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Hypoxia-Inducible Factor 1- α (HIF-1 α) Induces Apoptosis of Human Uterosacral Ligament Fibroblasts Through the Death Receptor and Mitochondrial Pathways

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Background: Hypoxia induces cell apoptosis in the uterosacral ligaments of patients with pelvic organ prolapse by upregulation of hypoxia-inducible factor-1 α (HIF-1 α). This study aimed to investigate the effects of HIF-1 α on human uterosacral ligament fibroblasts (hUSLFs) following treatment with the chemical inducer of hypoxia, cobalt chloride (CoCl₂), and to explore the underlying mechanisms.

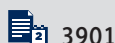
Material/Methods: Ten women who underwent hysterectomy for benign disease provided uterosacral ligament tissue for cell extraction. Following CoCl₂ treatment, cell viability of isolated and cultured hUSLFs was evaluated by the MTT assay. JC-1 fluorescence mitochondrial imaging was used to study the change in mitochondrial membrane potential. Cell apoptosis and expression of apoptosis-associated proteins and collagen type I alpha 1 (COL1A1) were measured by flow cytometry, TUNEL and Western blot, respectively.

Results: Hypoxia increased the expression of HIF-1 α and increased cell apoptosis, decreased cell viability and expression levels of COL1A1. The JC-1 assay showed that the mitochondrial membrane potential was reduced and caspase-8, and -9 inhibitors partly reduced hUSLF apoptosis. HIF-1 α treatment downregulated the expression of cellular FLICE inhibitory protein (c-FLIP), decoy receptor 2 (Dcr2), and the ratio of Bcl-2 to Bax, and upregulated the expression tumor necrosis factor related apoptosis-inducing ligand (TRAIL), death receptor 5 (DR5) or TRAIL-R2, Fas, Bcl-2 interacting protein 3 (BNIP3), and cytochrome C, and increased the activation of caspase-3, caspase-8, and caspase-9, all of which were reversed by knockdown of HIF-1 α .

Conclusions: HIF-1 α significantly induced apoptosis of hUSLFs through both the cell death receptor and the mitochondrial-associated apoptosis pathways.

MeSH Keywords: **Apoptosis • Cell Hypoxia • Hypoxia-Inducible Factor 1 • Pelvic Organ Prolapse**

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Background

Pelvic organ prolapse is a global and distressing illness identified by the descent of the pelvic organs such as uterus, bladder, rectum or all into the vagina resulting from damage to the supporting ligaments and muscles [1,2]. Pelvic organ prolapse increases with age, is associated with a history of childbirth [3], results in a reduced quality of life, and represents a global economic burden to healthcare systems [4].

Epidemiological studies have identified several risk factors for pelvic organ prolapse, including race, senescence, obesity, a history of vaginal delivery and pelvic surgery [5]. Previously published studies on the mechanisms of pelvic organ prolapse have focused on injury to the pelvic muscles and ligaments [6], metabolic changes in the extracellular matrix (ECM) [7–9], and the expression of matrix metalloproteinases (MMPs) [10,11]. Currently, increased apoptosis of fibroblasts in pelvic connective tissue is considered to be an important factor in the pathogenesis of pelvic organ prolapse [12,13]. However, the underlying molecular mechanism remains unknown.

Hypoxia is a common phenomenon observed in many physiological and pathological conditions, which can limit and even halt the physiological function of cells, tissues, and organs, due to cell apoptosis [14]. Hypoxia-inducible factors (HIFs) are key transcriptional factors leading to downstream effects of hypoxia [15]. HIF-1 α is the main regulator of the cellular response to hypoxia [16], and is associated with the expression of several downstream target genes that are associated with cell proliferation [17], angiogenesis [18], energy metabolism [19] and cell death [20]. HIF-1 α upregulates the expression of MMPs, and these proteins are associated with tumor invasion [21]. HIF-1 α also regulates some pro-apoptotic genes, including Bcl-2 interacting protein 3 (BNIP3) [22], and stabilizes the tumor suppressor, p53, to induce cell apoptosis [23]. Therefore, hypoxia may contribute to the pathogenesis of pelvic organ prolapse by increasing apoptosis through pathways associated with HIF-1 α expression.

A previously reported preliminary study by our research group collected tissue samples of human uterosacral ligament to compare the expression of HIF-1 α , to determine whether there were differences between women with or without pelvic organ prolapse [24]. The findings of this previous study showed that expression levels of HIF-1 α and apoptosis-associated proteins were significantly increased in the study group with pelvic organ prolapse compared with a normal group of women [24]. Correlation analysis showed a significant correlation between the percentage of apoptotic cells and expression levels of HIF-1 α protein [24].

Therefore, in view of these previous findings, this study aimed to investigate the effects of HIF-1 α on human uterosacral ligament fibroblasts (hUSLFs) following treatment with the chemical inducer of hypoxia, cobalt chloride (CoCl₂), and to explore the underlying mechanisms.

Material and Methods

Sample collection

The study was approved by the Ethics Committee of Qilu Hospital of Shandong University. The study included 10 women, who underwent hysterectomy for benign disease. Following surgery, the patients did not have any complications, including cancer, cardiovascular disease, diabetes, or immunological disorders. Women who received hormone replacement therapy (HRT) were not included in the study.

Primary cell culture

Fresh sterile uterosacral ligament tissues, measuring approximately 0.5×0.5×0.5 cm were collected and then cut into pieces with a diameter <0.1cm for primary culture [25]. After collection, tissues were immediately washed with phosphate-buffered saline (PBS). The human uterosacral ligament fibroblasts (hUSLFs) were isolated following digestion with type I collagenase (Solarbio Science & Technology Co., Ltd., Beijing, China) and 0.25% trypsin (Sigma-Aldrich, St. Louis MO, USA). After tissue digestion, the medium was centrifuged at 1500 rpm for 5 minutes. The isolated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, ThermoFisher Scientific, Waltham, MA, USA) containing 20% fetal bovine serum (FBS) and 1% antibiotics. The hUSLFs at 3–8 generations of exponential growth phase were selected for the subsequent experiments.

Immunofluorescence (IF)

The hUSLF cells were fixed with cold methyl alcohol for 15 min and then permeabilized with 0.5% Triton X-100 (Solarbio Science & Technology Co., Ltd., Beijing, China) for 10 min, and blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature. After washing, the cells were incubated with primary antibodies to vimentin and keratin at 4°C overnight, followed by incubation with secondary antibody (ZsBio, Shanghai, China) at 37°C for 1 h. Then, 4',6-diamidino-2-phenylindole (DAPI) (Solarbio Science & Technology Co., Ltd., Beijing, China) was used for counterstaining of the cell nuclei. Photomicrographs were acquired using an Olympus IX51 inverted microscope (Olympus, Tokyo, Japan). There was no cytokeratin expression by the isolated cells, which confirmed that the hUSLF cells did not contain cells of epithelial origin,

while the biomarker for the mesenchymal origin of the hUSLF cells, vimentin, showed strong expression.

