Assessing the protective effects of cryptotanshinone on $CoCl_2$-induced hypoxia in RPE cells

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Abstract. The development of several retinal diseases is closely related to hypoxia. As a component of the Traditional Chinese medicine *Salvia miltiorrhiza*, the effects of cryptotanshinone (CT) on retinal cells under hypoxic conditions are not well understood. The aim of the present study was to explore how CT exerted its protective effects on retinal pigment epithelium (RPE) cells under hypoxic conditions induced by cobalt chloride ($CoCl_2$). The effects of CT were investigated using a Cell Counting Kit-8 assay, Annexin V-FITC/PI staining, reverse transcription-quantitative PCR and western blotting in ARPE-19 cells. CT (10 and 20 μM) reduced the $CoCl_2$-induced increase in vascular endothelial growth factor expression and hypoxia-inducible transcription factor-1α expression in ARPE-19 cells. Additionally, CT alleviated hypoxia-induced apoptosis by regulating Bcl-2 and Bax protein expression. CT treatment also reduced the increase in the mRNA levels of IL-6, IL-1β and TNF-α induced by $CoCl_2$. In summary, CT may protect RPE cells against apoptosis and inflammation in $CoCl_2$-induced hypoxia, and these results warrant further *in vivo* study into its value as a drug for treating hypoxic eye diseases.

Introduction

The oxygen utilization rate of the retina is ~9.7 ml O_2/100 ml tissue/min, which is 2-3 times that of the brain (1). In order to maintain the high metabolic requirements required for normal vision, the retina is the most oxygen-dependent part of the human body (2). Retinal hypoxia, when retinal blood circulation is not sufficient for meeting the metabolic needs of the retina, serves an important role in the development of retinal artery and vein occlusion, diabetic retinopathy and age-related macular degeneration (3,4). Hypoxia-inducible transcription factor-1 (HIF-1) is an important factor involved in the hypoxic reactions that affect several biological functions, including angiogenesis, cell proliferation and inflammation (5,6).

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Retinal pigment epithelium (RPE) cells form a monolayer of cells that are located between the photoreceptors and Bruch membrane-choroid complex. They act as an external barrier to the blood-retina system. RPE cells regulate delivery of nutrients and oxygen to the retina, and also remove metabolic waste from photoreceptor cells (7,8). As RPE cells are adjacent to the choroidal capillaries, they are susceptible to ischemia or hypoxia (9). The tissues formed by the RPE cells have also been reported to be the most metabolically active of all tissues in the human body, and are extremely sensitive to any changes in oxygen tension (10). The functional properties of RPE cells have been extensively studied under appropriate culture conditions *in vitro* (11). A previous study showed that in hypoxic RPE cells, HIF-1α expression is stable and may lead to the production of several angiogenic factors, including vascular endothelial growth factor (VEGF) (12).

Cryptotanshinone (CT), an active component of *Salvia miltiorrhiza*, exerts a protective effect against several diseases, such as ischemia, atherosclerosis and Alzheimer's disease, without any notable side effects (13). CT has several pharmacological effects, including anti-oxidant, anti-inflammatory and anti-angiogenic effects (14). Moreover, Feng *et al* (15) showed that combined treatment with CT and albendazole significantly improved ganglion cell injury and reduced optic nerve demyelination caused by infection by *Angiostrongylus cantonensis*. Jian *et al* (16) found that, as one of the components of *Fufang Xueshuantong* capsules, CT reduced the retinal damage induced by streptozotocin in rats. Considering that there have been no studies assessing the effects of CT on the retinal cells under hypoxic conditions to the best of our knowledge, in the present study, the potential protective effects of CT on RPE cells in the presence of cobalt chloride ($CoCl_2$)-induced chemical hypoxia was assessed.

