

## ORIGINAL ARTICLE

# TNF $\alpha$ -induced abnormal activation of TNFR/NF- $\kappa$ B/FTH1 in endometrium is involved in the pathogenesis of early spontaneous abortion

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## Abstract

Early spontaneous abortion (ESA) is one of the most common complications during pregnancy and the inflammation condition in uterine environment such as long-term exposure to high TNF $\alpha$  plays an essential role in the aetiology. Ferritin heavy chain (FTH1) is considered to be closely associated with inflammation and very important in normal pregnancy, yet the underlying mechanism of how TNF $\alpha$  induced abortion and its relationship with FTH1 remain elusive. In this study, we found that TNF $\alpha$  and FTH1 were positively expressed in decidual stromal cells and increased significantly in the ESA group compared with the normal pregnancy group (NP group). Besides, TNF $\alpha$  expression was positively correlated with FTH1 expression. Furthermore, in vitro cell model demonstrated that high TNF $\alpha$  could induce the abnormal signals of TNFR/NF- $\kappa$ B/FTH1 and activate apoptosis both in human endometrium stromal cells (hESCs) and in local decidual tissues. Taken together, the present findings suggest that the excessive apoptosis in response to TNF $\alpha$ -induced upregulation of FTH1 may be responsible for the occurrence of ESA, and thus provide a possible therapeutic target for the treatment of ESA.

## KEYWORDS

early spontaneous abortion, FTH1, inflammation, TNF $\alpha$

## 1 | INTRODUCTION

Pregnancy is a physiological process that is often accompanied by a variety of complications, and it has significant effects on the physical and mental health in women of reproductive age.

Spontaneous abortion is one of the most common pregnancy complications with a clinical incidence of around 15%, in which more than 80% cases occur before 12 weeks, also known as early spontaneous abortion (ESA).<sup>1,2</sup> Many factors may lead to the occurrence of ESA, including chromosomal abnormalities of the

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embryo, endocrine disorders of the mother, uterine dysplasia or malformation, immune dysfunction, and reproductive tract infection.<sup>2-6</sup> However, about 50% of the aetiology and pathogenesis of the ESA are still elusive.

Researches have indicated that successful pregnancy requires the participation of inflammatory reaction, which is accompanied by the increase of cytokines and chemokines.<sup>7-9</sup> Tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) is thought to be one of the key cytokines and plays a vital role in the establishment and maintenance of pregnancy, whereas excessive TNF $\alpha$  has been linked to adverse pregnancy outcomes. Abnormally elevated levels of TNF $\alpha$  have been detected in both the local intrauterine and the serum of people and animals suffering abortion.<sup>7,10-12</sup> Previous studies have proposed using the TNF $\alpha$  expression as a diagnostic indicator of miscarriage and applying to guide its clinical treatment.<sup>12,13</sup> Although TNF $\alpha$  blocking agents have been applied for some chronic immune-mediated inflammatory diseases during pregnancy, such as inflammatory bowel disease and psoriasis, and seem no harm for pregnancy and newborns, it is still controversial for its clinical safety.<sup>14,15</sup> Therefore, better understanding of the association between TNF $\alpha$  and ESA and its possible underlying mechanism will be helpful for avoiding the development of ESA.

Iron and iron-binding proteins such as ferritin are also closely related to inflammation regulation during pregnancy. Ferritin is a ubiquitous and highly conserved protein that can bind to numerous irons and convert them into a nontoxic and bioavailable form to store inside its protein shell.<sup>16,17</sup> Abnormal alteration of ferritin content is usually linked with a variety of diseases, such as anaemia, pregnancy complications, kidney disease and Parkinson's disease.<sup>18-23</sup> It has been reported that ferritin concentration is higher in women with ESA than women with healthy pregnancy.<sup>24,25</sup> Besides, increased serum ferritin is also associated with higher rate of preterm birth.<sup>21,22</sup> As far as we know that mammalian ferritins are consist of two subunits named ferritin heavy chain (FTH1) and ferritin light chain (FTL), of them, FTH1 was considered to play an essential role in the process of pregnancy because its expression continues to increase throughout the gestation in response to progesterone.<sup>26</sup> Besides, Ferreira, et al. delete the *Fth* gene in mice using homologous recombination and discover that the mice undergo early embryonic lethality at 9.5 gestation days.<sup>16</sup> However, whether FTH1 fulfils functions in normal embryo implantation and the dysregulation of FTH1 poses implications on ESA remain unexplored.

Herein, we examined the changes of TNF $\alpha$  and FTH1 expression in patients suffered from ESA, and find that the expression of both TNF $\alpha$  and FTH1 were increased in local decidual tissues and show a strong correlation. In vitro experiments demonstrate that the activation of the TNFR/NF- $\kappa$ B/FTH1 pathway induced by TNF $\alpha$  would lead to excessive apoptosis, and the activation was further confirmed in the patients; thus, the dysregulated pathway may represent a novel therapeutic target of ESA.

## 2 | MATERIALS AND METHODS

### 2.1 | Subject sample collection

Eighteen women with normal pregnancy who chose induced abortion were enrolled as NP group, and seventeen women suffered from early spontaneous abortion were enrolled as ESA group. Their decidual tissues were collected via artificial abortion-vacuum aspiration. Ultrasound examination, menstrual cycle and blood hCG levels were used to confirm the viable intrauterine pregnancy and the gestational age of all subjects. All the subjects were enrolled according to the inclusion criteria as follows: i) The gestational age was ranged from 6 weeks to 12 weeks, ii) no malformation in reproductive tract and uterine, iii) no reproductive endocrine diseases or tract infections, and iv) no family history of heredity. This study was approved by the Ethical Review Board of West China Second University Hospital, Sichuan University. Informed consent was obtained from each subject in our study.

### 2.2 | Cell culture

Human endometrium stromal cells (hESCs) were purchased from iCell Bioscience Inc (Shanghai, China) and cultured in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 nM penicillin/streptomycin under an incubator with 5% CO<sub>2</sub> and 37°C.

### 2.3 | Real-time PCR (RT-PCR)

Total RNA was isolated from human decidual tissues or hESCs by the TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was converted to cDNAs using PrimeScript RT Reagent (Takara, Japan) in accordance with the manufacturer's instruction. SYBR Green Fast qPCR Mix (Bimake, China) was used to conduct RT-PCR on an iCycler RT-PCR Detection System (Bio-Rad Laboratories). Each assay was performed in triplicates for each sample, and *GAPDH* was applied as an internal control. The relative mRNA expression was quantified using the 2<sup>- $\Delta\Delta$ Ct</sup> method.<sup>27</sup> The primers for RT-PCR were listed in Table S1.

