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TUDCA attenuates intestinal injury and inhibits endoplasmic reticulum stress-mediated intestinal cell apoptosis in necrotizing enterocolitis

Peng Li[a,1], Dong Fu[a,1], Qingfeng Sheng[a], Shenghua Yu[a], Xingqi Bao[b], Zhibao Lv[a,*]

[a] Department of General Surgery, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai 200040, China
[b] Department of Urology Surgery, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai 200040, China

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ABSTRACT

Neonatal necrotizing enterocolitis (NEC) is a life-threatening disease with severe inflammation and intestinal cell apoptosis. Tauroursodeoxycholic acid (TUDCA) is a recognized endoplasmic reticulum stress (ERS) inhibitor which can inhibit cell apoptosis. Recently, intestinal cell apoptosis has been demonstrated to be vital for the pathogenesis of NEC. The purpose of the present study was to investigate the potential of TUDCA in the treatment of NEC and the possible mechanisms *in vivo* and *in vitro*. Our results showed that TUDCA reduced mortality rates, prolonged survival times, significantly diminished intestinal damage, and inhibited intestinal inflammation in the mouse model of NEC. The protective effect of TUDCA on the NEC mouse model was realized through inhibiting the expression levels of ERS markers and inhibiting the apoptosis of intestinal cells. In addition, TUDCA increased the expression of phospho-Akt (p-Akt). Furthermore, we confirmed that TUDCA inhibited the apoptosis of intestinal cells by modulating the PERK-eIF2α ERS pathway and the Akt pathway *in vitro* studies. Besides, TUDCA effects were impaired by AKT specific inhibitor MK2206 *in vitro* studies. Therefore, these results indicated that TUDCA alleviated intestinal injury in a mouse model of NEC and inhibited ERS-mediated intestinal cell apoptosis by activating the Akt pathway.

1. Introduction

Neonatal necrotizing enterocolitis (NEC) is one of the most serious and common diseases during the neonatal period and is the leading cause of gastrointestinal disease deaths in premature infants [1]. To date, there is still a lack of effective treatments for NEC in clinical practice. Children with severe NEC often need surgery. However, the incidence of severe complications, which include intestinal stenosis, short bowel syndrome, and neurodevelopmental delay [2,3], is often high after surgery. Therefore, seeking safer and more effective treatments for children with NEC has been a hot topic in related research fields worldwide.

The endoplasmic reticulum (ER) is a membranous organelle in eukaryotic cells that plays an important role in maintaining their physiological functions. ER is involved in a variety of intracellular biochemical reactions, such as the synthesis of lipids and steroids, the maintenance of calcium ion homeostasis, and the synthesis, folding and post-translational modification of proteins [4]. External stimuli or cell function abnormalities may lead to an increase in unfolded and misfolded proteins. If the levels of the unfolded and misfolded proteins exceed the processing capacity of the ER, endoplasmic reticulum stress (ERS) occurs. Sustained ERS activates apoptotic pathways and promotes apoptosis [5]. ERS is mainly characterized by the unfolded protein response (UPR). UPR enhances correct protein folding, inhibits protein production and accumulation, accelerates the degradation of non-functional and potentially toxic proteins, induces the transcription of ERS-related genes, and enhances the self-healing capability of ER [6–8]. Generally, ERS refers to UPR, which mainly consists of 3 signaling pathways: the inositol-requiring enzyme 1 (Ire1), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (Atf6) pathways. Moreover, ERS regulates inflammatory responses in intestinal cells. Studies have shown that intestinal ERS markers are upregulated in children with NEC, and ERS may represent one of the pathogenic mechanisms of NEC [9,10]. Inhibition of ERS may have a protective effect on NEC intestine.

Tauroursodeoxycholic acid (TUDCA) is an ERS inhibitor. Studies have found that this drug controls inflammation and inhibits apoptosis through inhibiting ERS [11,12]. However, to date, there are few studies related to the effects of TUDCA on NEC and the underlying mechanisms. TUDCA may have a protective effect on the NEC model.

* Corresponding author.
E-mail address: zhibaolv@126.com (Z. Lv).
[1] These authors contributed equally to this work.

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Therefore, investigation of the therapeutic effect of this clinical drug on NEC is of great significance for the prevention and treatment of NEC. The purpose of the present study was to investigate the effect of TUDCA on the apoptosis of the cultured intestinal epithelial cells and the intestinal cells of the NEC mouse model. In the present study, we reported that TUDCA activated the Akt pathway to inhibit ERS-mediated intestinal cell apoptosis. In addition, we found that TUDCA treatment reduced the incidence and severity of NEC and decreased the expression of inflammatory factors.

2. Materials and methods

2.1. Antibodies and other reagents

Sources of antibodies and other reagents included: mouse anti-BiP (Abcam, Cambridge, MA); rabbit anti-CHOP (Abcam); rabbit anti-Cleaved-caspase-3 (Abcam); rabbit anti-phospho-PERK (Cell Signaling Technology, Beverly, MA); rabbit anti-phospho-eIF2α (Cell Signaling Technology); rabbit anti-AKT (Cell Signaling Technology); rabbit anti-phospho-AKT (Cell Signaling Technology) and mouse anti-GAPDH (Cell Signaling Technology); lipopolysaccharide (LPS) (Sigma, St Louis, MO); TUDCA(Sigma); MK2206 (Selleck Chemicals, Houston, TX).

2.2. Cell culture and treatment

The IEC-6 cell line was obtained from the American Type Culture Collection (ATCC). IEC-6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). IEC-6 cells were treated with LPS (50 μg/ml) for 6 h, followed by cotreatment with TUDCA (50, 100, or 200 μM) or MK2206(5 μM). Cell proliferation was evaluated using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) method (Beyotime, Shanghai, China), while apoptosis was examined using the lactate dehydrogenase (LDH) assay kit (Beyotime).

2.3. Establishment of an animal model of NEC

All animal experimental protocols were approved by the Animal Protection Committee of Shanghai Children's Hospital. The NEC model was induced in 5–10-day-old mice by artificial feeding combined with hypoxia-cold stimulation as described [9,13,14]. The formula consisted of 15 g Similac Advance infant formula (Abbott Laboratories, Chicago, USA) in 75 ml canine milk replacement and mice pups were fed 5 times/day with a 1.9F central venous catheter (Togo Medikit, Miyazaki, Japan). Mice pups were subjected to hypoxia stress (60 s exposure to 99.99% nitrogen) followed by cold exposure (4 °C for 10 min) twice daily for 4 days.

The experimental grouping was as follows: 7 C57BL/6 female mice gave birth to 53 pups, which were randomly divided into four groups using the digital table method. Group 1 was the normal control group (CON) and contained 10 mouse pups that were fed by the mother mice and were intraperitoneally injected once daily with 0.1 ml of normal saline. Group 2 was the NEC model group (NEC) and contained 15 mouse pups that were artificially reared, exposed to hypoxia-cold stimulation, and intraperitoneally injected once daily with 0.1 ml of normal saline 30 min before the hypoxia-cold stimulation. Group 3 was the TUDCA intervention control group (CON + TUDCA) and consisted of 10 mouse pups that were fed by the mother mice and intraperitoneally injected once daily with TUDCA (500 mg/kg [15], dissolved in 0.1 ml of normal saline). Group 4 was the TUDCA intervention NEC model group (NEC + TUDCA) and consisted of 18 mouse pups that were intraperitoneally injected once daily with TUDCA (500 mg/kg, at 30 min before hypoxia-cold stimulation). The animals were sacrificed after the clinical symptoms of NEC (abdominal distension, abdominal wall discoloration, or bloody stool) developed or after 96 h. The ileal tissues and blood samples were then collected for further analysis.