MTT assay following treatment of hUSLFs with the chemical inducer of hypoxia, cobalt chloride (CoCl₂)

The hUSLFs were treated with cobalt chloride (CoCl₂) at different times and concentrations. Cells in the exponential phase were seeded in 96-well microplate at 1×10⁵ cells/well and incubated with 0, 50, 100, 150, 200, and 300 μ M CoCl₂ for 24, 48, 60, and 72 h before the MTT assay. Cytotoxicity was expressed as the concentration of CoCl₂ inhibiting cell growth by 50% (IC₅₀).

Cell transfection

After hUSLFs were cultured overnight to reach 60–70% confluence, cells were transfected with small-interfering RNA (siRNA) for HIF-1 α using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The siRNA HIF-1 α was designed by GenePharma Company (Shanghai, China). The siRNA HIF-1 α and negative control sequences were as follows: 5'-GCCGCUCAUUUUGAAUATT-UAUUCAUAAAUGAGCGGCTT-3', 5'-UUCUCCGAACGUGUCACGUTT-ACGUGACACGUUCGGAGAATT-3'.

Cell viability assay

The MTT assay was used to evaluate the viability of hUSLF cells following CoCl₂ treatment and transfection. The hUSLF cells were plated into a 96-well microplate (1×10⁴ cells/well) separately and treated with CoCl₂ or transfected cells. Then, 10 μ L of MTT solution (5 mg/ml) was added to each well prior to incubation for 4 h. The formazan precipitate was dissolved in 100 μ L of dimethyl sulfoxide (DMSO), and the absorbance was measured using a microplate reader (ThermoFisher Scientific, Waltham, MA, USA) at 490 nm.

Detection of cell apoptosis using flow cytometry

An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (BestBio, Shanghai, China) was used for flow cytometry to measure the percentage of apoptotic cells. After treatment, hUSLFs were washed twice with PBS and then stained with Annexin V-FITC/PI. After filtration, the suspension of each group was analyzed within 1 h using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

TUNEL assay for cell apoptosis

The hUSLF cells were fixed with paraformaldehyde for 30 minutes and treated with a permeabilizing solution (0.1% Triton X-100) at 4°C for 2 min and incubated in TUNEL reaction mixture for 60 min at 37 °C in the dark. The hUSLFs were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Four fields

were randomly selected from each sample, and at least 100 cells were counted to calculate the percentage of apoptotic cells.

Lactate dehydrogenase (LDH) assay

The degree of apoptosis in a cell population can also be evaluated by measuring plasma membrane damage, which results in the release of lactate dehydrogenase (LDH). Therefore, cytotoxicity was determined by measuring the release of LDH from hUSLFs into the culture supernatant, using an LDH cytotoxicity assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The LDH reagent and culture medium were mixed and incubated in the dark at room temperature for 30 min and the colorimetric result was read at 490 nm.

Measurement of caspase activity

The activity of caspase-3, caspase-8, and caspase-9 was detected using a Caspase Activity Assay Kit (Beyotime, China) according to the manufacturer's protocol. The hUSLFs were treated and the reagents were added to each well, and the positive and negative controls were measured at 405 nm. An increase in absorbance at 405 nm was used to quantify the caspase activities.

Effects of caspase inhibitors on the viability of hUSLF cells

After pretreated with and without 50 μ M of the caspase-8 inhibitor, Z-IETD-FMK (MedChem Express, Monmouth Junction, NJ, USA), and treatment with and without 100 μ M of the caspase-9 inhibitor, Z-LEHD-FMK (KeyGen Biotech Co. Ltd., Nanjing, China) for 2 h, cells were treated with CoCl₂ 48 h later. Change in cell viability of hUSLFs was assessed by the MTT assay.

Western blot

Total protein was extracted from the hUSLF cells for Western blot. The primary antibodies used for immune detection included antibodies to HIF-1 α (1: 1000) (Abcam, Cambridge, MA, USA), COL1A1 (1: 200) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), Fas (1: 500) (Affinity Biologicals, Hamilton, ON, Canada), TRAIL (1: 500) (Affinity Biologicals, Hamilton, ON, Canada), DcR2 (1: 500) (Affinity Biologicals, Hamilton, ON, Canada), c-FLIP (1: 500) (Affinity Biologicals, Hamilton, ON, Canada), DR5 (1: 500) (Affinity Biologicals, Hamilton, ON, Canada), cleaved caspase-8 (1: 500) (Cell Signaling Technology, Danvers, MA, USA), Bax (1: 200) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), Bcl-2 (1: 200) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), BNIP3 (1: 500) (Abcam, Cambridge, MA, USA), cytochrome C (1: 500) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), cleaved caspase-3 (1: 500) (CST), cleaved caspase-9 (1: 500) (Cell Signaling Technology, Danvers, MA, USA), and β -actin (1: 1000) (Cell Signaling Technology, Danvers, MA,

USA). The membranes were incubated with secondary antibodies (Merck Millipore, Burlington, MA, USA) and visualized by enhanced chemiluminescence (ECL) using Image Quant LAS 4000 (GE Healthcare Life Sciences, Logan, UT, USA). β -actin was used as an internal reference control. The results were assessed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

JC-1 fluorescence measurement of the mitochondrial membrane potential

The mitochondrial membrane potential was detected using JC-1 fluorescence mitochondrial imaging. The hUSLFs were incubated with JC-1 solution for 20 minutes at 37°C. The cells were then washed twice with JC-1 buffer and medium was added to each well. Images were taken using a fluorescence microscope (Olympus, Tokyo, Japan). The ratio of red to green fluorescence represented the mitochondrial membrane potential.

Statistical analysis

Data were shown as the mean \pm SEM from at least three independent experiments. The Student's t-test and one-way analysis of variance (ANOVA) were used to analyze the differences between groups. GraphPad Prism version 6.07 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis and graph plotting. A *P*-value <0.05 was regarded as statistically significant.

Results

Human uterosacral ligament fibroblast (hUSLF) viability was reduced by treatment with the chemical inducer of hypoxia, cobalt chloride (CoCl₂)

The growth of human uterosacral ligament fibroblasts (hUSLFs) was observed using an inverted microscope after between 3–5 days following inoculation of the tissue explants and isolated cells in the culture flask. The fibroblasts were seen to become elongated and formed long, spindle, stellate, bipolar, or polygonal shapes. Cells in the zone that surrounded the tissue specimens accumulated when cultured continuously, and cell fusion was completed in more than 20 days (Figure 1A). Following passage to the third generation, cell immunofluorescence indicated that more than 95.6 \pm 2.4% of the cells were vimentin-positive and cytokeratin-negative, which indicated most of the cells were fibroblasts (Figure 1B).

