégien, France). These experiments were repeated at least 3 times. The band intensities were quantified using ImageJ software (NIH). The results were normalized to α-Tubulin protein levels and were expressed as fold changes over the mock-infection con-
trol group.

Preparation of mitochondrial and cytosolic fractions
Mitochondrial and cytosolic fractions of FHs 74 Int cells were prepared as described previously [PMID: 32547023]. For cytosolic extracts free of nuclei and mitochondria, the cells were washed in ice-cold PBS (pH 7.2) and then in hypotonic extraction buffer (HEB; 50 mM PIPES, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) and harvested by centrifugation. Pellets were resuspended in HEB and lysed with a Dounce homogenizer. These cell lysates were then centrifuged at 100,000 × g for 60 min at 4°C, and the supernatants were flash-frozen in cold ethanol, aliquoted, and stored at −80°C. Mitochondrial fractions were prepared by washing the cells in ice-cold PBS and then resuspending them in an isotonic homogenization buffer (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, EDTA-free complete cocktail of protease inhibitors [Roche]). After 60 strokes in a Dounce homogenizer, unbroken cells were removed by centrifugation at 1,000 × g for 10 min, and the supernatants were centrifuged at 14,000 × g for 20 min. The supernatants were collected as the mitochondrial fraction. The expression of cytochrome c expression was determined by western blotting. COX IV and α-tubulin were used as mitochondria and cytosol markers, respectively.

Statistical analysis
All assays were performed in triplicate, and at least 3 independent experiments were conducted per test series. The results are presented as mean ± SD. Statical analysis of the data was performed using unpaired, 2-tailed Student’s t-tests. A P-value less than 0.05 indicated statistical significance.

RESULTS

T. gondii infection and ESP treatment induced cytotoxicity and apoptosis to FHs 74 Int cells
The cytotoxicity of T. gondii RH tachyzoites to FHs 74 Int cells were 4.25 ± 0.63%, 10.33 ± 0.46%, 24.72 ± 0.31%, and 44.42 ± 1.05% at 0, 2, 8, and 24 hr postinfection, respectively. Next, we examined whether T. gondii ESP is toxic to FHs 74 Int cells. ESP (1 µg/ml) was added to the host cells for the indicated time, and the cytotoxicity was dramatically increased to 12.62 ± 1.25%, 32.68 ± 2.57%, and 41.98 ± 0.94% at 2, 8, and 24 hr, respectively (Fig. 1A). To verify whether the cytotoxicity of the T. gondii RH strain or ESP to FHs 74 Int cells was associated with apoptosis, we measured the level of apoptosis indicator proteins in RH-infected or ESP-treated FHs 74 Int cells. Our results showed that the protein levels of cleaved PARP and cleaved caspase 3 were significantly elevated in response to RH and ESP, and their increase was time dependent (Fig. 1B). Similarly, T. gondii infection and ESP treatment time-dependently elevated release of cytochrome c from mitochondria into cytoplasm (Fig. 1C, D). Collectively, our data indicate that T. gondii RH strain tachyzoites and ESP induced cytotoxicity and apoptosis in FHs 74 Int cells in a time-dependent manner.

T. gondii and ESP stimulation derived mitochondrial apoptosis in FHs 74 Int cells
Western blot analysis data showed that the protein levels of Bax, Bak and Bim were obviously increased with T. gondii infection, whereas the protein levels of BID, Mcl-1, Bcl-2, and Bcl-xl were drastically reduced in a parasite infection time-dependent manner (Fig. 2A). JC-1 dye staining revealed that T. gondii time-dependently induced MMP depolarization (Fig. 2C).

Upon ESP treatment, the protein levels of Bax, Bak, and Bim were apparently increased and those of BID, Mcl-1, Bcl-2, and Bcl-xl were diminished in a time-dependent manner (Fig. 2B). Surprisingly, we also found that the PUMA protein level was reduced after 2 and 8 hr of T. gondii infection and then slightly increased at 24 hr (Fig. 2A), but with ESP treatment, PUMA was dramatically reduced at 2 hr and remained low until 24 hr (Fig. 2B). In addition, both T. gondii and ESP induced modest phosphorylation of Bcl-2 at 24 hr (Fig. 2A, B). ESP treatment also caused mitochondrial dysfunction in a time-dependent manner (Fig. 2D). These findings suggest that T. gondii RH strain tachyzoites derived mitochondrial dysfunction in FHs 74 Int cells in a time-dependent manner. Speculation is not Results.

T. gondii and ESP challenge caused ER stress in FHs 74 Int cells
The ER-resident transmembrane sensor proteins IRE1, PERK and ATF6 were activated, evidenced by the dramatic increase in phospho-IRE1α, phospho-PERK, phospho-eIF2α and cleaved ATF6 upon T. gondii infection (Fig. 3A, C). CHOP, which plays an important role in ER stress-induced apoptosis,
was also elevated. However, the protein level of caspase 12, which is essential for carrying out the caspase-dependent UPR, was visibly reduced compared with that in the uninfected control group. All the above events occurred in a parasite infection time-dependent manner (Fig. 3A, C).