2.4. Histopathological examination of intestinal tissues

The collected terminal ileum specimens were fixed in 4% paraformaldehyde solution, embedded in paraffin, sectioned, hematoxylin and eosin (H&E) stained, and pathologically scored (double blind method) according to the published scoring criteria by pathologists [13,16]. A score equal to or > 2 points suggests the occurrence of NEC.

2.5. Immunofluorescence assay

The frozen sections prepared from frozen tissues or cells grown on coverslips were blocked at room temperature (RT) for 1 h, incubated with primary antibodies at 4 °C overnight and secondary antibodies for 1 h, and then stained with 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime). The staining results were observed under a fluorescence microscope.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The TUNEL assay was performed to detect the enterocyte apoptosis *in vivo and vitro* using the *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics, Mannheim, Germany). The staining results were observed under a fluorescence microscope. The number of TUNEL positive cells *in vitro* was identified by double blind method and expressed as the number of TUNEL-positive cells per high power field, with > 10 fields/experiment studied and > 100 cells/field.

2.7. Enzyme-linked immunosorbent assay (ELISA) analysis

IL-6, TNF-α and IL-1β ELISA kits (R&D Systems, Minneapolis, MN) were used to measure the inflammatory factors in the serum or supernatants, according to the manufacturer's instructions.

2.8. Caspase-3 activity analysis

To examine the activity of caspase-3 in intestine tissues, intestine homogenates were analyzed using a caspase-3 activity assay kit (Beyotime) according to the manufacturer's instructions.

2.9. Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from snap-frozen ileal tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was used for cDNA synthesis. Initial RT-PCR amplification was done with a denaturation step at 95 °C for 10 min, followed by 28 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 45 s. Upon completion of the cycling steps, a final extension at 72 °C for 5 min was conducted. Reactions were run in triplicate for three independent experiments. The primer sequences are provided in Table 1. Expression data were normalized to the geometric mean of the housekeeping gene GAPDH to control the variability in expression levels and were analyzed using the $2^{-\triangle\triangle}$CT method.

2.10. Flow cytometric analysis

The annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining assay (R&D Systems) was used to quantify apoptosis of IEC-6 cells by flow cytometric analysis. The experimental method followed the manufacturer's instructions.

2.11. Western blotting analysis

Snap-frozen tissue samples were homogenized in lysis buffer (Beyotime). Protein concentrations of samples were determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Equal

Table 1
Primers used for qRT-PCR.

Target gene	Primer sequences (5′ to 3′)
GAPDH (mouse)	(F) AGGTCGGAGTCAACGGATTT
	(R) TGGAAGATGGTGATGGGATTT
IL-1β (mouse)	(F) GTGGTGTGTGACGTTCCCATTA
	(R) CCGACAGCACGAGGCTTT
IL-6 (mouse)	(F) GATACCACTCCCAACAGAC
	(R) CTTTTCTCATTTCCACGAT
BiP (mouse)	(F) TGCAGCAGGACATCAAGTTC
	(R) TACGCCTCAGCAGTCTCCTT
CHOP (mouse)	(F) AAGATGAGCGGGTGGCAGCG
	(R) GCACGTGGACCAGGTTCTGCT
GAPDH (rat)	(F) CGTATTGGGCGCCTGGTCACC
	(R) GGGATGATGTTCTGGAGAGCCC
TNF-α (rat)	(F) TGGGTCCAACTCCGGGCTCA
	(R) TGGAATCCTTGCCGGTGGCG
IL-6 (rat)	(F) GTCTCGAGCCCACCAGGAACG
	(R) AGGGAAGGCAGTGGCTGTCAAC
BiP (rat)	(F) TGCAGCAGGACATCAAGTTC
	(R) AAAGAAGACCCCGTTTACAG
CHOP (rat)	(F) CCAGCAGAGGTCACAAGCAC
	(R) CGCACTGACCACTCTGTTTC

amounts of total protein were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel. Gels were blotted on polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes were blocked in 5% BSA and incubated overnight at 4 °C with primary antibody. After washing, membranes were incubated with appropriate secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). After washing, the membrane was developed using ECL Plus reagents (Thermo Scientific). The protein expression level in each lane was determined by normalizing the band intensities to the GAPDH band intensity.

2.12. Statistical methods

Statistical analysis was performed using the SPSS 22.0 statistical software. The measurement data are expressed as mean ±SD. The within-group comparisons were performed using one-way analysis of variance (ANOVA), whereas the between-group comparisons were conducted using the Student-Newman-Keuls (SNK) test. The log-rank test was used for survival analysis. A P value of < 0.05 indicated that the difference was statistically significant.

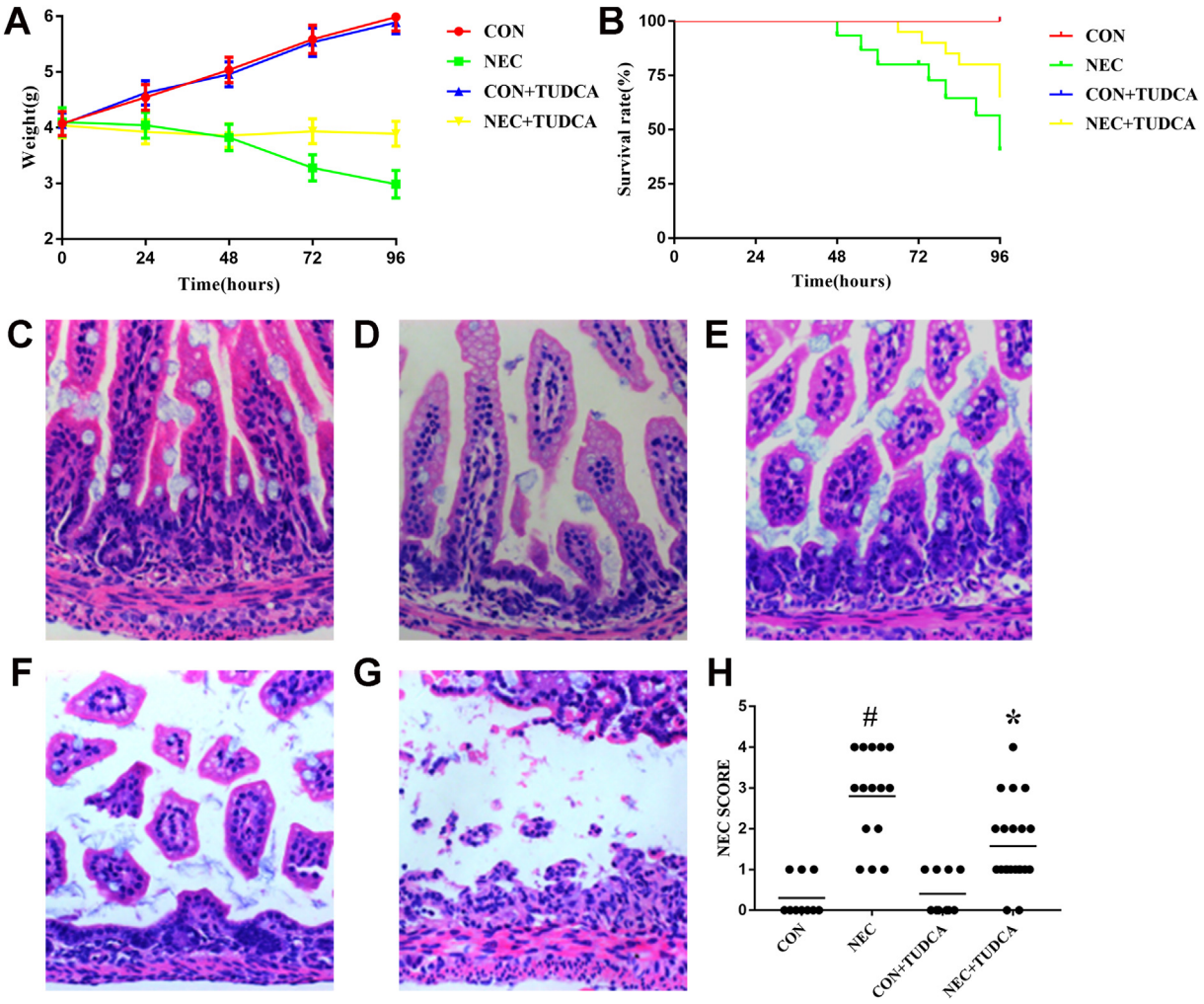

Fig. 1. The effect of TUDCA on body weight, survival, and intestinal injury in a mouse model of NEC.
A, Changes in the body weight of the surviving mice in each experimental group. B, Kaplan-Meier survival analysis of the survival time. And the histological scores of the distal ileum of neonatal mice: C, Grade 0, normal ileum. D, Grade 1, mild injury to the tip of the villus. E, Grade 2, partial loss of the villus. F, Grade 3, severe submucosal injury. G, Grade 4, complete necrosis. H, Summary of the histological NEC scores in four groups of mice. Data are expressed as mean ± SD. $^{\#}P < 0.05$ *vs.* CON group. $^{*}P < 0.05$ *vs.* LPS group. *Scale bars*, 20 μm.