Materials and methods

Reagents and antibodies. CT (cat. no. SC8640) was purchased from Beijing Solarbio Science & Technology Co., Ltd. $CoCl_2$ (cat. no. C8661) was purchased from Sigma-Aldrich; Merck KGaA. DMEM/F-12 (cat. no. 11330032) and FBS (cat. no. 16140071) were purchased from Gibco; Thermo Fisher Scientific, Inc. Streptomycin-penicillin (cat. no. C0222) and an Annexin V-FITC Apoptosis Detection kit (cat. no. C1062M) were purchased from Beyotime Institute of Biotechnology. Primers were synthesized by Genewiz, Inc. The Cell Counting Kit-8 (CCK-8) assay was purchased from Dojindo Molecular Technologies, Inc. An AxyPrep Multisource Total RNA Miniprep kit (cat. no. AP-MN-MS-RNA-50) was purchased from Axygen; Corning, Inc. The Prime Script RT Master Mix (cat. no. RR036A-1) and TB Green Premix Ex Taq (Perfect Real Time; cat. no. RR420) were purchased from Takara Bio, Inc.

The following antibodies were used in the present study: VEGF rabbit PolyAb (cat. no. 19003-1-AP; ProteinTech Group, Inc.), HIF1-α rabbit PolyAb (cat. no. 20960-1-AP; ProteinTech Group, Inc.), Bcl-2 rabbit PolyAb (cat. no. AB112; Beyotime Institute of Biotechnology), Bax (D2E11) rabbit mAb (cat. no. 5023; Cell Signaling Technology, Inc.), β-actin rabbit mAb (cat. no. AB0035; Abways Technology), and the secondary horseradish peroxidase-conjugated goat anti-rabbit IgG PolyAb (cat. no. SE134; Beijing Solarbio Science & Technology Co., Ltd.).

Cell culture. Human RPE cells (ARPE-19) were obtained from The Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in DMEM/F-12 supplemented with 10% FBS and 1% streptomycin-penicillin, and cultured in a humidified incubator with 5% CO_2 at 37˚C.

Preparation of CT stock solution. A 10 mM stock solution was prepared by dissolving CT in DMSO, and further diluted to 5, 10 and 20 µM using serum-free medium.

Cell viability. Cell viability was assessed using a CCK-8 assay. A total of $5x10^3$ cells/well were seeded in 96-well plates. Cells were grown to 70-80% confluence, and then treated with $CoCl_2$ (200, 400, 600 or 800 µM) or CT (5, 10 or 20 µM). After determining the concentration of $CoCl_2$ needed for the subsequent experiments, cells were exposed to 5, 10 or 20 µM CT with or without 600 µM $CoCl_2$. The negative control cells (NC) were treated with serum-free DMEM/F12. After 24 h of incubation, the morphology of cells was imaged using a light microscope (magnification, x200) (Olympus Corporation), then medium was aspirated from the cells, and then a mixture containing 10 µl CCK-8 and 100 µl serum-free medium was added to the cells and cultured for a further 2 h. Subsequently, the absorbance of each well was measured using a Multiskan FC plate reader (Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm (17).

Cell apoptosis. Apoptosis of ARPE-19 was detected using an Annexin V-FITC Apoptosis Detection kit. Briefly, cells were exposed to CT (5, 10 or 20 µM) with 600 µM $CoCl_2$ for 24 h, then cells were harvested, washed once with PBS, and stained with Annexin V-FITC and PI at room temperature for 30 min. The apoptotic rate was detected on a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Inc.), and the proportion of early and late apoptotic cells were further analyzed using FlowJo version 7.6.3 (FlowJo LLC) (18,19).