Cobalt chloride (CoCl₂) is commonly used as a hypoxic in cell culture studies. To evaluate whether hypoxic conditions were cytotoxic to primary hUSLFs, the fibroblasts were treated with 0, 50, 100, 150, 200 and 300 μ M of CoCl₂ for 24, 48, 60, and

72 h, respectively, following which cell viability was examined using the MTT assay. As shown in Figure 1C, following treatment for 48, 72, 96 h, the cell group treated with 50 μ M of CoCl₂ showed a significant decrease in cell viability ($p<0.01$). However, no significant differences were observed at 24 h. Also, the cell viability in the cell groups treated with CoCl₂ at concentrations >50 μ M were significantly different from the control group at 24h. The IC₅₀ values were 163.60, 133.70, 64.99, and 44.52 μ M after 24, 48, 60, and 72 h of treatment, respectively. The hUSLF cell viability decreased following CoCl₂ treatment in a time-dependent and dose-dependent manner. Following these results, a concentration of 100 μ M CoCl₂ was used for subsequent experiments.

CoCl₂ treatment resulted in apoptosis of hUSLFs, increased hypoxia-inducible factor-1 α (HIF-1 α) expression, and decreased collagen type I alpha 1 (COL1A1) expression

After incubating the hUSLF cells with CoCl₂ for increasing periods of time, the TUNEL and flow cytometry assays were performed to determine whether hypoxia had an effect on apoptosis of hUSLFs (Figure 2A, 2C). Apoptosis of hUSLF cells of 24, 48, 60, and 72h following treatment with CoCl₂ was observed, and when hUSLFs were stimulated with CoCl₂ at 24 h, no significant difference in the percentage of apoptotic cells was detected using either the TUNEL method or flow cytometry analysis ($p>0.05$) (Figure 2B, 2D).

However, 48 h later, a gradual increase in the number of apoptotic cells were found after CoCl₂ treatment (Figure 2B, 2D), supporting the effects of hypoxia on promoting the apoptosis of hUSLFs. By 48 hrs of CoCl₂ treatment, more than 20% of the hUSLFs were apoptotic and severe damage were observed after hypoxia treatment for 60 h. No significant differences were found between the flow cytometry assay and the TUNEL staining (Figure 2B, 2D).

Following the addition of CoCl₂, a significant increase in the HIF-1 α protein level was detected after 48 h of CoCl₂ treatment ($p<0.01$) (Figure 2E, 2F). However, the expression of COL1A1 was significantly decreased by treatment with CoCl₂ by 48 h ($p<0.05$) (Figure 2E, 2G). These results showed that hypoxia promoted the apoptosis of hUSLFs. Following these results, 48 h of CoCl₂ treatment was chosen for the hypoxic conditions of hUSLFs for subsequent experiments.

Involvement of the death receptor-associated pathway in CoCl₂-treated hUSLFs

To explore the potential effect of death receptor activation in apoptosis in CoCl₂-treated hUSLFs, the expression of apoptotic proteins was studied. When hUSLFs were incubated with 100 μ M of CoCl₂ for 48 h, increased expression of Fas, death