Table 2
Body weight (g) of mice during the experiment.

Group	Day1	Day2	Day3	Day4
CON	4.54 ± 0.23	5.04 ± 0.22	5.58 ± 0.29	5.99 ± 0.25
NEC	4.04 ± 0.24	3.83 ± 0.22[a]	3.28 ± 0.31[a]	2.98 ± 0.25[a]
CON + TUDCA	4.62 ± 0.20	4.96 ± 0.23	5.53 ± 0.29	5.88 ± 0.21
NEC + TUDCA	3.99 ± 0.22	3.86 ± 0.25[a]	3.94 ± 0.30[a,b]	3.89 ± 0.22[a,b]

[a] $P < 0.05$:NEC and NEC + TUDCA *vs.* CON or CON + TUDCA.
[b] $P < 0.05$:NEC + TUDCA *vs.* NEC.

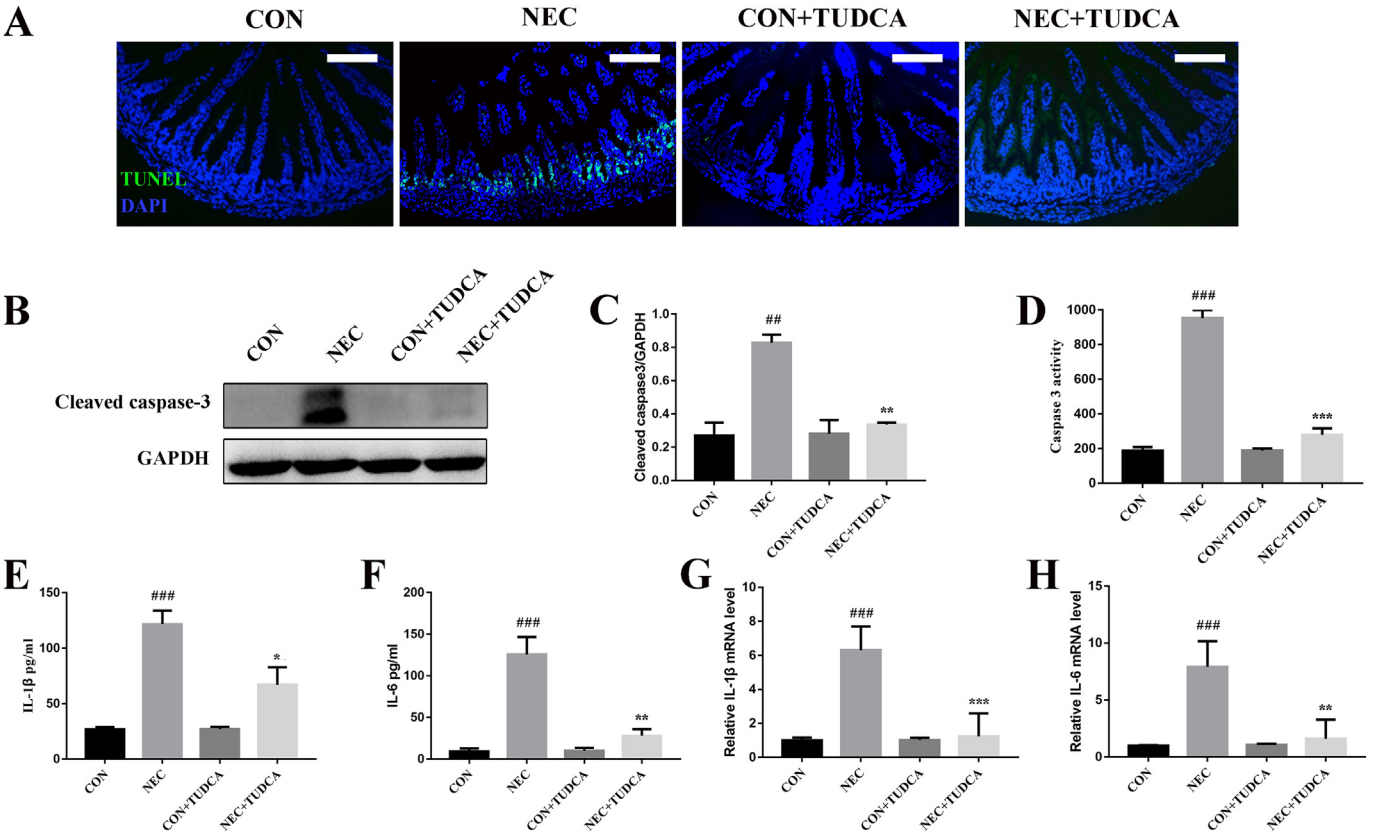

Fig. 2. The effect of TUDCA on apoptosis and inflammatory factors in a mouse model of NEC.
A, TUNEL staining (green) of the ileal tissues of mice among four groups. DAPI (blue) was used as a nuclear marker. B, Western blot analysis of the expression levels of Cleaved caspase-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in ileal tissues of mice among four groups. C, Densitometric analysis was used to quantify the levels of Cleaved caspase-3 and GAPDH. D, Examination of caspase-3 activity in the ileal tissues of mice among four groups. E and F, ELISA analysis of the serum expression of the inflammatory factors IL-1β and IL-6. G and H, qRT-PCR analysis of the mRNA expression levels of IL-1β, IL-6 and GAPDH in the ileal tissues. Data are expressed as mean ± SD for three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs.* CON group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* LPS group. *Scale bars*, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. The protective effect of TUDCA on the NEC mouse model

To determine whether TUDCA intervention exerted a protective effect on experimental NEC, we first monitored the body weight changes and survival rates in various groups of neonatal mice. NEC induction gradually reduced the body weight of the neonatal mice in NEC group during the experiment of establishing the model. And differences between NEC and NEC + TUDCA groups in the body weight were statistically significant at day 3 and 4 ($P = 0.021$, $P = 0.011$). While TUDCA treatment can significantly attenuated the NEC-induced weight loss, the body weight of the NEC + TUDCA group did not return to the level of the CON + TUDCA group at day 4 ($P = 0.015$). There was no significant difference in body weight between CON and CON + TUDCA groups (Fig. 1A, Table 2). Since technical death is not caused by disease, this study has eliminated technical death and then counted the survival rate. Notably, the NEC group exhibited a lower survival rate (46.67%) at 96 h after establishing the NEC model. In contrast, TUDCA treatment protected the neonatal mice from NEC-related mortality and enhanced the survival rate of NEC + TUDCA group to 66.67%. TUDCA treatment significantly improved the survival rate ($P = 0.0232$). Both CON group and CON + TUDCA group had a survival rate of 100% (Fig. 1B).