### 2.4 | Western blotting

The proteins of human decidual tissues or the cultured cells were extracted and separated using 12.5% SDS-polyacrylamide gels. Whereafter, the proteins were blotted into a polyvinylidene difluoride (PVDF) membrane (Millipore) and blocked with 5% skim milk for 1 h at room temperature. Subsequently, the membrane was incubated with primary antibodies, including anti-GAPDH, anti-TNF $\alpha$

and anti-FTH1, for 4°C overnight, and then incubated with corresponding secondary antibodies (goat anti-mouse IgG or goat anti-rabbit IgG, Abcam, US) for 1 h at room temperature. ECL Western blotting kit (Millipore) was applied to develop the blots.

## 2.5 | Enzyme-linked immunosorbent assay (ELISA)

The proteins were isolated from human decidual tissues, and the concentration of TNF $\alpha$  and FTH1 of the protein lysis buffers was detected by using Human TNF alpha Uncoated ELISA (Invitrogen, USA) and Human Ferritin Heavy chain 1 (FTH1) ELISA Kit (Mlbio, China). The procedure followed the instructions strictly. 3,3',5,5'-Tetramethylbenzidine (TMB) was applied to colour reaction. Absorbance was read at 450 nm after adding Stop Solution within 15 min. The concentration was calculated according to the standard curve.

## 2.6 | Immunofluorescence staining (IF)

For the immunofluorescence staining of human decidual tissues, the deparaffinized and rehydrated slides were boiled in 10 mM citrate buffer (pH 6.0) to enable antigen retrieval. Then, the slides were blocked with 10% normal donkey serum for 1 h at room temperature and incubated with primary antibodies which included anti-TNF $\alpha$  (Abclonal, China), anti-FTH1 (Santa Cruz, USA), anti-TNFR1 (Abcam, USA), anti-TNFR2 (Proteintech, China), anti-NF- $\kappa$ B (Proteintech, China) and anti-caspase3 (Abclonal, China) for 4°C overnight. After that, the slides were washed by 1 $\times$  PBS three times for 10 min each time, followed by 1-hour room temperature incubation of corresponding fluorescent secondary antibodies (AlexaFluor 594 anti-Rabbit, AlexaFluor 488 anti-Mouse, Invitrogen, USA) and 4,6-diamidino-2-phenylindole (DAPI). The slides were scanned and imaged by a digital slice scanner (Pannoramic MIDI, 3DHIESTECH, Hungary).

For the immunofluorescent staining of cultured cells, the slides were fixed with 4% paraformaldehyde and punched with 0.3% Triton X-100 in PBS for 15 min. After that, the slides were blocked with 5% BSA and incubated with primary antibodies including anti-NF- $\kappa$ B (Proteintech, China). The following steps were consistent with the immunofluorescent staining of decidual tissues. The slides were observed and imaged by a laser scanning confocal microscope (Olympus, Japan).

## 2.7 | Cell counting kit-8 analysis

The proliferation and cell viability of hESCs were evaluated by cell counting kit-8 (CCK-8, Bimake, China) according to the manufacturer's instructions. 5000 cells were seeded in each well of a 96-well plate and cultured overnight. After being treated with drugs, 10  $\mu$ L CCK-8 was added into each well, and the optical densities which reflected the cell numbers were determined at the absorbance of 450 nm after the incubation for 2 hours.

## 2.8 | Apoptosis assays

After hESCs were treated with or without 10 ng/ml TNF $\alpha$  for 72 or 96 h, the cells were collected to stain with FITC-conjugated Annexin V and propidium iodide (PI) using Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) in accordance with the recommendation of the manufacturer. The percentage of apoptotic cells was analysed by flow cytometry (BD Biosciences).

## 2.9 | TUNEL assay

TUNEL assay was carried out in accordance with the guideline of TUNEL BrightGreen Apoptosis Detection Kit (Vazyme). After the sections of decidual tissues were carefully deparaffinized and rehydrated, the slides were permeabilized with 20  $\mu$ g/ml proteinase K, washed by 1 $\times$  PBS three times and balanced with 1 $\times$  Equilibration Buffer for 20 min. Then, BrightGreen Labeling Mix was applied to mark the apoptotic cells by incubating at 37°C for 1 h. Subsequently, the slides were washed by 1 $\times$  PBS, stained with DAPI, and scanned and imaged by a digital slice scanner (Pannoramic MIDI, 3DHIESTECH, Hungary).

## 2.10 | Cell cycle analysis

Detection of the cell cycle of hESCs treated with or without 10 ng/ml TNF $\alpha$  for 72 or 96 h follows the instructions of Cell Cycle Analysis Kit (4A Biotech). After being treated with or without TNF $\alpha$ , the cells were collected and fixed with 75% ethanol overnight. Then, PI was applied to stain the cells at 37°C for 30 min. The determination of the cell cycle was conducted by Flow cytometry (BD Biosciences).