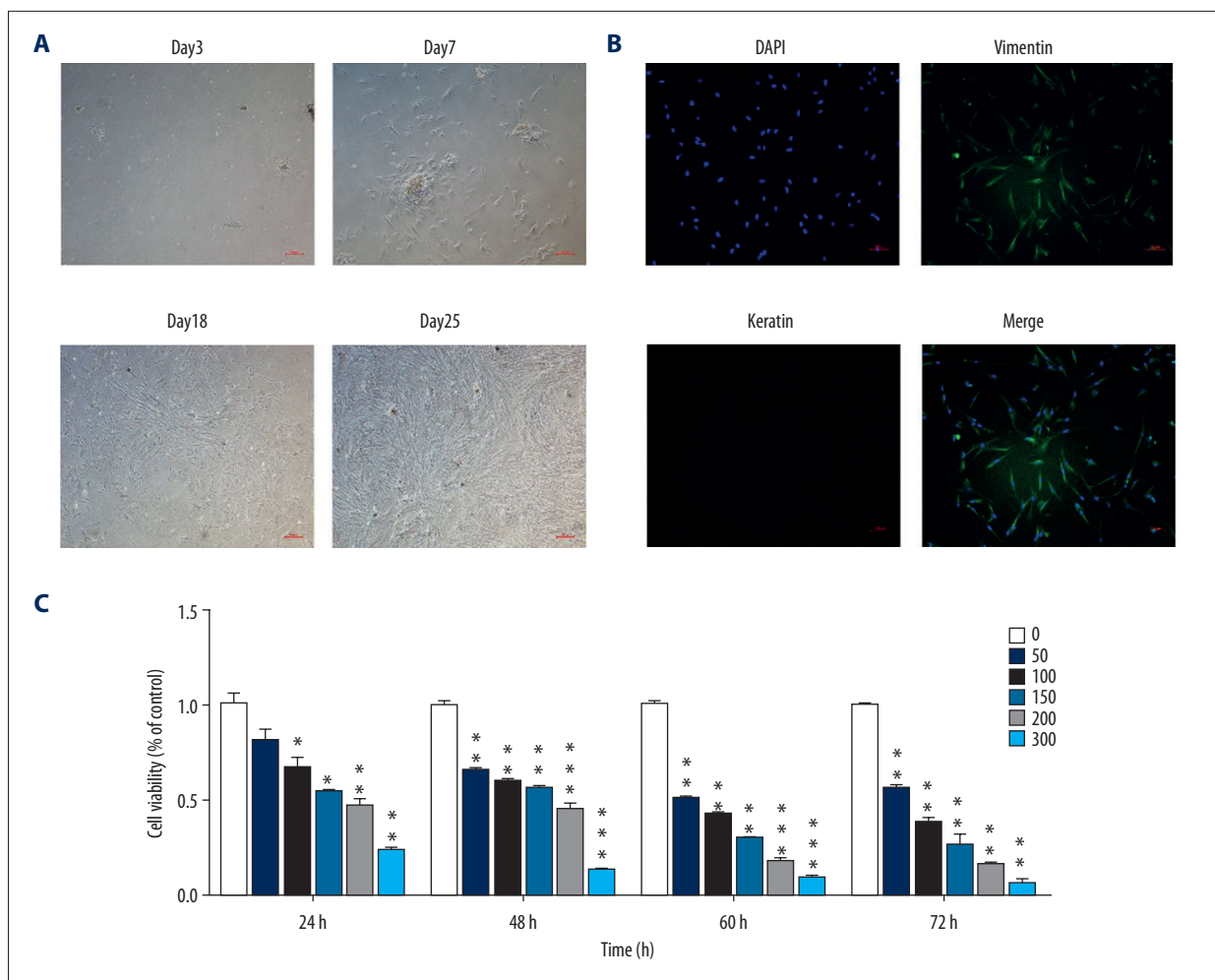


Figure 1. Primary culture of human uterosacral ligament fibroblasts (hUSLFs) and cell viability assays. **(A)** Human uterosacral ligament fibroblasts (hUSLFs) cultured *in vitro* at 25 days. Magnification, $\times 100$. Scale bar=100 μ m. **(B)** Immunofluorescence (IF) shows positive intracellular expression of vimentin and negative expression of keratin, supporting an interstitial origin. Magnification, $\times 100$. Scale bar=100 μ m. **(C)** The hUSLFs exposed to 0, 50, 100, 150, 200, and 300 μ M of the chemical inducer of hypoxia, cobalt chloride (CoCl_2), for 24, 48, 60, 72 h, and cell viability measured by the MTT assay. Data are presented compared with the controls as a percentage. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

receptor 5 (DR5), tumor necrosis factor related apoptosis-inducing ligand (TRAIL), and cleaved caspase-8 were detected by Western blot. However, cellular FLICE inhibitory protein (c-FLIP) and decoy receptor 2 (DcR2) protein expression levels were significantly decreased (Figure 3A, 3B). The activity of caspase-8 was increased in the hypoxia group and levels of lactate dehydrogenase (LDH) were also increased (Figure 3C, 3D). Also, when the caspase-8 inhibitor Z-IETD-FMK was added before treatment with CoCl_2 , the cell viability significantly increased compared to the hUSLFs under the same hypoxic conditions (Figure 3E). These results indicated that the death receptor-associated pathway was involved in apoptosis induced by hypoxia in hUSLFs by CoCl_2 .

The mitochondrial apoptotic pathway in hUSLFs treated with CoCl_2

To determine whether the mitochondrial apoptotic pathway was involved in CoCl_2 -induced apoptosis of hUSLFs, the expression of caspases and the members of the Bcl-2 family was evaluated by Western blot. There was a significant increase in the expression of cytochrome C, Bcl-2 interacting protein 3 (BNIP3), cleaved caspase-3, and cleaved caspase-9, and decreased expression of Bcl-2/Bax (Figure 4A, 4B). Also, CoCl_2 treatment significantly increased the activities of caspase-3 and caspase-9 (Figure 4C). As shown in Figure 4D, the cell viability rate was significantly increased when hUSLFs were seeded with CoCl_2 and the caspase-9 inhibitor, Z-LEHD-FMK, compared to treatment with Z-LEHD-FMK alone. However, Z-IETD-FMK further