Since NEC is characterized by acute intestinal injury, we next collected specimens of terminal ileum tissue post NEC to assess the severity of intestinal damage. Intestinal macroscopic and microscopic examination revealed clear evidence of intestinal injury similar to NEC (Fig. 1C–G). In contrast, CON group and CON + TUDCA group showed no change or abnormality in small intestinal structure. TUDCA was able to reduce the NEC pathologic score, and the reduction was statistically significant ($P < 0.001$). Specifically, the NEC group had a median

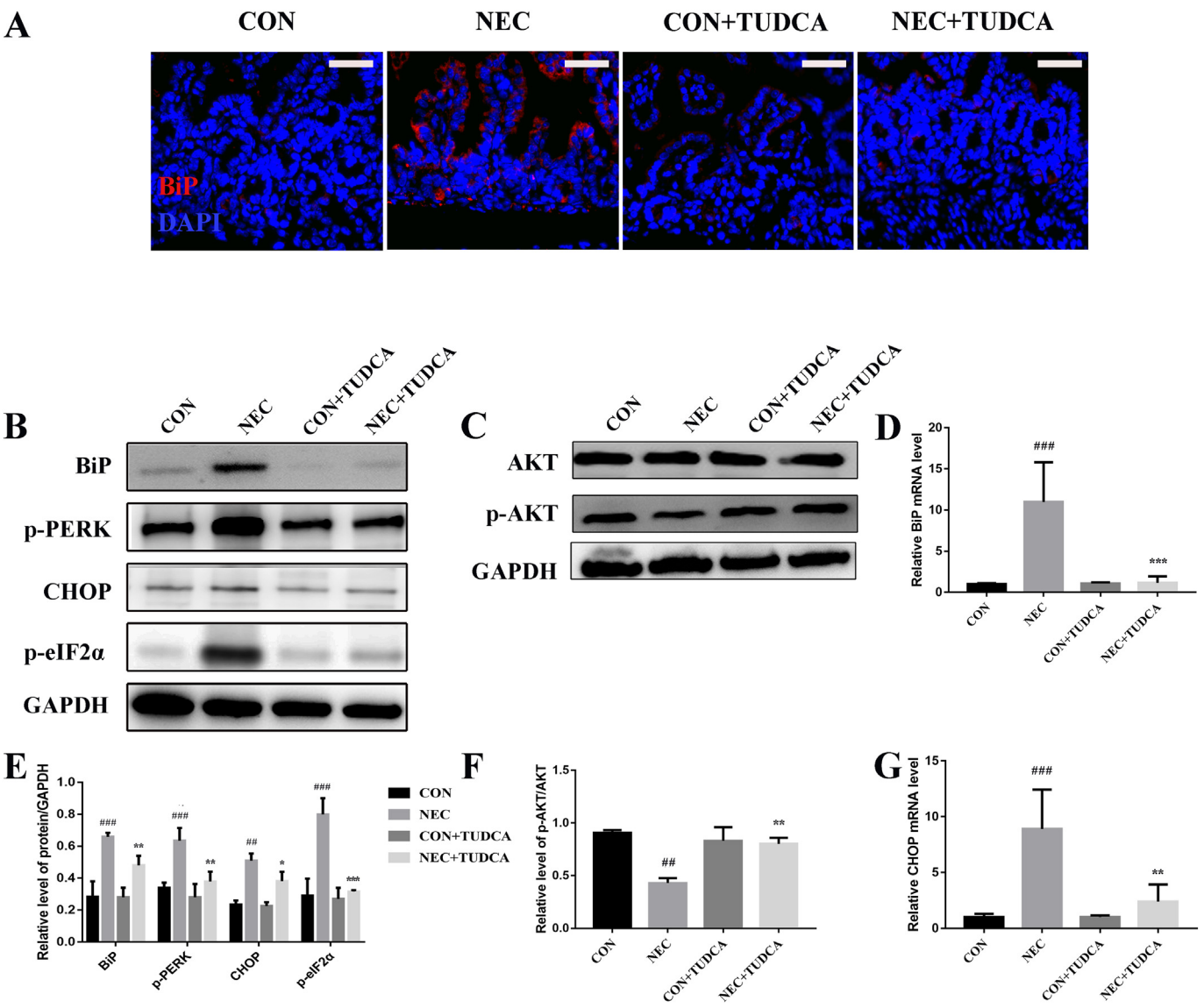

Fig. 3. The effect of TUDCA on ERS-related proteins in a mouse model of NEC.
A, Immunofluorescence examination of the expression level of BiP protein (red) in the ileal tissues of various groups of mice. DAPI (blue) was used as a nuclear marker. B and C, Western blot analysis of the expression levels of BiP, p-PERK, p-eIF2α, CHOP, p-AKT, AKT, and GAPDH proteins in the ileal tissues of mice among four groups. E and F, Densitometric analysis was used to quantify the levels of BiP, p-PERK, p-eIF2α, CHOP, p-AKT, AKT, and GAPDH. D and G, qRT-PCR analysis of the gene expression levels of ERS-related proteins (including BiP, CHOP, and GAPDH) in the ileal tissues of mice. Data are expressed as mean ± SD for three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs.* CON group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* LPS group. *Scale bars*, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

score of 3 (1–4), while NEC + TUDCA group had a median score of 1.5 (0–4) (Fig. 1H).

3.2. TUDCA exerts a protective effect on the NEC mouse model through inhibiting the apoptosis and reducing inflammation in intestinal cells

Apoptosis is an important indicator of NEC-induced intestinal injury. Therefore, further investigation is required to determine whether TUDCA reduces NEC-induced intestinal damage through inhibiting the apoptosis of small intestinal epithelial cells. The TUNEL assay results showed that the number of apoptotic small intestinal epithelial cells was significantly increased in the NEC group. In contrast, TUDCA intervention significantly reduced the apoptosis of small intestinal epithelial cells (Fig. 2A). The above findings were supported by Western blot analysis of the cleaved caspase-3 levels in mouse ileal tissues (Fig. 2B, C). In addition, TUDCA intervention significantly reduced the activity of caspase-3 (Fig. 2D).

Inflammation is another important feature of NEC-induced intestinal damage. Therefore, we evaluated the severity of intestinal infection by examining the mRNA and protein expression levels of tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) in ileal tissues. The expression levels of TNF-α and IL-6 mRNAs and proteins were increased in ileal tissues after NEC induction compared with CON group. Surprisingly, TUDCA intervention significantly reduced the NEC-stimulated release of inflammatory cytokines to a level virtually similar to that observed in CON and CON + TUDCA groups compared with the NEC group (Fig. 2E–H).

In summary, the above results demonstrated that TUDCA inhibited the apoptosis of small intestinal epithelial cells and attenuated the inflammatory response in the NEC mouse model.

