Yinjia pill inhibits persistent *Chlamydia trachomatis* infection

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To the Editor: Chlamydia trachomatis (C. trachomatis) is obligate intracellular pathogens, often resulting in development of chronic disease and tissue damage as a consequence of the host inflammatory immune response as well as persistent infections,[1] which is associated with the activation of mitogen-activated protein kinase (MAPK) and extracellular regulated protein kinases (ERK) mediated by Toll-like receptors (TLRs) and myeloid differentiation primary response gene 88 (MyD88).[2] Furthermore, *C. trachomatis* has evolved the ability to block the apoptosis of its host cells involving the interaction of autophagy and apoptosis.[3] Novel strategies to prevent and treat *C. trachomatis* infection effectively are needed.

Yinjia pill (YJP) decoction, consisting of Honeysuckle (Yinghua), Forsythia suspense (Lianqiao), Platycodon grandifloras (Jigeng), and other Chinese herbs, is a classic prescription used in the clinic to treat *C. trachomatis*-induced chronic pelvic inflammatory with the syndrome of "spleen deficiency and dampness-heat stasis."[4] However, the underlying mechanism is presently unknown. In this study, we aimed to investigate the potential effects of YJP decoction on the apoptosis, autophagy, and TLRs-MAPK/ERK inflammatory response in interferon-γ (IFN-γ) induced persistent *C. trachomatis* infection of HeLa cells. Future dissection of anti-Chlamydia and anti-inflammatory mechanism of YJP decoction may provide identification of novel therapeutic targets.

Here, HeLa 229 cells were cultured to establish a persistent *in vitro C. trachomatis* infection model by IFN-γ. The persistent *C. trachomatis* infected cells were treated with the drug-containing serum of YJP from rats (4, 8, and 16 g/kg) for 24 h. The production of infectious particles of *C. trachomatis* in HeLa cells was detected using immunofluorescence and inclusion-forming unit assays; cell apoptosis was measured by flow cytometry; inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were detected by the enzyme immunoassays; and the protein expressions related to apoptosis, autophagy, and TLRs-MAPK/ERK signaling pathway were measured by western blotting. All experiments were in triplicate.

Statistical analyses were performed using the GraphPad PRISM (GraphPad Software, San Diego, CA, USA). One way analysis of variance with a Bonferroni post-test was used for the differences between YJP-exposed and non-exposed groups. $P < 0.05$ was considered statistically significant.

It is identified that the persistent *C. trachomatis* infection of HeLa Cells was successfully established by IFN-γ induction, with typical aberrant intracellular reticulate bodies (RBs). After co-incubation with YJP serum, the productions of elementary bodies and RBs as well as the number of chlamydial inclusions in HeLa cells was decreased in a dose-dependent [Figure 1A]. These results indicated that YJP inhibited *C. trachomatis* growth and replication.

The staurosporine (STS)-induced apoptosis assay was used to analyze the apoptosis resistance of persistent *C. trachomatis* infection. The persistent *C. trachomatis*-infected cells were resistant to apoptosis induced by STS with a higher anti-apoptotic (Bcl-2)/pro-apoptotic (Bax) ratio.

However, YJP serum promoted the apoptosis of persistent *C. trachomatis* infected HeLa cells and decreased the Bcl-2/ Bax ratio in a dose-dependent manner [Figure 1B and 1C].

The results revealed that YJP attenuated the apoptosis resistance of persistent *C. trachomatis* infection by reducing the Bcl-2/Bax ratio.