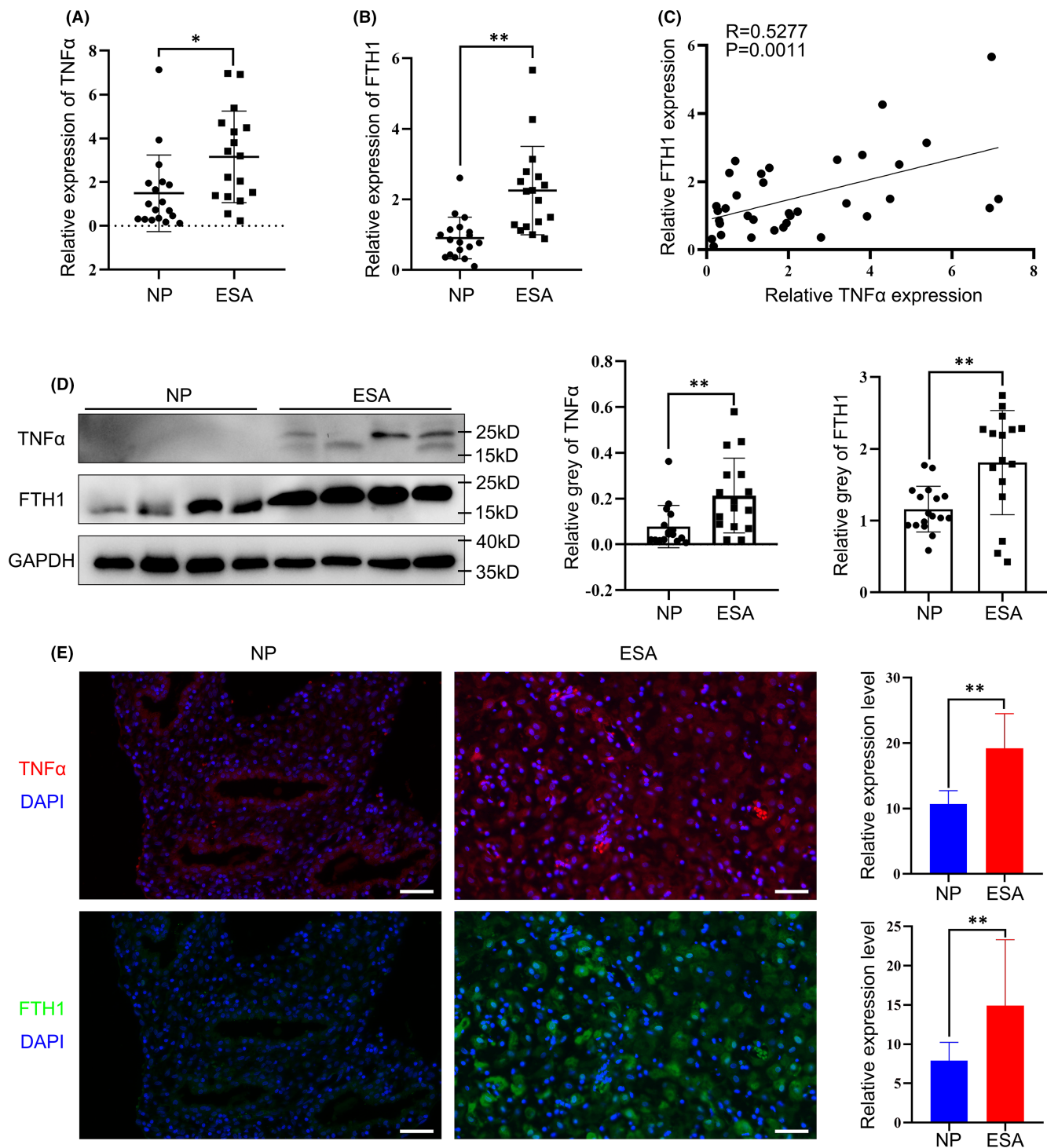
## 2.11 | Statistical analysis

Statistical analysis was performed using SPSS 25.0; all the data were presented as means  $\pm$  standard deviation (SD). For the analysis of two groups, a two-tailed unpaired Student's t-test was used; for the analysis of multiple comparisons, one-way ANOVA with Tukey's

TABLE 1 Clinical characteristics of subjects

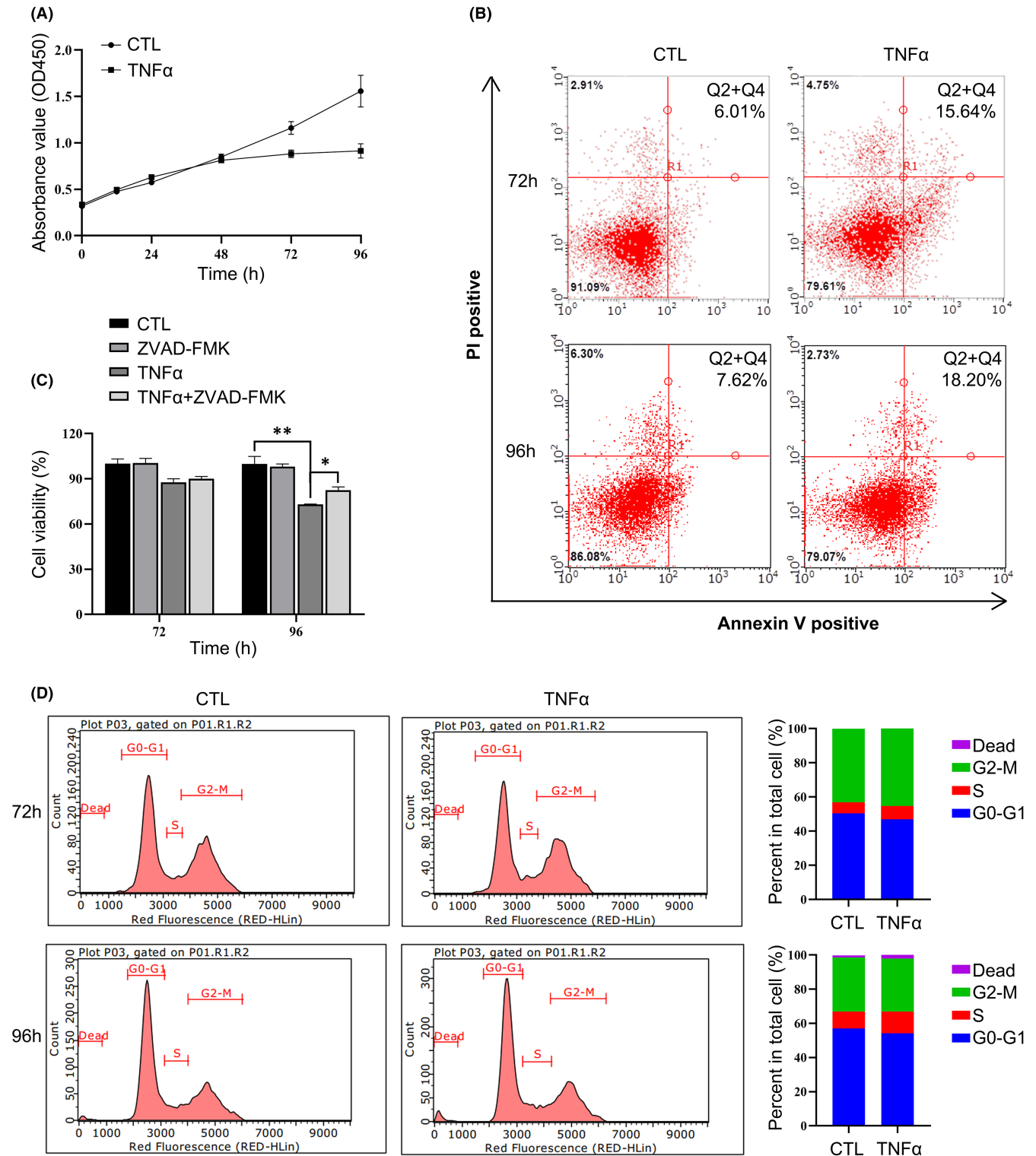
	NP	ESA	<i>p</i>
N	18	17	—
Age (year)	30.29 $\pm$ 3.34	30.56 $\pm$ 3.48	ns
BMI (kg/m <sup>2</sup> )	20.94 $\pm$ 2.44	21.06 $\pm$ 2.29	ns
Gestational age (week)	7.55 $\pm$ 1.20	9.66 $\pm$ 1.41	**

Note: All values were presented as means  $\pm$  standard deviation. ns, no significance; \*\**p* < 0.01.