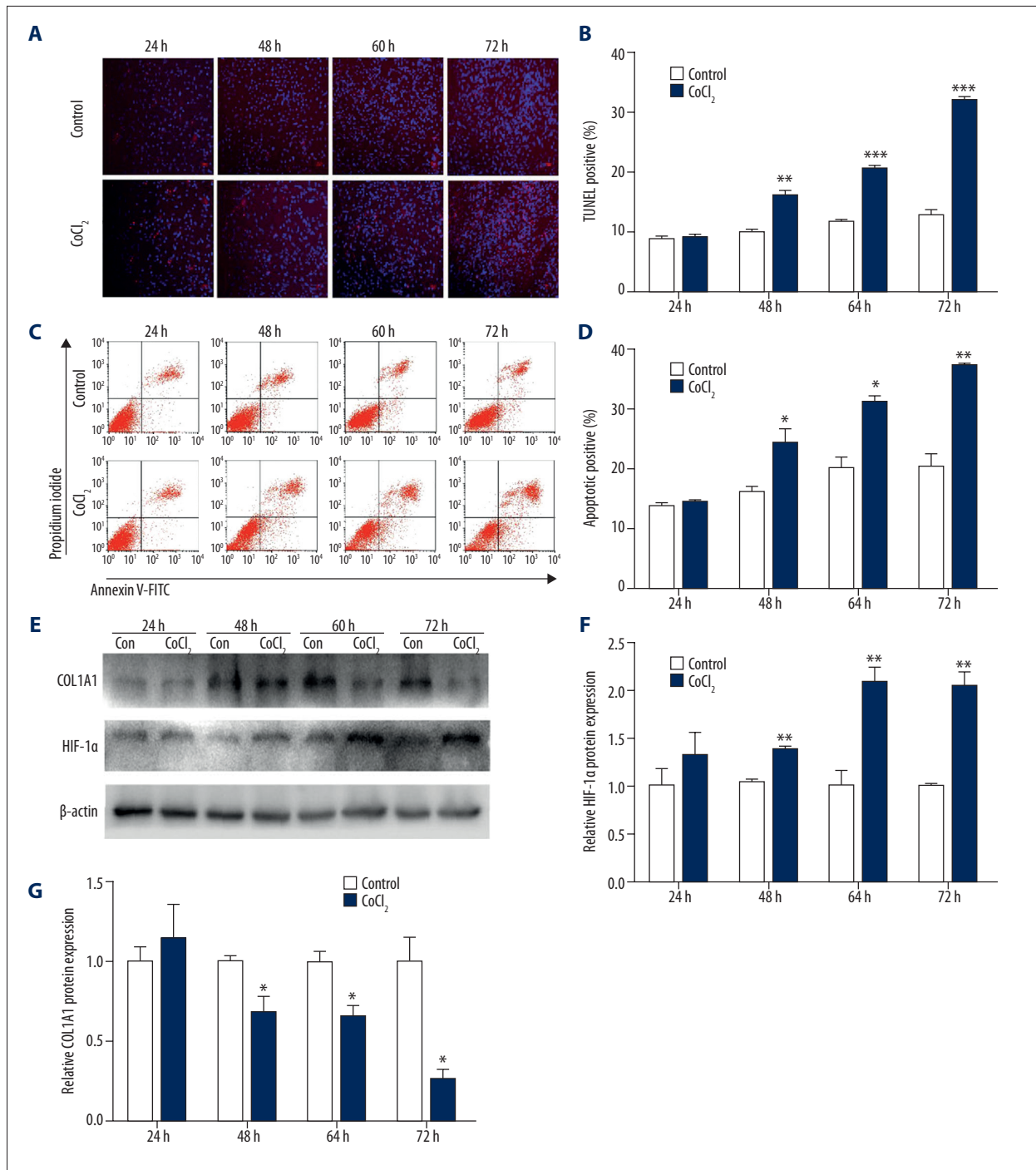


Figure 2. Hypoxia-induced cell apoptosis in human uterosacral ligament fibroblasts (hUSLFs) cultured *in vitro* at different times. (A) Images of TUNEL staining images showing apoptotic cell staining (red) and double-stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Magnification, $\times 100$. Scale bar=100 μ m. (B) Quantitative TUNEL-positive cell rates, evaluated by TUNEL staining under high power magnification. (C) Flow cytometry (FCM) images obtained of the hUSLFs after treatment with 100 μ M the chemical inducer of hypoxia, cobalt chloride (CoCl₂), at different times. (D) The quantitative apoptotic rate of hUSLFs, measured by double-staining with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI). (E) Representative Western blot assays for hypoxia-inducible factor-1 α (HIF-1 α) and collagen type I alpha 1 (COL1A1) protein expression in hUSLFs in the control group (normoxia) and hypoxia group at 24, 48, 60, and 72 h. (F) Quantitative analysis of the expression of HIF-1 α by Western blot. (G) The statistical analysis of COL1A1 expression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Con – control.

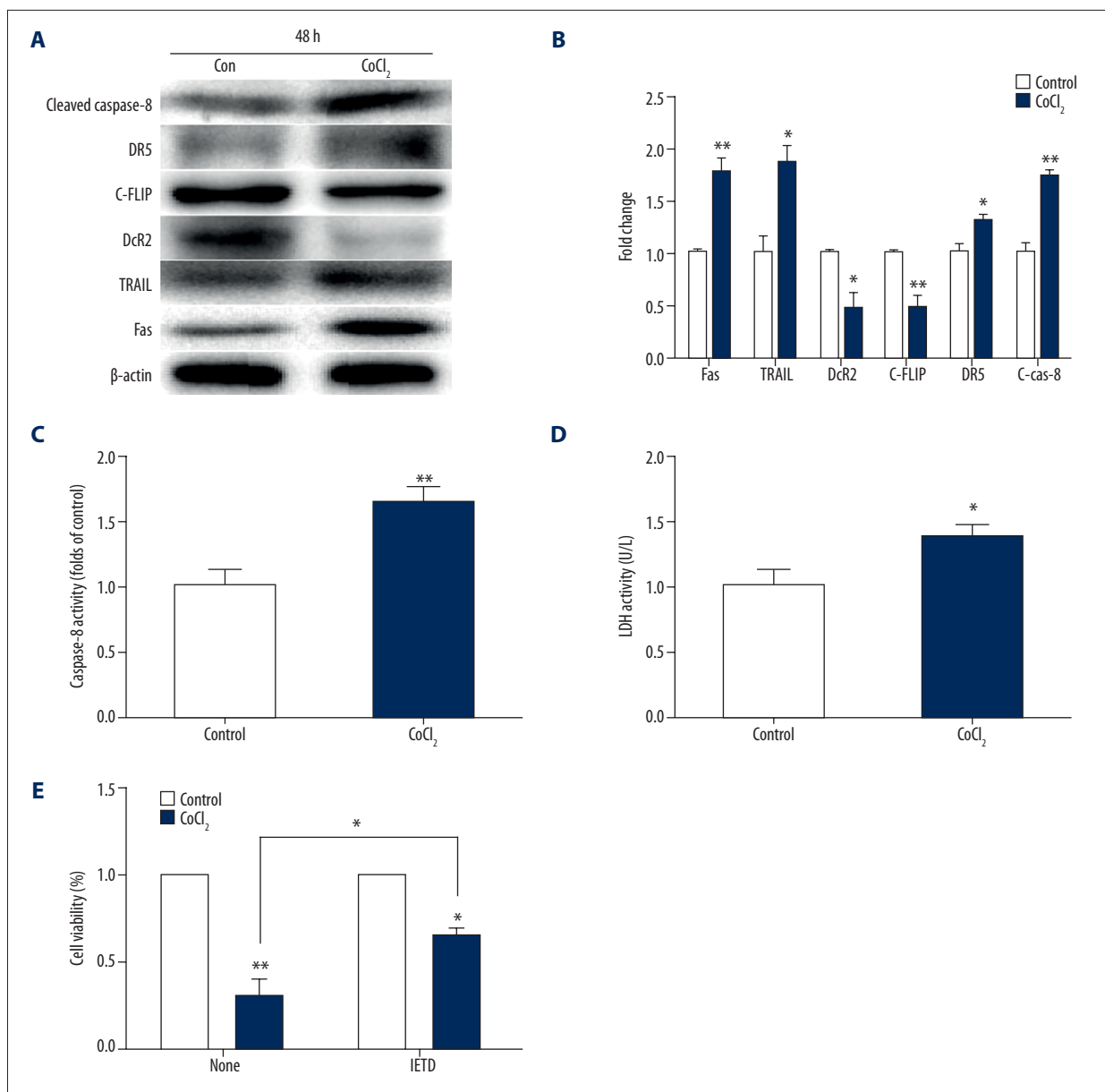


Figure 3. Expression of death receptor-associated apoptotic markers in the cobalt chloride (CoCl₂)-treated human uterosaral ligament fibroblasts (hUSLFs). **(A)** Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), Fas, decoy receptor 2 (DcR2), cellular FLICE inhibitory protein (c-FLIP), death receptor 5 (DR5), and cleaved caspase-8 protein levels by Western blot analysis in hUSLFs treated with the chemical inducer of hypoxia, cobalt chloride (CoCl₂), at 48 h. **(B)** Quantitative analysis of Fas, TRAIL, DcR2, c-FLIP, and DR5 expression standardized to β -actin. **(C)** Caspase-8 activity measured in hUSLFs treated with CoCl₂ for 48 h. **(D)** After 48 h, the effects of CoCl₂ on the levels of lactate dehydrogenase (LDH) in hUSLFs were measured. **(E)** Results of the MTT assay for cell viability following treatment with CoCl₂ for 48 h with or without the presence of 50 μ M of the caspase-8 inhibitor, Z-IETD-FMK. * $P < 0.05$, ** $P < 0.01$. Con – control. C-cas-8 – cleaved caspase-8.

increased the cell viability rate when added to each cell group. JC-1 fluorescence mitochondrial imaging was used to study the change in mitochondrial membrane potential and examined with a fluorescence microscope. When the mitochondrial membrane potential was depolarized, JC-1 formed a monomer and emitted green fluorescence, and JC-1 aggregates emitted red

fluorescence. Figures 4E and 4F show the rate of breakdown of red to green fluorescence. Hypoxia induced the depletion of the mitochondrial membrane potential, and this finding provided an insight into the mechanism of apoptosis in hUSLFs.