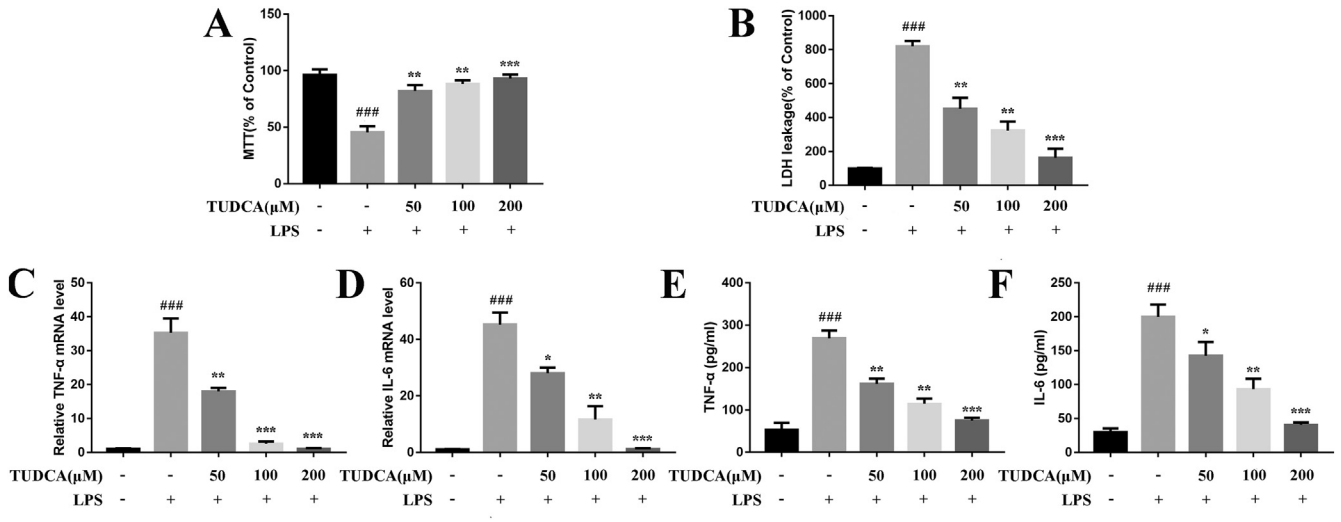

Fig. 4. The effect of TUDCA on LPS-induced IEC-6 cell death and inflammation *in vitro*.
A, Examination of cell survival rate by MTT assay. B, Examination of cell death by LDH activity analysis. C and D, Examination of the mRNA expression levels of TNF-α, IL-6, and GAPDH in IEC-6 cells by qRT-PCR. E and F, Examination of the expression of the inflammatory factors TNF-α and IL-6 in cells supernatants by ELISA. IEC-6 cells were exposed to LPS (50 μg/ml) in the absence or presence of TUDCA for 6 h. Data are expressed as mean ± SD for three independent experiments. $^{###}P < 0.001$ *vs.* CON group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* LPS group.

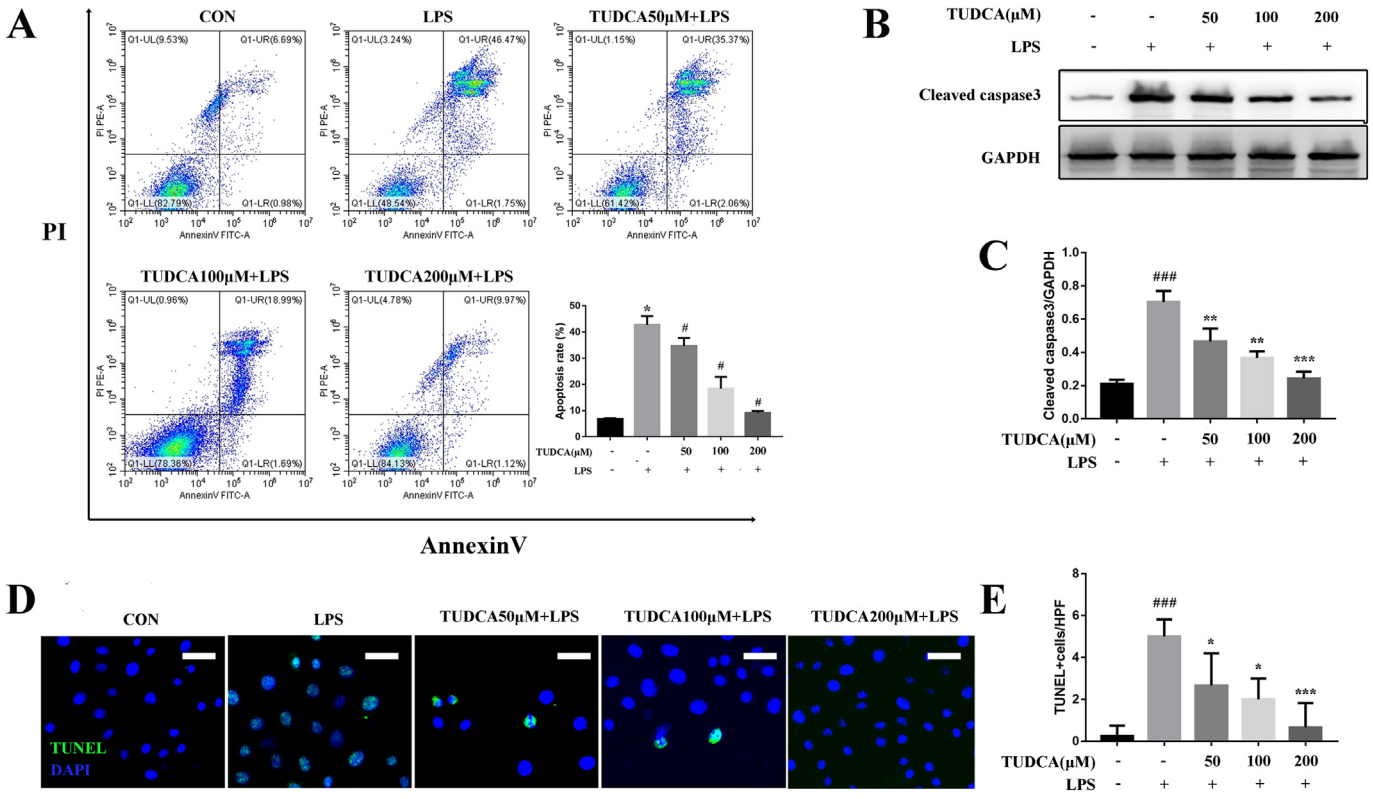

Fig. 5. The effect of TUDCA on LPS-induced apoptosis of IEC-6 cells *in vitro*.
A, Examination and quantification of apoptotic rates by flow cytometry. B, Western blot analysis of the expression levels of Cleaved caspase-3, and GAPDH in IEC-6 cells exposed to LPS (50 μg/ml) in the absence or presence of TUDCA for 6 h. C, Densitometric analysis was used to quantify the levels of cleaved caspase-3, and GAPDH. D, Examination of apoptosis by TUNEL staining (green). DAPI (blue) was used as a nuclear marker. E, Quantification of the TUNEL-stained apoptotic cells. Data are expressed as mean ± SD for three independent experiments. $^{###}P < 0.001$ *vs.* CON group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* LPS group. *Scale bars*, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. TUDCA exerts a protective effect on the NEC mouse model by activating the AKT pathway and inhibiting ERS in intestinal cells

Studies have shown that the levels of intestinal ERS markers are increased in children with NEC and in animal models, and ERS may play an important role in the occurrence of NEC. Therefore, we investigated the effect of TUDCA on intestinal ERS in a mouse model of NEC. BiP and C/EBP homologous protein (CHOP) are important markers of ERS. The RT-PCR and Western blot results showed that TUDCA intervention inhibited the mRNA and protein levels of BiP and CHOP in