**FIGURE 1** Expression of TNF $\alpha$  and FTH1 was upregulated in the ESA group compared with the NP group. (A and B) RT-PCR results showed that the mRNA expression of TNF $\alpha$  (A) and FTH1 (B) was increased in the decidua from the ESA group ( $n = 17$ ) compared with the NP ( $n = 18$ ) group. NP, normal control; ESA, early spontaneous abortion. \* $p < 0.05$ , \*\* $p < 0.01$ . (C) Correlation analysis showed a significant positive correlation between the mRNA expression of TNF $\alpha$  and FTH1. (D) The protein expression of TNF $\alpha$  and FTH1 was increased in the decidua from the ESA group ( $n = 16$ ) compared with the NP ( $n = 17$ ) group by Western blotting. The middle and right figures were the statistical analysis of the normalized expression of TNF $\alpha$  and FTH1. \*\* $p < 0.01$ . (E) The immunofluorescence staining of the decidua from the NP group and ESA group showed that TNF $\alpha$  and FTH1 were positively expressed in decidual stromal cells, and manifested stronger intensities in the ESA group compared with the NP group. The statistical diagrams indicated total immunofluorescence staining intensities evaluated by ImageJ. NP,  $n = 4$ ; ESA,  $n = 4$ . \*\* $p < 0.01$ . Scale bar = 50  $\mu$ m





**FIGURE 2** TNF $\alpha$  inhibited cell proliferation of stromal cells by inducing apoptosis but not altering the cell cycle. (A) Long-time treatment of 10 ng/ml TNF $\alpha$  inhibited the proliferation of stromal cells. (B) Long-time exposure of TNF $\alpha$  (10 ng/ml, 72 or 96 h) induced apoptosis of cells. (C) The partial rescue effect of pan-caspase inhibitor ZVAD-FMK on TNF $\alpha$ -induced cell death in hESCs. Cells were exposed to TNF $\alpha$  (10 ng/ml, 72 or 96 h) with or without ZVAD-FMK (10  $\mu$ M). \* $p$  < 0.05, \*\* $p$  < 0.01. D, The cell cycle of the stromal cells was not affected by the treatment of TNF $\alpha$  (10 ng/ml, 72 or 96 h). The left pattern showed the cell cycle detected by flow cytometry, and the right pattern manifested the corresponding statistical graphs of cell proportion at different cycle stages

post hoc test was applied; for the analysis of the correlation between two groups, Person's correlation was conducted. All the in vitro experiments were repeated three times. The  $p$  values less than 0.05 were determined as significant difference.

### 3 | RESULTS

#### 3.1 | Increased expression of TNF $\alpha$ and FTH1 were found in the ESA group

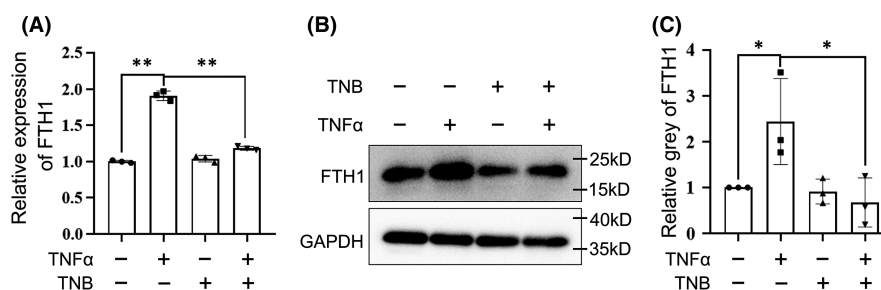
The clinical characteristics of patients from the NP group and the ESA group were investigated and no significant differences in age and BMI between the two groups were found (Table 1), suggesting the samples from the two groups could be used for next analysis. To understand the relationship between TNF $\alpha$ , FTH1 and ESA, RT-PCR was performed to detect the mRNA expression levels of TNF $\alpha$  and FTH1 in decidual tissues from the two groups, and results showed significant increases of TNF $\alpha$  and FTH1 relative expression in the ESA group compared with the NP group (Figure 1A,B). Interestingly, further correlation analysis revealed a remarkably positive correlation between the relative mRNA expression of TNF $\alpha$  and FTH1 ( $N = 18$  for the group of NP,  $N = 17$  for the group of ESA;  $p = 0.0011$ ) (Figure 1C). Consistently, Western blotting and ELISA displayed similar results, which further confirmed that the upregulation of TNF $\alpha$  and FTH1 in the ESA group and their positive correlation with each other (Figure 1D; Figure S1A-C). Additionally, immunofluorescence staining of decidual tissues was applied to demonstrate that both TNF $\alpha$  and FTH1 were located and upregulated in the stromal cells (Figure 1E). Since FTH1 and FTL made up ferritin together, we further explored the expression of FTL in decidual tissues, and similarly discovered an obvious elevation of FTL in the ESA group and a positive correlation with relative TNF $\alpha$  expression (Figure S1D,E). Taken altogether, we speculated that the development of ESA may result from the unusual expression of TNF $\alpha$  and ferritin.

#### 3.2 | TNF $\alpha$ induced the apoptosis of human endometrium stromal cells

To further understand the effect of abnormal expression of TNF $\alpha$  in decidual stromal cells, 10 ng/ml TNF $\alpha$  was used to stimulate hESCs to imitate the intrauterine environment of patients with ESA. After being treated with TNF $\alpha$  for 72 h, the cell proliferation was inhibited and the inhibition was more obvious for 96 h (Figure 2A). Previous studies suggested that the inhibition of cell proliferation may be due to the increase of cell death and the arrest of cell cycles.<sup>28</sup> Hence, the alteration of cell death and cell cycle of hESCs treated with or without TNF $\alpha$  for 72 or 96 h were analysed, and the percentage of apoptotic cells were found to be increased (Figure 2B), besides, it could be partially rescued by apoptosis inhibitor ZVAD-FMK, but not ferroptosis inhibitor ferrostatin-1 (Fer-1) or necroptosis inhibitor necrostatin-1 (Nec-1) (Figure 2C; Figure S2A). While there was no effect on the cell cycle after being treated with TNF $\alpha$  (Figure 2D), these findings indicated that the inhibition of hESC cell proliferation induced by TNF $\alpha$  resulted from the increase of apoptosis rather than the arrest of cell cycle. Some studies have reported toxic effects of TNF $\alpha$  on trophoblast cells during pregnancy<sup>29</sup>; thus, we further investigated the impact of TNF $\alpha$  on the cell viability of human first-trimester extravillous trophoblast cells (HTR8/SVneo); however, the HTR8/SVneo showed a normal growth after long-time treatment of TNF $\alpha$  (Figure S2B), indicating that the stromal cells were more likely to be affected by TNF $\alpha$  treatment.