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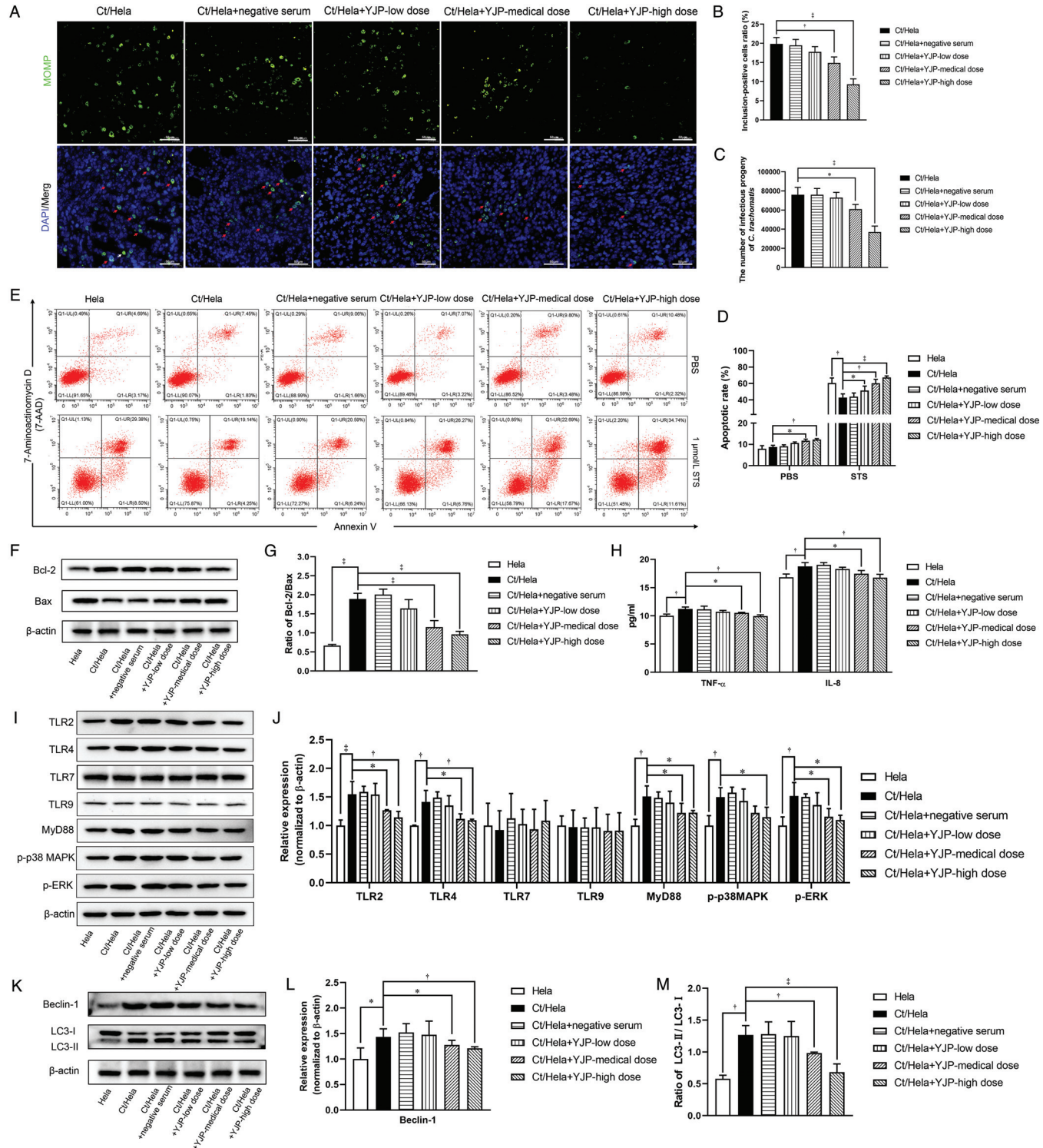

Figure 1: (A) YJP decoction inhibited the production of infectious particles of C. trachomatis in HeLa Cells. The yellow arrow shows the elementary bodies. The red arrow shows the RBs. (B) The inclusion-positive cells ratio and (C) C. *trachomatis* infection were quantified. (D-E) YJP decoction weakens the anti-apoptotic effect of persistent C. *trachomatis* infection of HeLa Cells. (F-G) WB for detection of the expression of Bcl-2 and BCL2-Associated X protein (Bax). (H) The secretion of TNF-α and IL-1β from HeLa cells with persistent C. *trachomatis* infection was determined by ELISA. (I-J) WB for detection of the expression of TLR 2, TLR4, TLR7, TLR9, MyD88, p-p38 MAPK, and p-ERK. (K-M) YJP decoction inhibited the autophagy activation in persistent C. *trachomatis* infected HeLa cells. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$. Bcl-2: B-cell lymphoma-2; Ct: C. trachomatis; ELISA: Enzyme-linked immunosorbent assay; ERK: Extracellular regulated protein kinases; IL-1β: Interleukin-1β; LC3: Light Chain 3; MAPK: Mitogen-activated protein kinase; MyD88: Myeloid differentiation primary response gene 88; PBS: Phosphate Buffered Saline; RBs: Reticulate bodies; STS: Staurosporine; TNF-α: Tumor necrosis factor-α; TLR: Toll-like receptor; WB: Western blotting; YJP: Yinjia pill.