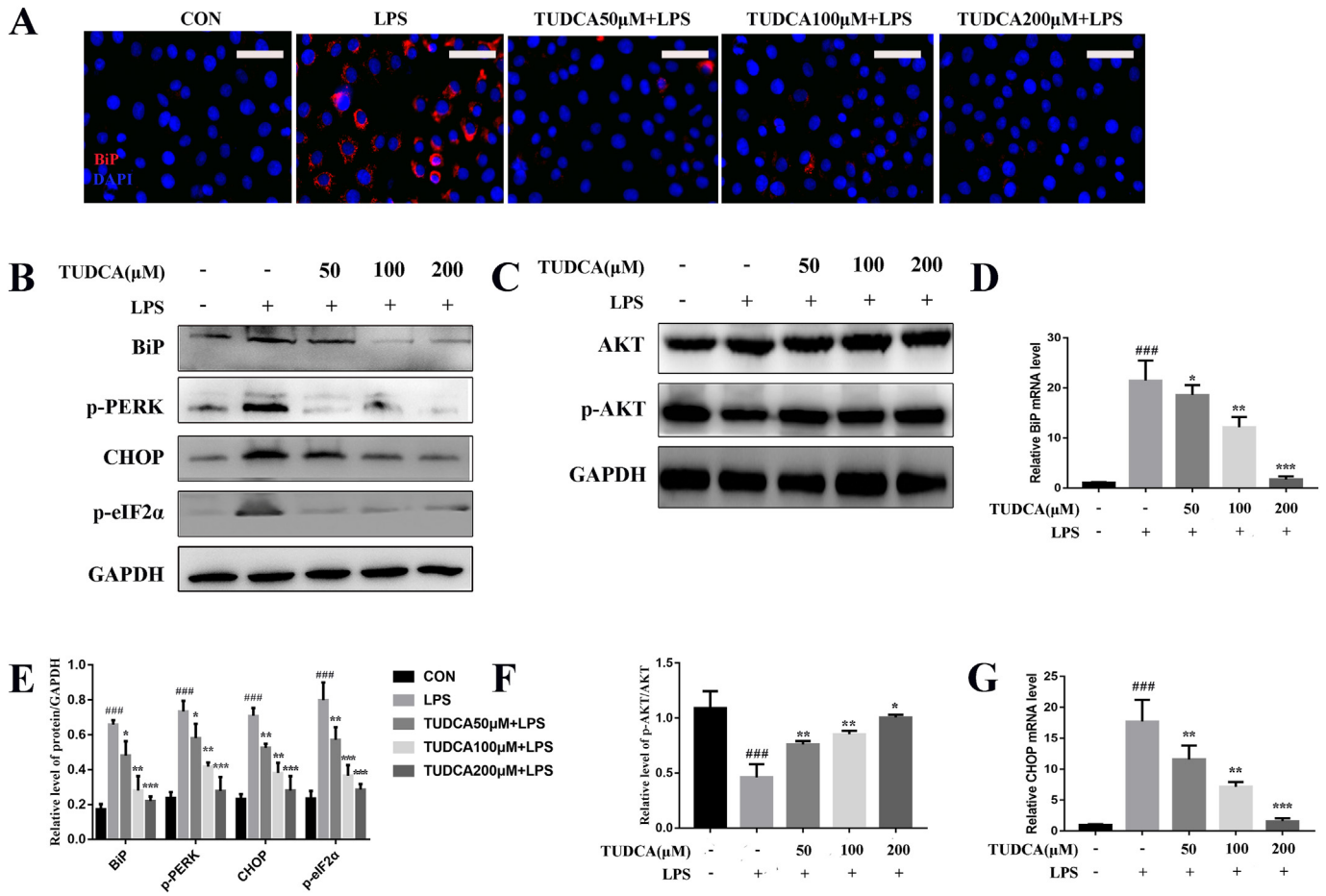

Fig. 6. The effect of TUDCA on the PERK- eIF2α ERS pathway in IEC-6 cells *in vitro*.
A, Examination of the expression of BiP protein (red) by immunofluorescence staining in IEC-6 cells exposed to LPS (50 μg/ml) in the absence or presence of TUDCA for 6 h. DAPI (blue) was used as a nuclear marker. B and C, The protein levels of BiP, p-PERK, p-eIF2α, CHOP, p-AKT, AKT, and GAPDH were evaluated by Western blotting in IEC-6 cells exposed to LPS (50 μg/ml) in the absence or presence of TUDCA for 6 h. E and F, Densitometric analysis was used to quantify the levels of BiP, p-PERK, p-eIF2α, CHOP, p-AKT, AKT, and GAPDH. D and G, Determination of the mRNA expression levels of BiP, CHOP, and GAPDH by qRT-PCR. Data are expressed as mean ± SD for three independent experiments. $^{###}P < 0.001$ *vs.* CON group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* LPS group. *Scale bars*, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NEC + TUDCA group. Therefore, TUDCA treatment was able to improve ERS in the ileal tissue of the NEC mouse model (Fig. 3B–G). Immunofluorescence analysis of BiP confirmed the Western blot and RT-PCR results (Fig. 3A). Subsequently, we examined the effects of TUDCA on several other key proteins related to ERS. The Western blot results showed that TUDCA intervention inhibited the expression levels of phospho-PERK(p-PERK) and phospho-eIF2α(p-eIF2α) proteins in NEC + TUDCA group; moreover, TUDCA intervention increased the protein level of p-AKT in NEC + TUDCA group.

3.4. TUDCA reduces cell death and relieves inflammatory response in IEC-6 cells

To further verify the above results, we examined the effect of TUDCA on LPS-treated IEC-6 cells *in vitro*. Exposure of the IEC-6 cells that had been treated with LPS for 6 h to TUDCA intervention (50, 100 and 200 μM) decreased the release of LDH, enhanced cell viability, reduced cell death (Fig. 4A, B), and diminished the release of inflammatory factors (Fig. 4C–F). Therefore, TUDCA protected the LPS-treated IEC-6 cells from death.

3.5. TUDCA inhibits the apoptosis of IEC-6 cells

Next, we further examined the effect of TUDCA on the apoptosis of the LPS-treated IEC-6 cells. The results of TUNEL assays showed that treatment with TUDCA (50, 100 and 200 μM) significantly reduced the apoptosis in IEC-6 cells (Fig. 5D, E). This result was supported by Western blot and flow cytometric analyses of the levels of cleaved caspase-3 in IEC-6 cells (Fig. 5A–C).

3.6. TUDCA inhibits ERS by activating the AKT pathway in IEC-6 cells

To investigate the molecular mechanism of action of TUDCA in ERS-induced apoptosis of IEC-6 cells, we first examined *in vitro* the protein and mRNA expression levels of the ERS markers BiP and CHOP. The qRT-PCR and Western blotting results showed that treatment with TUDCA (50, 100 and 200 μM) reduced the mRNA and protein levels of BiP and CHOP (Fig. 6C–E). Immunofluorescence result also showed that TUDCA treatment reduced the expression of BiP (Fig. 6A). To further investigate the pathways through which TUDCA improves ERS, the protein levels of p-PERK and p-eIF2α were measured *in vitro*. The Western blot results showed that treatment with TUDCA (50, 100 and 200 μM) reduced the levels of p-PERK, and p-eIF2α proteins. In addition, the Western blot results showed that TUDCA treatment increased p-AKT protein levels (Fig. 6B–G). We further used AKT inhibitor MK2206 to investigate the connection between activation of the Akt pathway and TUDCA. Results indicated that the reduction of LPS-induced ERS and apoptosis by TUDCA were significantly impaired by co-