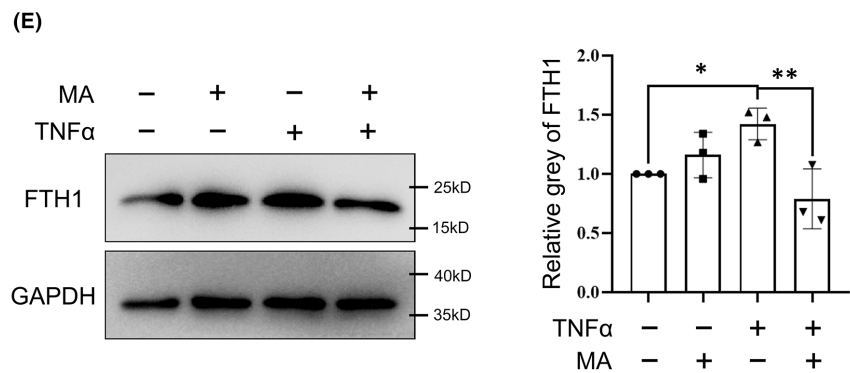
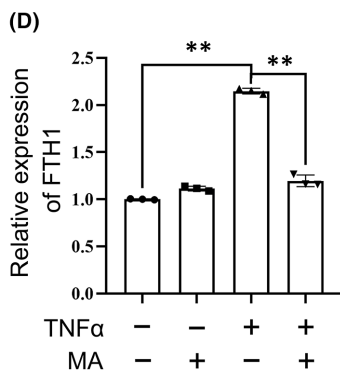
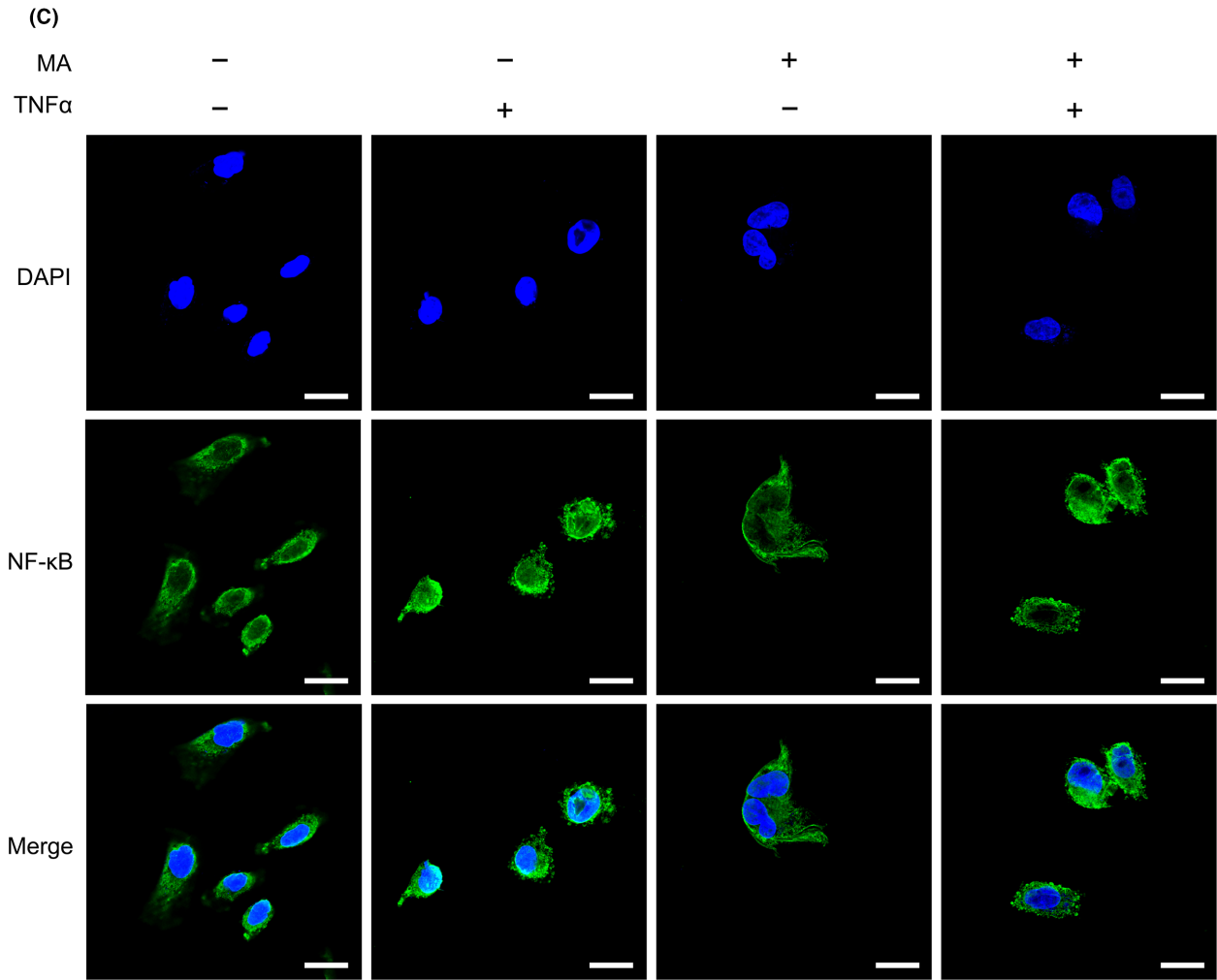
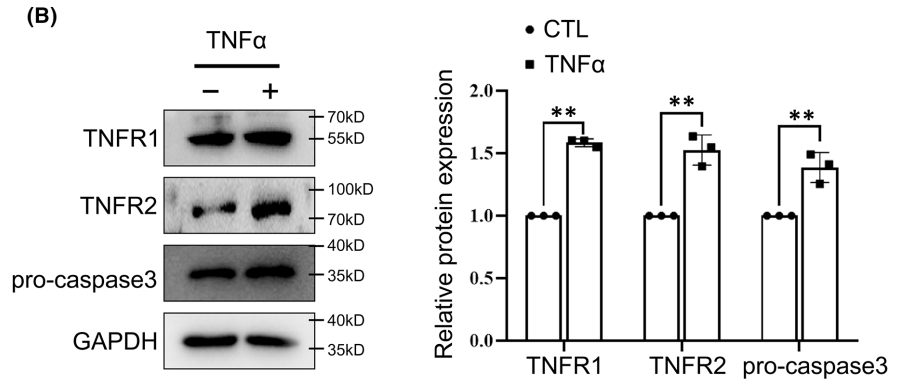
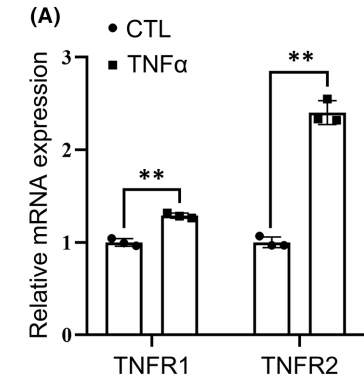
#### 3.3 | The expression of FTH1 was upregulated by TNF $\alpha$ in hESCs