Reverse transcription-quantitative (RT-qPCR). Cells were treated with CT (5, 10 or 20 µM) with or without 600 µM $CoCl_2$ for 12 h. The expression of a target protein is usually expressed later than that of its mRNA (20). When referring to the previous references (21,22), 12 h was selected to carry out the experiments to study the mRNA expression of these cytokines. Total RNA from ARPE-19 cells was extracted using an AxyPrep Multisource Total RNA Miniprep kit according to the manufacturer's protocol. The RNA concentration was measured using a BioDrop µLITE PC spectrophotometer (BioDrop). Prime Script RT Master Mix was used to reverse transcribe the RNA into cDNA; the RT reaction conditions were 37˚C for 15 min, followed by 85˚C for 5 sec. qPCR was performed using a CFX96 Real-Time system (Bio-Rad Laboratories, Inc.), and the thermocycling conditions were: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. qPCR was performed using TB Green PCR Master Mix. mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (23). The sequences of the primers used are listed in Table I.

Western blotting. ARPE-19 cells were lysed in lysis buffer for 30 min, then the cells were centrifuged at 12,000 x g for 10 min at 4˚C, and the supernatant, which contained the total protein, was collected. The protein was loaded on a 10% SDS-gel, resolved using SDS-PAGE, and transferred to a PVDF membrane. Membranes were blocked using 5% non-fat milk, followed by incubation with one of the following antibodies at 4˚C overnight: Anti-β-actin (1:1,000), anti-Bcl-2 (1:800), anti-VEGF (1:1,000), anti-Bax (1:1,000) or anti-HIF-1α (1:500). After washing with PBS-Tween, the membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5,000) at room temperature for 45 min. Finally, enhanced chemiluminescence reagent was mixed in equal proportions and used to visualize the signals. Densitometry analysis was performed using ImageJ version 2.0.0 (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± standard error of the mean of at least three repeats. Differences between groups were compared using an unpaired Student's t-test or a one-way ANOVA followed by a post hoc Tukey's test. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

CT inhibits $CoCl_2$-induced cytotoxicity in ARPE-19 cells. First, whether $CoCl_2$ (200, 400, 600 or 800 µM) or CT (5, 10 or 20 µM) treatment induced cytotoxicity in ARPE-19 cells was determined. In the range of $CoCl_2$ concentrations used, cell viability decreased gradually in a dose-dependent manner. As the concentration that inhibited the cell viability of ARPE-19 cells by ~60% was 600 µM $CoCl_2$ (Fig. 1A), in all subsequent experiments, this concentration was used. Although CT did not affect the cell viability when 5, 10 or 20 µM was used (Fig. 1B), CT treatment did exert a protective effect on cell viability against $CoCl_2$-induced hypoxia (Fig. 1C and D).

Table I. Sequences of the primers used in the present study.

Gene name	Sequence, 5'-3'
VEGFA	
Forward	AGGGCAGAATCATCACGAAGT
Reverse	AGGGTCTCGATTGGATGGCA
HIF-1α	
Forward	ATCCATGTGACCATGAGGAAATG
Reverse	TCGGCTAGTTAGGGTACACTTC
TNF-α	
Forward	CCTCTCTCTAATCAGCCCTCTG
Reverse	GAGGACCTGGGAGTAGATGAG
IL-1β	
Forward	AGCTACGAATCTCCGACCAC
Reverse	CGTTATCCCATGTGTCGAAGAA
IL-6	
Forward	CCTGAACCTTCCAAAGATGGC
Reverse	TTCACCAGGCAAGTCTCCTCA
β-actin	
Forward	CATGTACGTTGCTATCCAGGC
Reverse	CTCCTTAATGTCACGCACGAT

HIF-1α, hypoxia-inducible transcription factor-1α.

CT inhibits $CoCl_2$-induced apoptosis of ARPE-19 cells by regulating Bax and Bcl-2 expression. Since CT could protect against $CoCl_2$-induced cytotoxicity, the effect of CT on apoptosis of ARPE-19 cells treated with $CoCl_2$ was assessed. Flow cytometry analysis showed that CT (10 or 20 μM) inhibited apoptosis of ARPE-19 cells induced by $CoCl_2$ (Fig. 2).