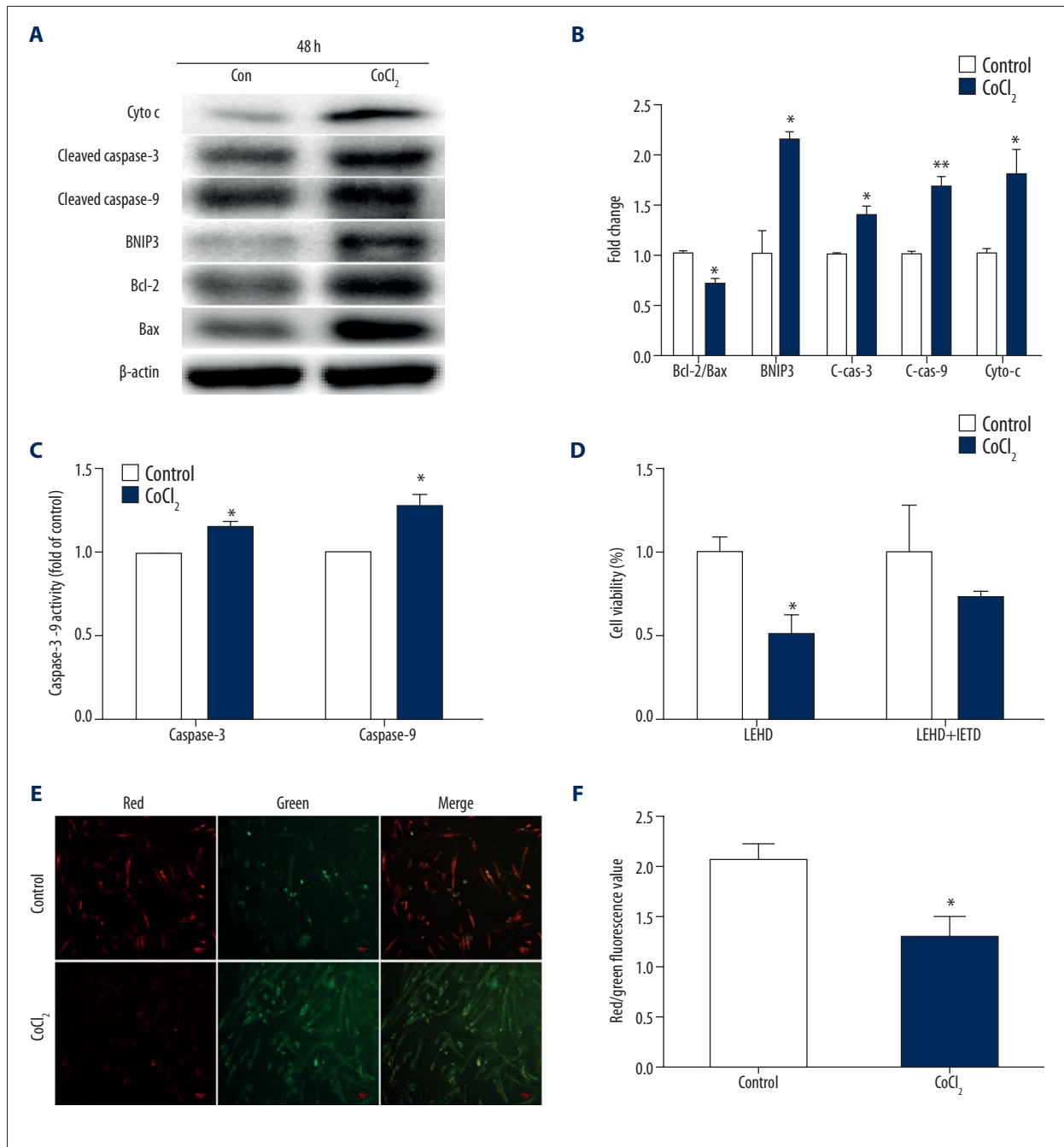


Figure 4. Expression of mitochondrial-associated apoptotic markers in the cobalt chloride (CoCl₂)-treated human uterosacral ligament fibroblasts (hUSLFs). **(A)** Bcl-2, Bax, cytochrome C, Bcl-2 interacting protein 3 (BNIP3), cleaved caspase-3, and cleaved caspase-9 protein levels were analyzed by Western blot in human uterosacral ligament fibroblasts (hUSLFs) that were treated with the chemical inducer of hypoxia, cobalt chloride (CoCl₂) at 48 h. **(B)** Quantitative analysis of Bcl-2/Bax, cytochrome C, BNIP3, cleaved caspase-3, and cleaved caspase-9 protein expression standardized to β -actin. **(C)** Caspase-3, and caspase-9 activity measured in hUSLFs that were treated with CoCl₂ for 48 h. **(D)** Results of the MTT assay for cell viability following treatment with CoCl₂ for 48 h, which were also exposed to 100 μ M of the caspase-9 inhibitor, Z-LEHD-FMK, with or without the presence of 50 μ M of the caspase-8 inhibitor, Z-IETD-FMK. **(E)** Photomicrograph of JC-1 fluorescence mitochondrial imaging used to study the change in mitochondrial membrane potential in the different groups. **(F)** The relative mitochondrial membrane potential level was evaluated by comparing the red fluorescence cells to green fluorescence cells in the groups. * $P < 0.05$, ** $P < 0.01$. Scale bar=100 μ m. Con – control; C-cas-3 – cleaved caspase-3; C-cas-9 – cleaved caspase-9; Cyto c – cytochrome C.