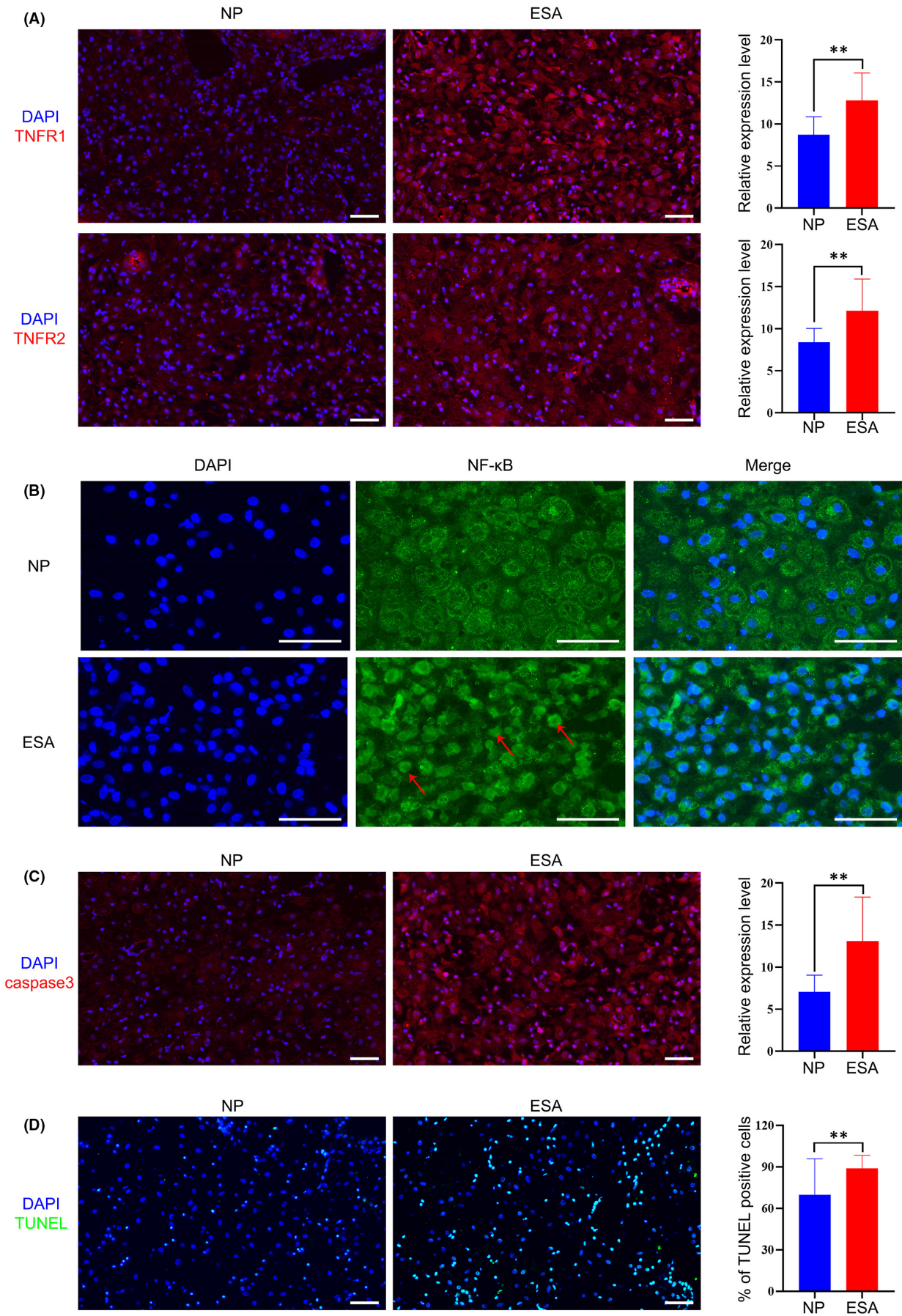
Given that the expression of FTH1 and FTL were significantly positively correlated with the expression of TNF $\alpha$  in the human decidual tissues, we therefore aim to examine whether TNF $\alpha$  could regulate FTL and FTH1 expression. As expected, the expression level of



**FIGURE 3** TNF $\alpha$  induced the increased expression of FTH1. (A and B) Cells were stimulated by TNF $\alpha$  (10 ng/ml) with or without TNB (2  $\mu$ g/ml) for 48 h. Both RT-PCR (A) and Western blotting (B) showed that the expression of FTH1 was upregulated by the treatment of TNF $\alpha$ , and could be neutralized by anti-TNF $\alpha$  TNB. (C) Statistical analysis of the Western blotting. \* $p < 0.05$ , \*\* $p < 0.01$

**FIGURE 4** Expression of FTH1 was mediated by the axis of TNF $\alpha$ /TNFR/NF- $\kappa$ B. A and B, RT-PCR (A) and Western blotting (B) showed that treatment of 10 ng/ml TNF $\alpha$  for 48 h stimulated the elevated expression of TNFR1, TNFR2 and pro-caspase3. C-E, Stromal cells were treated with MA (20  $\mu$ M) in the presence or absence of TNF $\alpha$  (10 ng/ml). The location of NF- $\kappa$ B was observed by immunofluorescence staining (C). The expression of FTH1 was detected through RT-PCR (D) and Western blotting (E). Scale bar = 20  $\mu$ m. \* $p < 0.05$ , \*\* $p < 0.01$