Furthermore, western blotting showed that Bax expression was increased and Bcl-2 expression was decreased following treatment with $CoCl_2$ when compared with the NC group (Fig. 3). In contrast, treatment with CT reversed these trends (Fig. 3). These results indicated that CT (10 and 20 μM) could inhibit $CoCl_2$-mediated apoptosis, at least partly through regulation of Bax and Bcl-2 expression. The current study also examined the effects of CT (5, 10 or 20 μM) alone on RPE cell apoptosis, as shown in Figs. S1 and S2, under normal conditions. It was found there were no statistically significant differences among them.

CT regulates inflammatory factors in hypoxic ARPE-19 cells. It has been previously shown that CT exerts an anti-inflammatory effect on different diseases (13). The mRNA levels of TNF-α, IL-1β and IL-6 in ARPE-19 cells treated with $CoCl_2$ were significantly increased compared with the NC group, and CT (5, 10 and 20 μM) treatment decreased the mRNA expression levels of these inflammatory factors in the $CoCl_2$ treated cells (Fig. 4). Furthermore, the transcriptional levels of these inflammatory factors were not statistically significant when RPE cells were treated with CT (5, 10 or 20 μM) alone (Fig. S3).

CT reduces VEGF expression in ARPE-19 cells under $CoCl_2$-induced hypoxic conditions. Since hypoxia is a major inducer of angiogenesis (24), the anti-angiogenic effects of CT under hypoxic conditions were assessed. Hypoxic conditions induced by $CoCl_2$ significantly increased the expression of VEGF and HIF-1α in ARPE-19 cells, both at the mRNA and protein level (Fig. 5). In contrast, CT treatment inhibited the CoCl2-induced increase of HIF-1α and VEGF expression (Fig. 5). However, under normal conditions, CT (5, 10 or 20 μM) alone treatment did not affect the gene and protein expression levels of HIF-1α and VEGF in RPE cells (Figs. S2 and S3).

Discussion

In the present study, a model of hypoxia in RPE cells using $CoCl_2$ was established, and it was shown that CT could protect RPE cells in the following three ways: i) CT exhibited an anti-apoptotic effect by regulating the expression of Bax and Bcl-2; ii) CT served an anti-inflammatory effect by reducing the transcriptional levels of the inflammatory factors TNF-α, IL-6 and IL-1β; and iii) CT inhibited the expression of HIF-1α and VEGF, which may inhibit the formation of new blood vessels.

HIF-1 is widely recognized as a major regulator of the response to hypoxia. HIF-1 is a transcription factor composed of HIF-1α and HIF-1β (25). HIF-1α is hydroxylated by prolyl hydroxylase under conditions of sufficient oxygen, leading to its degradation. However, under hypoxic conditions, the HIF-1α protein is stabilized and accumulates due to inhibition of prolyl hydroxylase (26). The hydroxylation of prolyl hydroxylase requires molecular oxygen, and cobalt can replace the ferrous ions bound to the active site, causing the inactivation of hydroxylase, thereby stabilizing the HIF-1α protein, and this has been widely used to simulate hypoxia *in vitro* (27).

Following treatment with $CoCl_2$, the apoptotic rate of RPE cells was increased as confirmed by Annexin V-FITC and PI double staining, and the addition of CT to cells reduced the apoptotic rates of cells. Next, the mechanism underlying the effects of CT were determined. Cytochrome *c* is an initiator of apoptosis, and is primarily associated with the Bcl-2 family of proteins, such as Bax and Bcl-2. Bax promotes the release of cytochrome *c* from the mitochondria, thereby activating a series of downstream caspase reactions leading to apoptosis, whereas Bcl-2 prevents the release of cytochrome C by maintaining the integrity of the mitochondrial membrane (26,28).