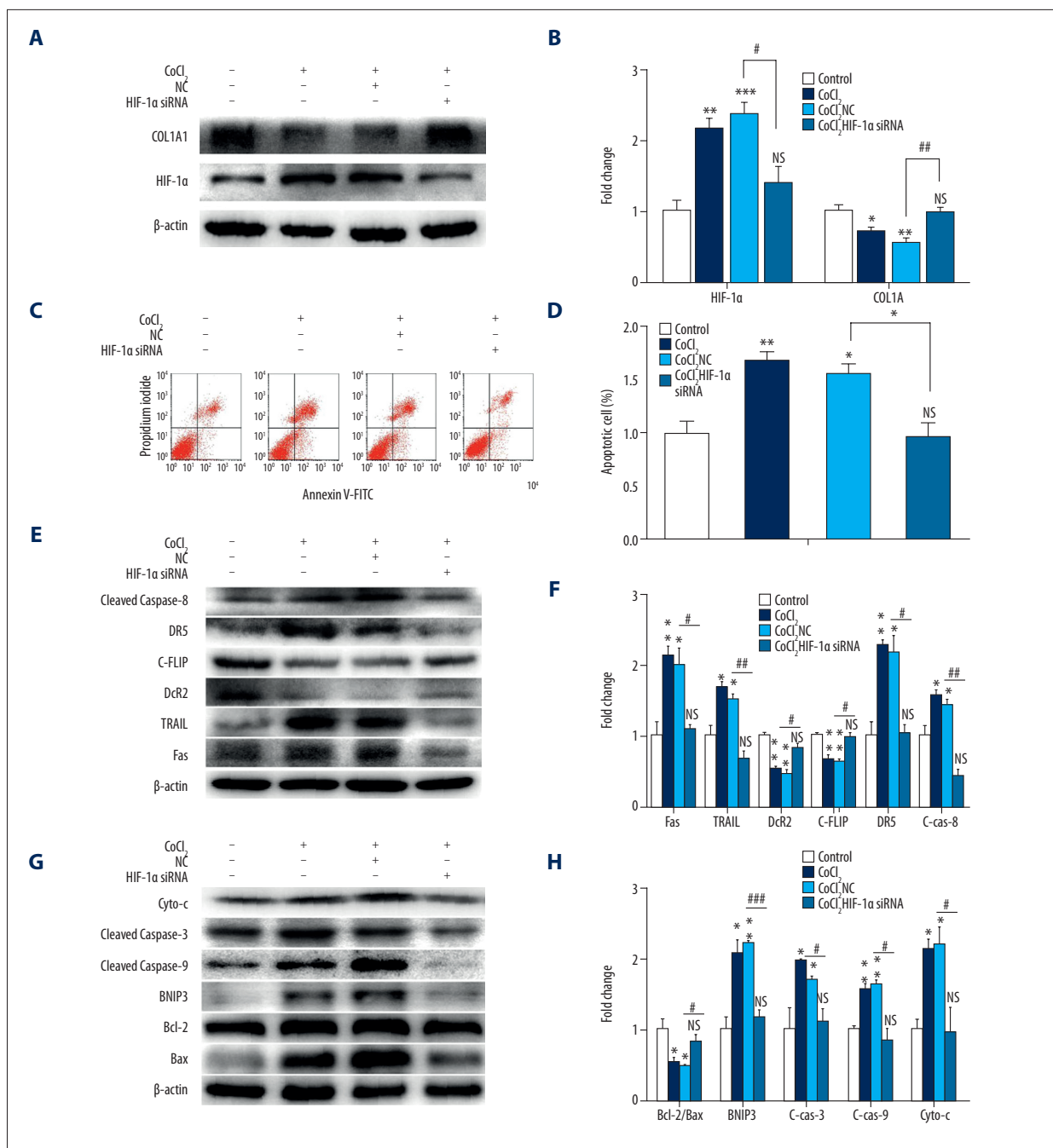


Figure 5. Knockdown of hypoxia-inducible factor-1 α (HIF-1 α) prevented cobalt chloride (CoCl₂)-induced apoptosis in human uterosacral ligament fibroblasts (hUSLFs) by normalizing the expression levels of apoptosis-associated markers, as shown by Western blot of cells with or without HIF-1 α knockdown treated with CoCl₂ for 48 h. **(A)** Western blot results for the expression of hypoxia-inducible factor-1 α (HIF-1 α) and collagen type I alpha 1 (COL1A1). **(B)** Western blot results show the relative band intensities of HIF-1 α and COL1A1. **(C)** Flow cytometry shows cell apoptosis by after double-staining with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI). **(D)** Flow cytometry shows the cell apoptosis rate. **(E)** Western blot results show the relative band intensities of death receptor-associated apoptotic markers. **(F)** Western blot results show the relative band intensities of death receptor-associated apoptotic markers. **(G)** Western blot results show the relative band intensities of mitochondrial-associated apoptotic markers. **(H)** Western blot results show the relative band intensities mitochondrial-associated apoptotic markers. * $P < 0.05$, ** $P < 0.01$, vs. control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, vs. CoCl₂ + NC. Con – control; C-cas-3 – cleaved caspase-3; C-cas-8 – cleaved caspase-8; C-cas-9 – cleaved caspase-9; Cyto-c – cytochrome C.

Inhibition of HIF-1 α down-regulated the apoptosis rate of hUSLFs and normalized the expression of apoptosis-associated proteins

Following transfection with small-interfering RNA (siRNA), the expression of siRNA-HIF-1 α , and COL1A1 expression were significantly reduced in hUSLFs (Figure 5A, 5B). Apoptosis of hUSLFs was reduced following HIF-1 α silencing by staining with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) (Figure 5C, 5D). However, it remained unclear whether HIF-1 α knockdown could reduce hUSLF apoptosis via the death receptor-associated pathway or the mitochondrial-associated apoptosis pathway. Therefore, the expression of apoptotic proteins was evaluated by Western blot. The protein levels of c-FLIP and DcR2 were increased, whereas the expressions of cleaved caspase-8, DR5, TRAIL, and Fas were reduced (Figure 5E, 5F). Also, the ratio of Bcl-2/Bax was upregulated, while cytochrome C, BNIP3, and cleaved caspase-3, and cleaved caspase-9 were downregulated in hUSLFs following HIF-1 α silencing (Figure 5G, 5H). Therefore, apoptosis was induced by hypoxia of hUSLFs through the death receptor-associated pathway and the mitochondrial apoptosis-associated pathway.

Discussion

There have been previous studies on the etiology of pelvic organ prolapse that have focused on cell apoptosis of pelvic supporting structures, including ligaments [26,27]. It has previously been reported that in the uterosacral ligament fibroblasts (USLs) of patients with pelvic organ prolapse, cell apoptosis was increased and the number of fibroblasts and the production of collagen and elastin was decreased [9]. However, the mechanisms involved in these changes in USLs have been poorly understood. A previously published study conducted by our group showed that in human uterosacral ligament tissues from patients with pelvic organ prolapse, the upregulation of hypoxia-inducible factor-1 α (HIF-1 α) increased cell apoptosis *in vivo* [24]. Based on these previous findings, an *in vitro* model using treatment with the chemical inducer of hypoxia, cobalt chloride (CoCl₂) in human uterosacral ligament fibroblasts (hUSLFs) provided further insight to the pathogenesis of pelvic organ prolapse [28]. The findings from the present study, which assessed the effect of hypoxia on apoptosis of hUSLFs, showed that hypoxia enhanced cell apoptosis by activating the death receptor-associated pathway and mitochondrial apoptosis-associated pathway.