**FIGURE 5** Validation of the activation of TNFR/NF- $\kappa$ B/apoptosis signalling in the decidual stromal cells of the patient in the ESA group. (A) The fluorescence staining of the decidua from patients in NP group and ESA group showed that the expression of TNFR1 and TNFR2 was increased in the patient of ESA group. NP,  $n = 4$ ; ESA,  $n = 4$ .  $**p < 0.01$ . Scare bar = 50  $\mu$ m. (B) The NF- $\kappa$ B signal was transactivated in the decidual stromal cells of the patient in the ESA group. Red arrow denotes the nuclear translocation of NF- $\kappa$ B. NP,  $n = 3$ ; ESA,  $n = 3$ . Scare bar = 50  $\mu$ m. (C) Significantly increased staining with caspase3 in decidua in the ESA group compared to the NP group. NP,  $n = 4$ ; ESA,  $n = 4$ .  $**p < 0.01$ . Scare bar = 50  $\mu$ m. (D) The TUNEL staining showed significant elevation of apoptotic cells in the decidua from the ESA group. NP,  $n = 4$ ; ESA,  $n = 4$ .  $**p < 0.01$ . Scare bar = 50  $\mu$ m

FTH1 was changed throughout processing time and rose to a peak after being stimulated with 10 ng/ml TNF $\alpha$  for 48 h (Figure S3A,B). However, 48 h treatment of TNF $\alpha$  did not induce the upregulation of FTL (Figure S3C). Subsequently, different concentrations of TNF $\alpha$  Nanobody (TNB) were used to attenuate the stimulation of TNF $\alpha$ , of which 2  $\mu$ g/ml TNB showed complete neutralization (Figure S3D). Therefore, 2  $\mu$ g/ml TNB was used to further verify the action of TNF $\alpha$  on FTH1. It was not surprising that the upregulation of FTH1 induced by TNF $\alpha$  was blocked by TNB (Figure 3), demonstrating that the expression of FTH1 was mediated by TNF $\alpha$ . Intriguingly, TNF $\alpha$  could not regulate FTH1 expression in HTR8/SVneo cells (Figure S3E), further implying the detrimental role of TNF $\alpha$ -induced FTH1 in stromal cells, which closely associated with ESA.

### 3.4 | Apoptosis was mediated by TNF $\alpha$ /TNFR/NF- $\kappa$ B/FTH1 axis in hESCs

As literature reported, engagement of TNF $\alpha$  with its cognate receptors TNFR1 and TNFR2 results in the recruitment of many important adaptor proteins like TRADD, TRAF2, RIP and FADD, thereby activating and initiating downstream events leading to apoptosis, as well as NF- $\kappa$ B activation, and so on.<sup>30,31</sup> To further understand the mechanism of TNF $\alpha$ -induced apoptosis and the function of FTH1 in it, the downstream molecules were explored in the hESCs stimulated by TNF $\alpha$ . RT-PCR and Western blotting showed that the expression of TNFR1 and TNFR2 was increased both at the transcription level and at the protein level (Figure 4A,B). In addition, NF- $\kappa$ B was confirmed to translocate from the cytoplasm to the nucleus by immunofluorescence staining (Figure 4C), suggesting the activation of NF- $\kappa$ B by TNF $\alpha$ . Furthermore, pro-caspase3 was also induced by TNF $\alpha$  (Figure 4C), indicating the occurrence of apoptosis. The above results had demonstrated that FTH1 was upregulated by TNF $\alpha$ ; thus, we speculated that FTH1 was the downstream target of NF- $\kappa$ B through transcriptional regulation. JASPAR database (<http://jaspar.genereg.net>) was used to predict the transcription factor-binding sites in the FTH1 promoter sequences,<sup>32</sup> and NF- $\kappa$ B was found to be likely to bind with the promoter of FTH1 (Table S2). To validate the binding, NF- $\kappa$ B inhibitor maslinic acid (MA) was applied to repress the activation of NF- $\kappa$ B. After being treated with MA, the nucleus translocation of NF- $\kappa$ B was suppressed (Figure 4C); meanwhile, the increased expression of FTH1 was inhibited (Figure 4D,E). Together, the results support the notion that TNF $\alpha$  induces apoptosis through mediating of TNFR/NF- $\kappa$ B/FTH1 axis in hESCs.

### 3.5 | Validation of abnormal TNFR/NF- $\kappa$ B/FTH1/apoptosis signals in human decidual tissues

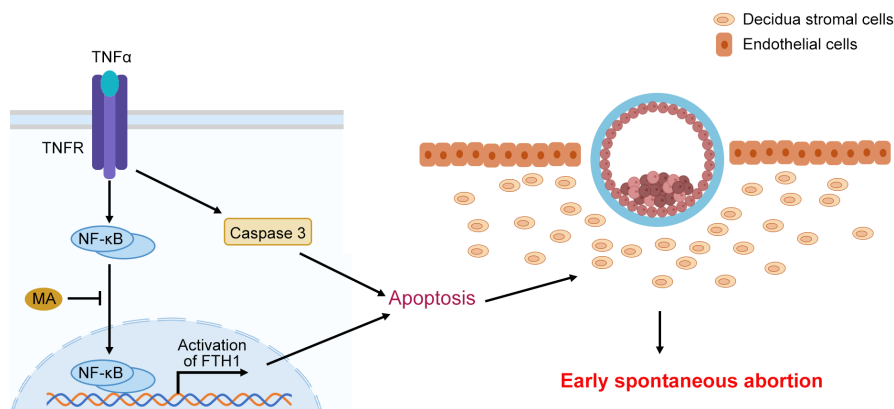
To elaborate that the occurrence of ESA was closely associated with the function of TNF $\alpha$ /TNFR/NF- $\kappa$ B/FTH1/apoptosis signalling, the induction of this pathway was further testified in human decidual tissues. The immunofluorescence staining showed a significant enhancement of TNFR1 and TNFR2 in human decidual tissues from the ESA group (Figure 5A). Additionally, NF- $\kappa$ B was nucleus translocated (Figure 5B), suggesting the activation of its transcriptional activity in the ESA group. Besides, decidual tissues from the ESA group exhibited a strongly positive staining of caspase3 (Figure 5C) and an obvious increase of TUNEL positive cells (Figure 5D), indicating the exacerbated apoptosis. These results were consistent with those data in hESCs and further verified the findings that the abnormal activation of TNF $\alpha$ /TNFR/NF- $\kappa$ B/FTH1/apoptosis signalling may account for the pathogenesis of ESA, at least in some cases (Figure 6).

## 4 | DISCUSSION

Previous evidences have emphasized the important role of ferritin in pregnancy; the imbalance of ferritin levels is often associated with adverse pregnancy outcomes.<sup>21,22,24,25</sup> Unfortunately, little researches focus on the functions of its subunits FTH1 during pregnancy. So far, just one literature has reported that FTH1 deletion would contribute to early embryonic lethality in mice,<sup>16</sup> while whether the deficiency or excess of FTH1 would result in other pregnancy consequences remains to be explored. In the current study, we have demonstrated several novel points which could clarify the relationship between inflammation and FTH1 function in ESA pathogenesis, including (1) TNF $\alpha$  and FTH1 are upregulated in the decidual tissues from patients undergoing ESA; (2) the expression of FTH1 is mediated by TNF $\alpha$ /TNFR/NF- $\kappa$ B axis; and (3) FTH1-induced excessive apoptosis in response to TNF $\alpha$  signals may contribute to the development of ESA.