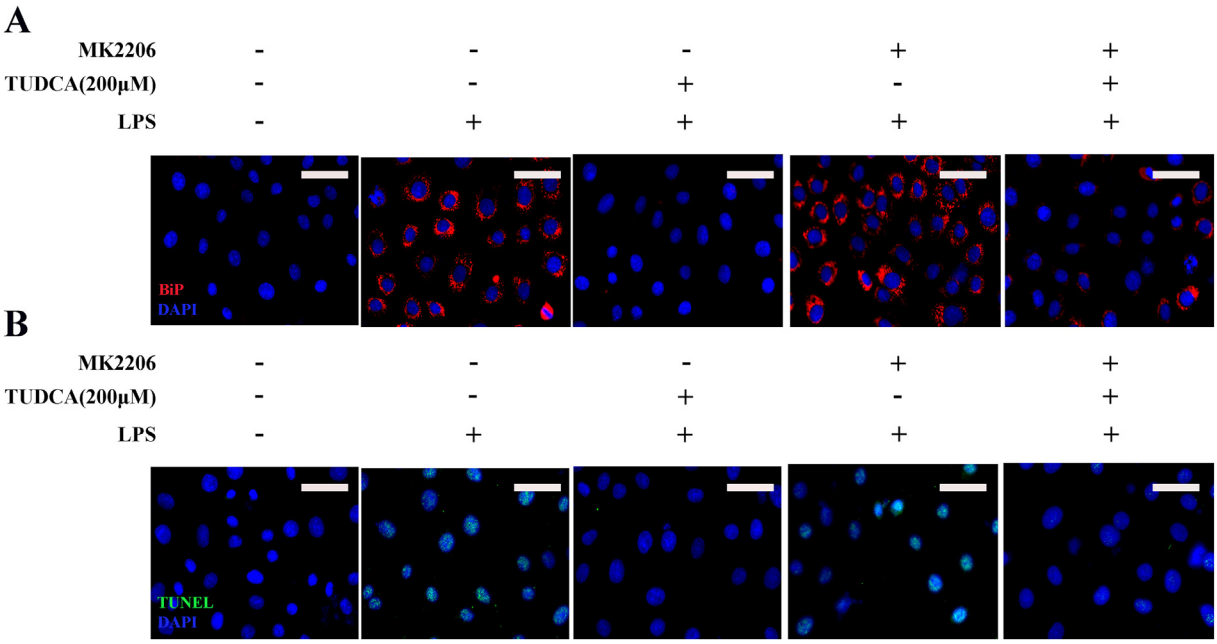

Fig. 7. The effect of TUDCA on the ERS and apoptosis by blocking the AKT in IEC-6 cells *in vitro*.
A, Examination of the expression of BiP protein (red) in various groups of cells by immunofluorescence staining in IEC-6 cells exposed to LPS (50 μg/ml) in the presence of TUDCA or MK2206 for 6 h. DAPI (blue) was used as a nuclear marker. B, Examination of apoptosis by TUNEL staining (green) in IEC-6 cells exposed to LPS (50 μg/ml) in the presence of TUDCA or MK2206 for 6 h. DAPI (blue) was used as a nuclear marker. *Scale bars*, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treated with MK2206 (Fig. 7A, B). The above results further demonstrated that TUDCA regulated the ERS-induced IEC-6 cell apoptosis through activating the AKT pathway and inhibiting the PERK-eIF2α pathway.

4. Discussion

In China, the incidence and mortality rate of NEC are currently very high among infants with very low birth weight [17]. The pathogenesis of NEC remains unclear. The development and progression of NEC involve complex pathophysiological processes in premature infants, such as intestinal immaturity, abnormal bacterial colonization and hyperimmune response [18,19]. Because the exact cause and pathogenic mechanism of NEC are unclear, there are currently no effective preventive and therapeutic measures for NEC in clinical practice. TUDCA is an effective ingredient of bear bile. Clinically, TUDCA is mainly used to treat diseases such as cholestatic liver disease and gallstones [20,21]. Studies have found that TUDCA inhibits apoptosis through inhibiting ERS. This drug is conducive to improving cardiac function and alleviating the progression of Parkinson's disease [22,23]. In addition, studies have found that TUDCA inhibits enteritis similar to Crohn's disease [24,25]. By establishing a neonatal mouse model of NEC and conducting *in vitro* experiments, the present study found that TUDCA improved inflammation and significantly inhibited apoptosis, thereby reducing intestinal damage in the NEC mouse models. The molecular mechanism underlying TUDCA mediated inhibition of intestinal cell apoptosis may involve the regulation of the PERK-eIF2α ERS pathway through activating the AKT pathway.

Intestinal epithelial cell apoptosis is an important cause of NEC [26]. Apoptosis is co-activated by the endogenous and the exogenous apoptotic signaling pathways. Endogenous apoptosis is initiated by a variety of microenvironmental disturbances, including DNA damage, ERS and reactive oxygen species (ROS) overload [27]. ERS-induced apoptosis is one of the endogenous apoptotic signaling pathways. Apoptotic mechanisms are activated during sustained ERS. Our study found that TUDCA protected the intestinal tract in the NEC mouse model by reducing the levels of BiP and CHOP, the key regulatory factors of ERS. The ER chaperone protein BiP is a major regulatory factor of ER homeostasis and stress response. It promotes protein folding and assembly and interacts with three major UPR sensors to jointly affect the ERS response. The three ER transmembrane effector proteins are PERK, IRE1 and ATF6. CHOP is a marker in the UPR and ERS response [28]. Under normal conditions, CHOP is expressed at a low level. However, apoptosis is initiated when the self-protection mechanism is overwhelmed. During ERS-induced apoptosis, CHOP accumulation in the nucleus is positively upregulated [29]. The results of the present study showed that TUDCA treatment inhibited the expression of CHOP. PERK induces the transcription of CHOP and the activation of the apoptotic machinery [30]. We further conducted *in vitro* and *in vivo* experiments and found that TUDCA reduced the levels of p-PERK and p-eIF2α proteins. These results demonstrate that TUDCA regulates ERS-induced apoptosis through regulating the PERK-eIF2α pathway, thereby protecting the intestinal tract in the NEC mouse model.

Akt is a serine threonine kinase. It plays key roles in the intracellular signal transduction pathways related to cell proliferation, cell survival, inflammation, and metabolism [12,31]. Previous studies have shown that intestinal epithelial cell apoptosis aggravates tissue damage and functional impairment. There is evidence that the activation of the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway antagonizes apoptosis and exerts an important protective effect on the intestinal tract in NEC [32]. After the onset of NEC, Akt phosphorylation attenuates intestinal epithelial cell apoptosis by altering the expression of the downstream molecules (such as caspase-3). To investigate the protective mechanism of TUDCA on NEC intestine, we measured the intestinal ratio of p-Akt/Akt in the NEC mouse model. The results showed that the ratio of p-Akt/Akt was significantly reduced after the occurrence of NEC. Compared with the NEC group, administration of TUDCA increased the ratio of p-Akt/Akt in the corresponding group. *In vitro*, experiments verified these results. In addition, TUDCA effects were impaired by AKT specific inhibitor MK2206 *in vitro* studies. In summary, the results of the present study demonstrate that TUDCA

inhibits PERK activity by activating the Akt signaling pathway, thereby attenuating intestinal damage in NEC.

Studies have shown that cytokines participate in the entire pathophysiological process of NEC by aggravating inflammatory response and cell damage. The interaction between these inflammatory factors exacerbates the inflammatory response, leading to the aggregation and infiltration of intestinal leukocytes and the aggravation of microcirculatory disorder. Many studies show that the upregulation of TNF-α and IL-6 in NEC directly causes intestinal injury by exacerbating the inflammatory response [33,34]. Our results further demonstrated that TUDCA treatment significantly reduced serum TNF-α and IL-6 levels and effectively relieved intestinal damage in NEC mice. Although previous studies have shown that ERS and inflammatory signaling pathways are intrinsically linked through multiple mechanisms and that ERS promotes the occurrence of inflammation in intestinal cells [35,36], the relationship between ERS and inflammation in NEC needs to be further investigated.

In summary, the present study investigated the effects of TUDCA on NEC intestinal injury *via in vivo* and *in vitro* experiments. The results indicated that TUDCA treatment reduced the severity of intestinal damage and inhibited the apoptosis of intestinal cells in NEC. The mechanism involved in the above process may be that TUDCA inhibits the PERK-eIF2α ERS pathway through activating the Akt pathway. The present study provides new ideas for future investigation of the pathogenesis and treatment of NEC.

Author contributions

Peng Li and Zhibao Lv designed the experiments and research project. Peng Li and Dong Fu performed the experiments and analyzed the data. Qingfeng Sheng, Shenghua Yu participated in the discussion. Peng Li and Zhibao Lv wrote the paper.

Declaration of Competing Interest

The authors declare no conflict of interests.