Elevated Bax and decreased Bcl-2 levels leads to initiation of apoptosis (29). In the present study, compared with the NC group, Bax protein levels were increased and Bcl-2 protein levels were decreased in the $CoCl_2$ group. CT inhibited the $CoCl_2$-induced apoptosis, suggesting that CT exerted an anti-apoptotic effect by regulating Bax and Bcl-2 protein expression levels in the hypoxic RPE cells. However, whether CT affects the release of caspase-activated enzymes requires further experimental study. Zhu *et al* (30) found that in a rat model of stroke, CT exerted an anti-apoptotic effect by increasing the levels of Bcl-2 in the cerebral cortex and peripheral blood. However, Kim *et al* (31) found that in non-small cell lung cancer cells, CT increased caspase-3 and Bax expression levels and inhibited Bcl-2, thereby promoting the activation of apoptosis and reducing cell proliferation.

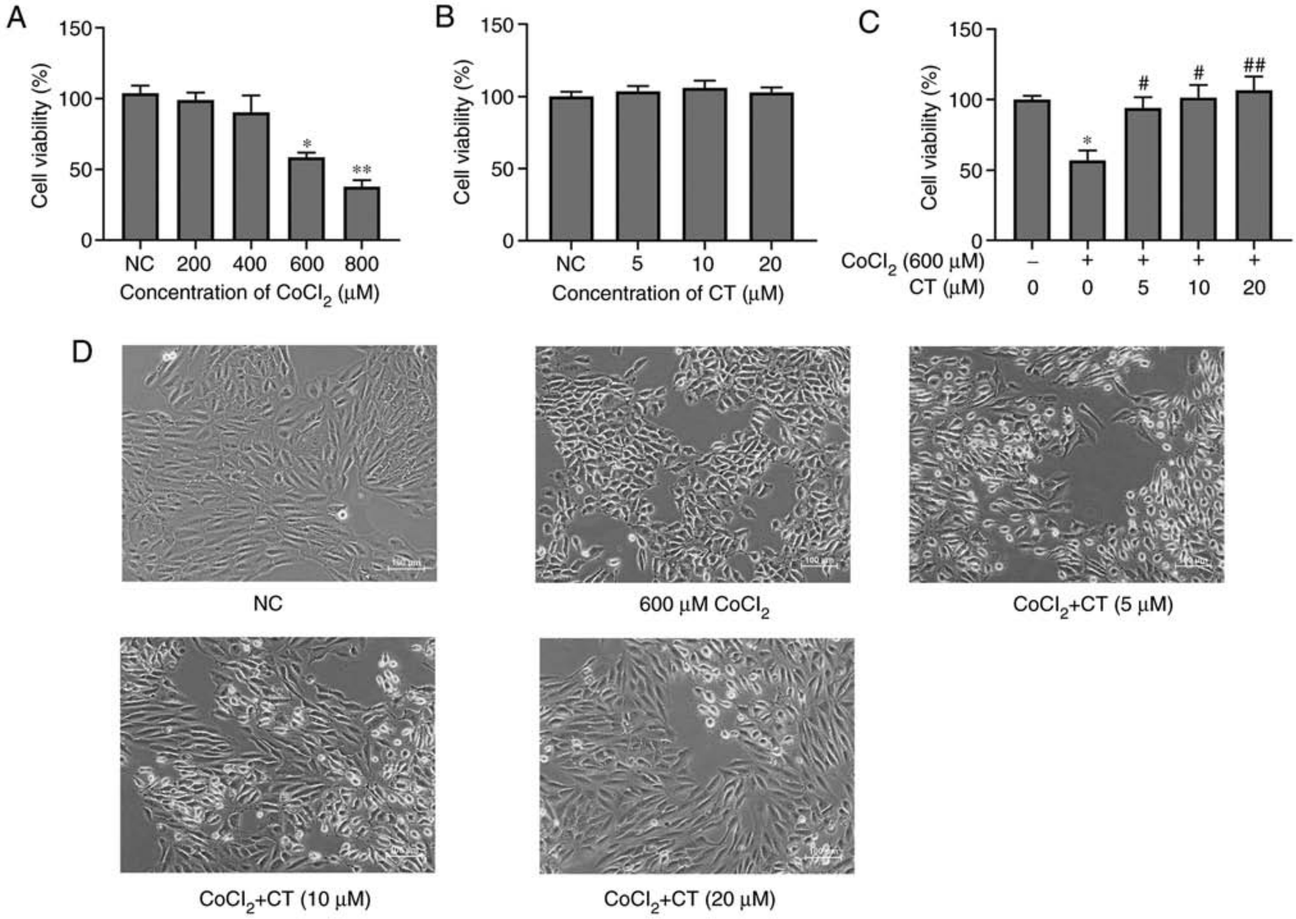

Figure 1. CT inhibits CoCl2-induced cytotoxicity in ARPE-19 cells. ARPE-19 cells were treated with different concentrations of (A) $CoCl_2$ or (B) CT for 24 h. (C and D) CT treatment increased the cellular viability of ARPE-19 cells treated with $CoCl_2$. Data are presented as the mean ± the standard error of the mean. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. 600 μM $CoCl_2$ alone; $^{*}P<0.05$, $^{**}P<0.01$ vs. NC. NC, negative control cells treated in serum-free DMEM/F12; CT, cryptotanshinone; $CoCl_2$, cobalt chloride.

These differences in effects mediated by CT may be attributed to the use of different cell lines, and the dysregulation of several signaling pathways in cancer cell lines compared with normal healthy cells.

In addition, hypoxia is also related to inflammation. Hypoxia can cause inflammation and tissue damage in certain diseases, such as rheumatoid arthritis and stroke (10). Just as hypoxia can trigger an inflammatory response, hypoxia often occurs at the site of inflammation. It has also been reported that under hypoxic conditions, HIF-1α and HIF-1β are bound together and translocate to the nucleus to activate the inflammatory cascade by promoting transcription of pro-inflammatory genes (32,33). $CoCl_2$ may increase HIF-1α