Many studies have claimed that ferritin especially FTH1 would be elevated together with aggravated infection or inflammation, which triggered by many pro-inflammatory cytokines, in particular TNF $\alpha$ .<sup>17,33-35</sup> As early as 1988, Torti, et al. have revealed that the expression of FTH1 could be regulated by TNF $\alpha$  in mouse TA1 adipocytes and human muscle cells.<sup>33</sup> Subsequently, similar conclusions are identified in lung macrophages,<sup>34</sup> hepatocellular carcinoma cells,<sup>35</sup> human periodontal ligament cells<sup>17</sup> and so on. Intriguingly, we





**FIGURE 6** Hypothesis of the underlying pathogenesis for ESA induced by TNF $\alpha$ -upregulated FTH1. The elevated TNF $\alpha$  induces the abnormally activated signals of TNFR/NF- $\kappa$ B/FTH1 and leads to the excessive apoptosis of decidual stromal cells, which further results in unstable implantation of embryos and contributes to ESA

detected increased TNF $\alpha$  expression in local intrauterine decidual tissues from the ESA group, and discovered a similar upregulation of FTH1 which obviously positively correlated with the level of TNF $\alpha$  in our study. In subsequent experiments on hESCs, the expression of FTH1 was confirmed to be modulated by TNF $\alpha$ . Moreover, the expression of another subunit of ferritin FTL was also detected, although it was increased in decidual tissues from the ESA group and showed a strong correlation with TNF $\alpha$ , it was not modulated by TNF $\alpha$  in hESCs, implying the specific role of TNF $\alpha$ -induced FTH1 in ESA.

To our knowledge, TNF $\alpha$  could activate apoptotic pathways via binding with TNFR1 containing the death domain, or enhance TNF $\alpha$ /TNFR1 mediated apoptotic signals by binding to TNFR2,<sup>36,37</sup> which may lead to embryo death, or unstable implantation of embryos, further contributing to ESA.<sup>38,39</sup> Meanwhile, the binding of TNF $\alpha$  to TNFR1 and TNFR2 induces the activation of NF- $\kappa$ B as well, whereas the function of NF- $\kappa$ B was controversial. Some researches suggest that NF- $\kappa$ B could act as a blocker of apoptosis to protect the embryo from stimuli including TNF $\alpha$ , while some indicate that the activation of NF- $\kappa$ B would aggravate apoptotic cell death through acting as a transcriptional factor to enhance the expression of target genes.<sup>5,36</sup> In the current study, we discovered the abnormal activation of TNF $\alpha$ -induced TNFR/NF- $\kappa$ B/FTH1/apoptosis signals both in hESCs and in decidual tissues from the ESA group, providing strong evidence that the dysregulated FTH1 signalling may be closely related with the occurrence of ESA. Nevertheless, long-term treatment with TNF $\alpha$  could not induce the cell death of HTR8/SVneo, indicating that trophoblast cells may resistant to apoptosis signalling induced by TNF $\alpha$ .

Up to now, FTH1 is generally considered to have antioxidant properties that could protect cells from reactive oxygen species (ROS) due to its ferroxidase activity which could catalyse the conversion of Fe<sup>2+</sup> into Fe<sup>3+</sup>.<sup>17,20</sup> Meanwhile, NF- $\kappa$ B-mediated FTH1 up-regulation is thought to protect cells from apoptosis.<sup>35,40</sup> However, our results showed that TNF $\alpha$  could induce the apoptosis through activating the signals of NF- $\kappa$ B/FTH1, which were opposite from the discovery reported by literatures. This may due to different diseases we focused on and different cell models we used, just like that the same concentration of TNF $\alpha$  could induce apoptosis in some cells, while showing a protective function in some cells.<sup>35,41</sup> Additionally,

some researches have revealed the anti-growth and apoptosis-promoting effects of FTH1 on breast cancer cells and non-small cell lung cancer cells, further illustrating the existence of inconsistency among different cells.<sup>42,43</sup>

Decidua (a specialized endometrium) is an abundant source of cytokines, including TNF $\alpha$ , interleukin-1 $\alpha$  (IL-1 $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), interferon- $\gamma$  (IFN- $\gamma$ ) and so on.<sup>44</sup> The disorders of these cytokines would lead to adverse outcomes, containing miscarriage, preeclampsia, premature labour and so on.<sup>45,46</sup> However, the content of cytokines other than TNF $\alpha$  was not detected in our study, especially interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and interferon- $\gamma$  (IFN- $\gamma$ ), which are also considered risk stimuli of apoptosis and pregnancy loss.<sup>7,47</sup> Moreover, it has been reported that the FTH1 could be triggered by IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IFN- $\gamma$  as well.<sup>17,48-50</sup> Therefore, it was difficult to conclude that the occurrence of ESA was just accounted for TNF $\alpha$ -induced upregulation of FTH1. Since the literatures have proposed that FTH1 could be regarded as a marker to reflect iron stores of the body, and plays an important role in balancing iron homeostasis,<sup>51</sup> it was suspectable that the increase of apoptosis may be related to the disorder of iron metabolism induced by dysregulation of FTH1. More studies are needed to understand the detailed mechanism underlying the association among inflammation, FTH1 and iron metabolism during pregnancy.

In conclusion, our study has found that the level of TNF $\alpha$  is significantly increased in the decidual tissues from the ESA group, and long-term stimulation of high TNF $\alpha$  level is proved to induce the aberrant activation of TNFR/NF- $\kappa$ B/FTH1 signals, which further led to the excessive apoptosis of the decidua, resulting in unstable implantation and ESA. This is the first study linking the TNF $\alpha$ -upregulated FTH1 to the development of ESA, providing a possible target of drug therapy for ESA.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTION

**Yuting Wen:** Formal analysis (equal); Validation (equal); Visualization (equal); Writing – original draft (equal). **Meng Cheng:** Resources (equal); Writing – review & editing (equal). **Lang Qin:** Supervision (equal); Writing – review & editing (equal). **Weinming xu:** Conceptualization (equal); Funding acquisition (equal); Supervision (equal); Writing – review & editing (equal).

## DATA AVAILABILITY STATEMENT

Data are available on reasonable request. All data relevant of this study are available from the corresponding author on reasonable request.

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