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References

[1] B.J. Stoll, N.I. Hansen, E.F. Bell, et al., Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993–2012[J], JAMA 314 (10) (2015) 1039–1051.
[2] J. Neu, W.W.A. Medical progress, necrotizing enterocolitis, J, N. Engl. J. Med. 364 (3) (2011) 255–264.
[3] S. Eaton, C.M. Rees, N.J. Hall, Current research on the epidemiology, pathogenesis, and management of necrotizing enterocolitis[J], Neonatology 111 (4) (2017) 423–430.
[4] T. Anelli, R. Sitia, Physiology and pathology of proteostasis in the early secretory compartment[J], Semin. Cell Dev. Biol. 21 (5) (2010) 520–525.
[5] E. Dufey, D. Sepulveda, D. Rojas-Rivera, et al., Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 1. An overview[J], Am. J. Phys. Cell Phys. 307 (7) (2014) C582–C594.
[6] S.S. Cao, Endoplasmic reticulum stress and unfolded protein response in inflammatory bowel disease[J], Inflamm. Bowel Dis. 21 (3) (2015) 636–644.
[7] Y.P. Liu, L. Zeng, A. Tian, et al., Endoplasmic reticulum stress regulates the innate immunity critical transcription factor IRF3[J], J. Immunol. 189 (9) (2012) 4630–4639.
[8] C. Hetz, F.R. Papa, The unfolded protein response and cell fate control[J], Mol. Cell. 69 (2) (2018) 169–181.
[9] A. Afrazi, M.F. Branca, C.P. Sodhi, et al., Toll-like receptor 4-mediated endoplasmic reticulum stress in intestinal crypts induces necrotizing enterocolitis[J], J. Biol. Chem. 289 (14) (2014) 9584–9599.
[10] P. Lu, M.C. Struijs, J. Mei, et al., Endoplasmic reticulum stress, unfolded protein response and altered T cell differentiation in necrotizing enterocolitis[J], PLoS One 8 (10) (2013) e78491.
[11] W. Wang, J. Zhao, W. Gui, et al., Tauroursodeoxycholic acid inhibits intestinal inflammation and barrier disruption in mice with non-alcoholic fatty liver disease [J], Br.J.Pharmacol. 175 (3) (2018) 469–484.
[12] D. Sun, G. Gu, J. Wang, et al., Administration of tauroursodeoxycholic acid attenuates early brain injury via Akt pathway activation[J], Front. Cell. Neurosci. 11 (2017) 193.
[13] Q.F. Sheng, Z.B. Lv, W. Cai, et al., Human beta-defensin-3 promotes intestinal epithelial cell migration and reduces the development of necrotizing enterocolitis in a neonatal rat model[J], Pediatr. Res. 76 (3) (2014) 269–279.
[14] Q. Sheng, Z. Lv, W. Cai, et al., Protective effects of hydrogen-rich saline on necrotizing enterocolitis in neonatal rats[J], J. Pediatr. Surg. 48 (8) (2013) 1697–1706.
[15] M. Doerflinger, J. Glab, C. Nedeva, et al., Chemical chaperone TUDCA prevents apoptosis and improves survival during polymicrobial sepsis in mice[J], Sci. Rep. 6 (2016) 34702.
[16] M. Ginzel, Y. Yu, C. Klemann, et al., The viral dsRNA analogue poly (I:C) induces necrotizing enterocolitis in neonatal mice[J], Pediatr. Res. 79 (4) (2016) 596–602.
[17] T. Qian, R. Zhang, L. Zhu, et al., Necrotizing enterocolitis in low birth weight infants in China: mortality risk factors expressed by birth weight categories[J], Pediatr. Neonatol. 58 (6) (2017) 509–515.
[18] D.F. Nino, C.P. Sodhi, D.J. Hackam, Necrotizing enterocolitis: new insights into pathogenesis and mechanisms[J], Nat. Rev. Gastroenterol. Hepatol. 13 (10) (2016) 590–600.
[19] B.B. Warner, P.I. Tarr, Necrotizing enterocolitis and preterm infant gut bacteria[J], Semin. Fetal Neonatal Med. 21 (6) (2016) 394–399.
[20] Y. Hou, H. Yang, Z. Cui, et al., Tauroursodeoxycholic acid attenuates endoplasmic reticulum stress and protects the liver from chronic intermittent hypoxia induced injury[J], Exp. Ther. Med. 14 (3) (2017) 2461–2468.
[21] P. Invernizzi, K.D. Setchell, A. Crosignani, et al., Differences in the metabolism and disposition of ursodeoxycholic acid and of its taurine-conjugated species in patients with primary biliary cirrhosis[J], Hepatology 29 (2) (1999) 320–327.
[22] S. Rani, P.K. Sreenivasaiah, J.O. Kim, et al., Tauroursodeoxycholic acid (TUDCA) attenuates pressure overload-induced cardiac remodeling by reducing endoplasmic reticulum stress[J], PLoS One 12 (4) (2017) e0176071.
[23] A.I. Rosa, I. Fonseca, M.J. Nunes, et al., Novel insights into the antioxidant role of tauroursodeoxycholic acid in experimental models of Parkinson's disease[J], Biochim. Biophys. Acta 1863 (9) (2017) 2171–2181.
[24] L. Van Den Bossche, D. Borsboom, S. Devriese, et al., Tauroursodeoxycholic acid protects bile acid homeostasis under inflammatory conditions and dampens Crohn's disease-like ileitis[J], Lab. Investig. 97 (5) (2017) 519–529.
[25] L. Van Den Bossche, P. Hindryckx, L. Devisscher, et al., Ursodeoxycholic acid and its taurine- or glycine-conjugated species reduce colitogenic dysbiosis and equally suppress experimental colitis in mice[J], Appl. Environ. Microbiol. 83 (7) (2017).
[26] Y. Zhou, Y. Li, B. Zhou, et al., Inflammation and apoptosis: dual mediator role for toll-like receptor 4 in the development of necrotizing enterocolitis[J], Inflamm. Bowel Dis. 23 (1) (2017) 44–56.
[27] L. Galluzzi, I. Vitale, S.A. Aaronson, et al., Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018[J], Cell Death Differ. 25 (3) (2018) 486–541.
[28] S.S. Cao, Epithelial ER stress in Crohn's disease and ulcerative colitis[J], Inflamm. Bowel Dis. 22 (4) (2016) 984–993.
[29] J. Han, S.H. Back, J. Hur, et al., ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death[J], Nat. Cell Biol. 15 (5) (2013) 481–490.
[30] K. Zhang, R.J. Kaufman, From endoplasmic-reticulum stress to the inflammatory response[J], Nature 454 (7203) (2008) 455–462.
[31] Z.F. Wang, Z.Y. Pan, C.S. Xu, et al., Activation of G-protein coupled estrogen receptor 1 improves early-onset cognitive impairment via PI3K/Akt pathway in rats with traumatic brain injury[J], Biochem. Biophys. Res. Commun. 482 (4) (2017) 948–953.
[32] S.R. Shiou, Y. Yu, S. Chen, et al., Erythropoietin protects intestinal epithelial barrier function and lowers the incidence of experimental neonatal necrotizing enterocolitis[J], J. Biol. Chem. 286 (14) (2011) 12123–12132.
[33] R. Drut, Up-regulation of macrophage migration inhibitory factor in infants with acute neonatal necrotizing enterocolitis[J], Histopathology 49 (1) (2006) 100–101 (author reply 101).
[34] N. Baregamian, J. Song, C.E. Bailey, et al., Tumor necrosis factor-alpha and apoptosis signal-regulating kinase 1 control reactive oxygen species release, mitochondrial autophagy, and c-Jun N-terminal kinase/p38 phosphorylation during necrotizing enterocolitis[J], Oxidative Med. Cell. Longev. 2 (5) (2009) 297–306.
[35] F. Martinon, X. Chen, A.H. Lee, et al., TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages[J], Nat. Immunol. 11 (5) (2010) 411–418.
[36] S. Hosomi, J. Grootjans, M. Tschurtschenthaler, et al., Intestinal epithelial cell endoplasmic reticulum stress promotes MULT1 up-regulation and NKG2D-mediated inflammation[J], J. Exp. Med. 214 (10) (2017) 2985–2997.