Previously published studies have shown that CoCl₂ can be used to mimic hypoxia by enhancing the expression of HIF-1 α in a variety of cells [29,30]. To investigate the effects of hypoxia in the pathogenesis of pelvic organ prolapse and the associated mechanisms, an *in vitro* cell model was established of chemical

hypoxia by treating hUSLFs with CoCl₂. In the present study, CoCl₂ treatment gradually decreased cell viability of hUSLFs and the expression of collagen type I alpha 1 (COL1A1) in a dose-dependent and time-dependent manner and upregulated the expression of HIF-1 α and the number of TUNEL-positive apoptotic cells. Collagen, which is secreted by fibroblasts, is an important component of uterosacral and cardinal ligaments. Previous studies have shown that COL1A1 expression levels were reduced in patients with pelvic organ prolapse, which may be due to changes in cell function or apoptosis [31,32]. These results indicate that hypoxia-induced HIF-1 α expression might have a key role in pelvic organ prolapse by promoting fibroblast apoptosis.

HIF-1 is a heterodimeric transcription factor composed of a constitutively expressed β subunit and an O₂-regulated α subunit, and it acts as a core factor in the hypoxic transcriptional response [33,34], which involves interaction with hypoxia response elements (HREs) to induce transcriptional activity [35]. Under normoxic conditions, HIF-1 α is unstable and is degraded by the proteasome [36]. Hypoxia induces HIF-1 α protein expression mainly due to inhibition of hydroxylation and proteasome degradation, followed by activation of a defined set of transcriptional programs [37]. HIF-1 activity is mainly determined by the HIF-1 α subunit in response to hypoxic and normoxic conditions. The target genes of HIF-1 α encode proteins that function in cellular processes that include angiogenesis, epithelial-mesenchymal transition (EMT), tumorigenesis, and chemotherapy resistance [38–40]. Also, HIF-1 α influences apoptosis by modulating the expression of pro-apoptotic proteins and anti-apoptotic protein, including Bcl-2, Bax, and NIX [41]. The results of the present study indicated that HIF-1 α might have a role in the pathogenesis of pelvic organ prolapse by facilitating the apoptosis of hUSLFs.

Recent studies of apoptotic pathways have shown that apoptosis is triggered when cell-surface death receptors, such as Fas, are bound by their ligands (the extrinsic pathway) [42] or when Bcl2-family of pro-apoptotic proteins cause the permeabilization of the mitochondrial outer membrane (the intrinsic pathway) [43]. Both pathways result in the activation of the caspase protease family, which is ultimately responsible for cell apoptosis. Caspase-3 has an important role in both the death receptor-associated pathway and the mitochondrial apoptosis-associated pathway and is a downstream component of apoptotic pathways [44]. One possible procedure for activating caspase-3 is the caspase-8-mediated process triggered by Fas and tumor necrosis factor related apoptosis-inducing ligand (TRAIL). TRAIL activates the extrinsic pathway and forms the death signaling complex (DISC) by binding and trimerizing death receptor 5 (DR5). Then, the Fas-associated protein with death domain (FADD) combines with the DISC complex and induces the activation of caspase-8 and caspase-10 [45]. Cellular FLICE

inhibitory protein (c-FLIP) is a caspase-8-like protein that can inhibit caspase-8 activation [46].

Several previously published studies have investigated the role of HIF-1 α in TRAIL-induced cancer cell death. Knockdown of HIF-1 α can induce apoptosis in human colon cancer, neuroblastoma, uterine cervical cancer, lung cancer, and gastric cancer cell apoptosis, which is TRAIL-mediated [47,48]. The findings of the present study showed that the activation of Fas, TRAIL, DR5, and caspase-8 in the hUSLFs treated with CoCl₂, which are markers of death receptor-associated and apoptosis-associated pathways. The findings of the present study showed downregulation of decoy receptor 2 (DcR2) and c-FLIP. Also, following transfection with small-interfering RNA (siRNA), the expression of siRNA-HIF-1 α reversed the expression of markers of apoptosis and cell viability, which was partially reversed by the use of the caspase-9 inhibitor, Z-LEHD-FMK. These results showed that HIF-1 α can activate the death receptor-associated apoptotic signaling pathways in hUSLFs treated with CoCl₂. These findings require further studies to determine whether active caspase-3 is associated with mitochondrial apoptotic signaling pathways.

A further possible mechanism for activating caspase-3 is the caspase-9-mediated process regulated by mitochondria after induction of HIF-1 α . Hypoxia impairs the integrity of the mitochondrial outer membrane, resulting in the release of proapoptotic factors into the cytosol. Cytochrome C induces the assembly of a caspase activation complex, the apoptosome, which can activate caspase-9 [49]. This process is controlled by the Bcl-2 protein family and subfamily, which contains anti-apoptotic proteins, including Bcl-2 and Bcl-xL, and pro-apoptotic proteins, including Bax and Bad [50,51]. The ratio of the anti-apoptotic and pro-apoptotic proteins may be a crucial signal required for the release of cytochrome C from the mitochondria into the cytosol. Bcl-2 interacting protein 3 (BNIP3) is a

member of the BH3 subfamily of the Bcl-2 superfamily and is a proapoptotic protein [52]. In the present study, the activities of caspase-3 and caspase-9 were increased and the ratio of Bcl-2 to Bax and mitochondrial membrane potential of hUSLFs were reduced after the addition of HIF-1 α . Treatment of the hUSLFs with the chemical inducer of hypoxia, CoCl₂, increased the release of cytochrome C from the mitochondria and up-regulated BNIP3 expression. Also, the use of specific inhibitors of caspase-8 and caspase-9 reversed the loss of cell viability. Overall, these results indicated that the mitochondrial-dependent pathway might be involved in hypoxia-induced apoptosis of hUSLFs *in vitro*.

Conclusions

The findings of this study showed that apoptosis of human uterosacral ligament fibroblasts (hUSLFs) occurred following treatment with the chemical inducer of hypoxia, cobalt chloride (CoCl₂), which was mediated by hypoxia-inducible factor-1 α (HIF-1 α). CoCl₂ treatment activated the Fas/TRAIL/caspase-8 signaling pathway which can lead to the activation of caspase-3 and upregulated the expression of Bcl-2 interacting protein 3 (BNIP3), leading to the release of cytochrome C from the mitochondria to the cytoplasm. The activation of caspase-3 and caspase-9 resulted in apoptosis of hUSLFs and downregulation of expression of collagen type I alpha 1 (COL1A1). These results supported that apoptosis was induced by hypoxia of hUSLFs through both mitochondrial apoptosis-associated and death receptor-associated pathways. These *in vitro* results might have implications for the pathogenesis of pelvic organ prolapse.

Conflict of interest

None.

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