Similar to *T. gondii* infection, ESP treatment also induced elevation of p-IRE1α, p-PERK, p-eIF2α, cleaved ATF6, and CHOP and attenuation of procaspase-12 in a time-dependent manner (Fig. 3B, D). Both *T. gondii* infection and ESP treatment induced an obvious increase in the BIP protein level at 8 hr and maintained the high level until 24 hr. In contrast, *T. gondii* infection reduced the protein levels of Ero1-Lα and PDI at 2 hr, which then slightly recovered, whereas ESP treatment gradually reduced the protein levels of Ero1-Lα and PDI.

*T. gondii* and ESP-motivated mitochondrial apoptosis is mediated by ER stress in FHs 74 Int cells

Our results showed that both *T. gondii* - and ESP-induced alterations in the expression of Bcl-2 family members (Fig. 4A, B) and ER stress-related proteins (Fig. 4C, D) were reversed with 4-PBA pretreatment. In addition, 4-PBA pretreatment attenuated the protein levels of cleaved PARP and cleaved caspase 3 in *T. gondii*-infected or ESP-treated FHs 74 Int cells (Fig. 4E, F). *T. gondii* infection- or ESP treatment-derived the release of cytochrome c from the mitochondria into the cytoplasm, which was also prevented by 4-PBA pretreatment (Fig. 4G and Supplementary Fig. S1A, B). Confocal microscopy imaging of JC-1 dye staining showed that *T. gondii* infection- or ESP treatment-induced MMP depolarization in FHs 74 Int cells was rescued by 4-PBA pretreatment (Fig. 4H, I and Supplementary Fig. S1C, D). These results demonstrate that the *T. gondii* RH strain and ESP regulated ER stress, which mediates host mitochondrial dysfunction and apoptosis.
DISCUSSION

The current study revealed that *T. gondii* infection and ESP treatment significantly induced cell cytotoxicity and caspase-3-dependent mitochondrial apoptosis in FHs 74 Int cells. In addition, *T. gondii* infection and ESP treatment dramatically increased Bax, Bak and Bim levels but decreased the Mcl-1 protein level in a time-dependent manner. ER stress induction by *T. gondii* infection and ESP was confirmed by detecting increases in BIP, calnexin, p-IRE1α, p-PERK, p-eIF2α, CHOP, cleaved ATF6 and cleaved ATF4. Moreover, pretreatment with the ER stress inhibitor 4-PBA attenuated the levels of Bcl-2-, ER stress- and apoptosis-related proteins. More importantly, *T. gondii* infection and ESP treatment caused a strong decrease in MMP, but 4-PBA pretreatment attenuated MMP depolarization. These data indicate that *T. gondii* infection and ESP treatment induced apoptosis via the PERK/eIF2α/ATF4/CHOP, ATF6/CHOP, and IRE1α/Caspase-12 ER stress-derived mitochondrial pathways in human FHs 74 Int cells.

*T. gondii* infection induces NLRP3 inflammasome activation

**Fig. 2.** *Toxoplasma gondii* and ESP derived mitochondrial apoptosis in FHs 74 Int cells. (A, B) Protein expression of proapoptotic Bcl-2 and prosurvival Bcl-2 family members. (C, D) JC-1 staining shows red fluorescence for high mitochondrial membrane potential, while green fluorescence for low mitochondrial membrane potential. Bar graph showing the ratio of JC-1 red and green fluorescence.
in FHs 74 Int cells [19]. Notably, the NLRP3 inflammasome can also cause apoptosis in response to a broad spectrum of proapoptotic stimuli [21,22]. Hence, we hypothesized that *T. gondii* infection may have a functional role in regulating FHs 74 cell apoptosis. Our results are consistent with those of previous reports indicating that *T. gondii* infection and ESP treatment induced FHs 74 cell cytotoxicity and caspase-dependent apoptosis in cells in a time-dependent manner [17,23]. However, our results were contrary to those of Nishikawa et al. [14], who reported that *T. gondii* inhibits ultraviolet light-induced apoptosis through multiple interactions with the mitochondrion-dependent programmed cell death pathway. These different effects on host cell effects may be a result of differences in cell type, treatment method, parasite infection time, and host cell microenvironments.