Enzyme linked immunosorbent assay (ELISA) results indicated that the secretion levels of TNF-α and IL-1β in cells with persistent C. *trachomatis* infection were decreased by YJP serum in a dose-dependent manner [Figure 1D]. Furthermore, YJP serum inhibited the protein expression of TLR2/4, MyD88, p-p38 MAPK, and p-ERK

with a dose-dependent manner, but there was no difference in the expression of TLR7/9 [Figure 1E]. These data suggested that YJP could inhibit the production of inflammatory cytokines TNF-α and IL-1β through down-regulating TLR2/4-mediated MAPK/ERK signaling pathway.

The Western blotting assay for autophagy-related proteins showed that persistent *C. trachomatis* infection increased the autophagy level in HeLa cells through increasing the beclin-1 expression and Microtubule-associated Protein1 Light Chain 3 (LC3)-II/LC3-I ratio, but YJP serum inhibited the beclin-1 expression and LC3-II/LC3-I ratio in a dose-dependent manner [Figure 1F]. These data suggested that YJP serum could inhibit the autophagy activation of persistent *C. trachomatis* infected HeLa cells.

The induction of apoptosis resistance has been regarded as an important immune escape mechanism for *C. trachomatis*.[5] We found that YJP promoted the apoptosis of *C. trachomatis*-infected cells and attenuated the apoptosis resistance of chlamydia against STS. Apoptosis is tightly regulated by anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) effector molecules.[6] We found that the ratio of Bcl-2/Bax in HeLa cells was increased after persistent *C. trachomatis* infection. Since Bcl-2 binds to Bax, altered interaction between Beclin-1 and Bcl-2 may affect Bax function, which is essential for apoptosis activation. Therefore, inhibition of autophagy by YJP might promote the activation of the Bax-mediated apoptosis pathway through altering the interaction of Beclin-1 and Bcl-2 in persistent *C. trachomatis* infection of HeLa cells.

The autophagy activation could limit the maturation of the chlamydial inclusion body and promote RBs formation, which is one of the mechanisms of persistent infection of *C. trachomatis*.[7] In this research, we found that YJP could promote the removal of chlamydial inclusion and decrease the number of infectious progenies in persistent *C. trachomatis* infection of HeLa cells. Moreover, YJP down-regulated the beclin-1 expression and LC3-II/LC3-I ratio in persistent *C. trachomatis* infected HeLa cells, which indicated that YJP could inhibit the autophagy activation. Recent study suggested that autophagy-specific protein Light Chain 3 (LC3) can help to obtain nutrients from the host cell through recruiting microtubules, while inhibition of autophagy has significant negative effects on infection and development of *C. trachomatis*,[3] Therefore, it is inferred that YJP may limit the nutrient supply of chlamydia growth by inhibiting autophagy, thereby inhibiting the growth and replication.

During the persistent infection of *C. trachomatis*, the abnormal activation of MAPK/ERK signaling mediated by TLRs and MyD88 played a vital role in initiating and maintaining host inflammation.[2,8] We found YJP inhibited the secretion of TNF-α and IL-1β, the activation of MAPK/ ERK signaling, and the expression of TLR2 and TLR4. Interestingly, there was no difference in TLR7/9 expression, which was consistent with the study of Yu *et al*.[8] Therefore, YJP could inhibit the production of inflammatory cytokines TNF-α and IL-1β through down-regulating TLR2/4-mediated MAPK/ERK signaling pathway, but independent of TLR7 and TLR9.

In conclusion, we demonstrated that YJP decoction could inhibit *C. trachomatis* growth and replication in a persistent infection state, which might be associated with inducing apoptosis and inhibiting autophagy. Moreover, YJP also could inhibit the production of inflammatory cytokines TNF-α and IL-1β through down-regulating TLR2/4-mediated MAPK/ERK signaling pathway.

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Conflicts of interest

None.

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