Bcl-2 plays an important role in maintenance of mitochondrial integrity by preventing apoptosis-induced cytochrome c release, and cytochrome c is involved in activating downstream caspases that trigger apoptosis [9,23]. Mitochondria are not only a checkpoint for apoptosis progression but also release toxic proteins controlled by Bcl-2 family members at the mitochondrial outer membrane [8]. We utilized a JC-1 MitoMP detection kit to detect mitochondrial membrane potential (MMP). Because JC-1 dye exhibits potential-dependent accumulation in mitochondria with a fluorescence shift from green (488 nm) to red (561 nm), mitochondrial depolarization is indicated by a decrease in the red:green fluorescence intensity ratio [24]. We found that *T. gondii* infection and ESP treatment apparently increased Bax, Bak, and Bim protein levels but that BID, Mcl-1, Bcl-2, and Bcl-xL levels were diminished in a time-dependent manner. JC-1 dye staining revealed that *T. gondii*
Fig. 4. Toxoplasma gondii- and ESP-motivated mitochondrial apoptosis in FHs 74 Int cells. FHs 74 Int cells were pretreated with various concentrations of the ER stress inhibitor 4-phenylbutyrate (4-PBA) for 4 hr and subsequently infected with T. gondii at an MOI of 10 or treated with 1 µg/ml ESP for 18 hr. (A, B) The Bcl-2 family member. (C, D) ER stress-related protein. (E, F) Cleaved PARP and cleaved caspase 3 protein levels. (G) Mitochondrial and cytosolic fractions analyzed for expression of cytochrome c. (H, I) JC-1 staining observed via confocal microscopy imaging.
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infection and ESP treatment caused a strong decrease in MMP, indicated by a reduction in red and increase in green JC-1 fluorescence signals. This event is governed by a complex interplay between Bcl-2 family member proteins [8,25].

The endoplasmic reticulum (ER) is an elaborate cellular organelle essential for cell function and survival. Conditions that interfere with ER function lead to accumulation and aggregation of unfolded proteins, which are detected by ER transmembrane receptors that initiate the unfolded protein response (UPR) to restore normal ER function. If ER stress is prolonged or the adaptive response fails, apoptotic cell death ensues [26]. T. gondii infection time-dependently increased phospho-IRE1α, phospho-PERK, phospho-eIF2α, CHOP, and cleaved-ATF6 protein levels. In addition, T. gondii ESP treatment also elevated p-IRE1α, p-PERK, p-eIF2α, CHOP, and cleaved ATF6 protein levels. Recently, Augusto et al. [27] described that T. gondii infection activates each of the UPR sensor proteins, including IRE1, via ER stress and IRE1 controls host cell migration in infected immune cells in vitro and also facilitates dissemination of T. gondii in vivo. The phosphorylation of ATF6β by T. gondii ROP18 induced its proteosomal degradation and reduction in ATF6β mediated gene expression after induction of UPR. ATF6β-deficient mice exhibit a high susceptibility to infection by the parasite, indicating that ATF6β has a key role in resistance against T. gondii infection [28]. Our results show that small intestinal epithelial cell apoptosis being initiated by endoplasmic reticulum stress via activation of caspase-12, CHOP, and the JNK pathway in T. gondii infection and ESP treatment.

The findings of the present study suggest that ER stress-related protein expression is regulated by T. gondii infection and ESP treatment and positively correlated with mitochondrial dysfunction in FHs 74 Int cells. Pretreatment with the ER stress inhibitor 4-PBA attenuated the levels of ER stress-, Bcl-2- and apoptosis-related proteins, such as p-IRE1α, p-PERK, p-eIF2α, cleaved ATF6, CHOP, Bax, Bak, Bim, cleaved PARP, and cleaved caspase-3, and MMP depolarization in T. gondii-infected or ESP-treated FHs 74 Int cells. Notably, the present results indi-

Fig. 5. Schematic model of mitochondrial apoptosis induced by T. gondii and ESP stimulation in FHs 74 Int cells. T. gondii and ESP induce ER stress-mediated mitochondrial dysfunction.
cate that *T. gondii* infection and ESP treatment caused Bcl-2 family proteins to regulate mitochondrial apoptosis in FHs 74 Int cells via the PERK/eIF2α/ATF4/CHOP, ATF6/CHOP, and IRE1q/caspase-12 pathways (Fig. 5). Therefore, ER stress response-regulated intracellular signaling pathways might play crucial roles in small intestinal immunopathology following oral infection with *T. gondii*.

**ACKNOWLEDGMENTS**

This work was supported by the National Natural Science Foundation of China (81771612), the Characteristic Innovation Projects of Guangdong Universities (2018KTSCX081), the Guangdong Basic and Applied Basic Research Foundation (2019A151011715), and the Competitive Allocation Project of Zhanjiang Municipal Science and Technology Development Special Fund (2020A01021).

**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest.

**REFERENCES**

11. Contreras-Ochoa CO, Lagunas-Martínez A, Belkind-Gerson J, Díaz-Chávez J, Córrea D. *Toxoplasma gondii* infection and ESP treatment caused Bcl-2 family proteins to regulate mitochondrial apoptosis in FHs 74 Int cells via the PERK/eIF2α/ATF4/CHOP, ATF6/CHOP, and IRE1q/caspase-12 pathways (Fig. 5). Therefore, ER stress response-regulated intracellular signaling pathways might play crucial roles in small intestinal immunopathology following oral infection with *T. gondii*.