expression, and subsequently affect activation of various inflammation-associated transcription factors, such as NF-κB, which in-turn increases the expression of pro-inflammatory cytokines, including TNF-α, IL-6 and NO in the retina (34,35). Since hypoxia and inflammation are the primary causes of several eye diseases (36), and CT has been reported to exert an anti-inflammatory effect (37), the mRNA levels of the inflammatory factors, IL-6, IL-1β and TNF-α, under hypoxic conditions were explored, and whether CT exerted anti-inflammatory effect was assessed.

The results showed that hypoxia was positively related to inflammation, as the expression levels of the inflammatory factors, IL-6, IL-1β and TNF-α, were elevated when the cells were treated with $CoCl_2$, suggesting that hypoxia could promote the transcription of inflammatory cytokines. Treatment with CT reduced the levels of these inflammatory factors, thus exerting an anti-inflammatory effect on hypoxic RPE cells. Zhang *et al* (38) reported that CT not only inhibited the secretion of IL-1, IL-8 and TNF-α in CT26 cells, but also inhibited the expression of IL-6, TNF-α and pro-IL-1 *in vivo*.

HIF-1α can upregulate the expression of several genes that encode proteins related to angiogenesis, such as VEGF, which serves a critical role in retinal angiogenesis (12). Studies have reported that patients with various eye diseases related to angiogenesis have significantly elevated levels of VEGF protein in the aqueous fluid or vitreous body (39,40). Currently available anti-VEGF drugs, such as bevacizumab, ranibizumab and abribercept, which are used for treatment of neovascular eye diseases, have notable side effects following repeated high dose administration, limiting their applicability (41). It has been reported that regulation of the HIF pathway may be more suitable for the management of neovascular eye diseases than drugs that only target VEGF (42).

In the present study, the data showed that both HIF-1α mRNA and protein levels were increased, and this increased VEGF production in RPE cells under hypoxic conditions induced by $CoCl_2$. It is generally hypothesized that HIF-1α mRNA expression is unaffected when the oxygen concentration

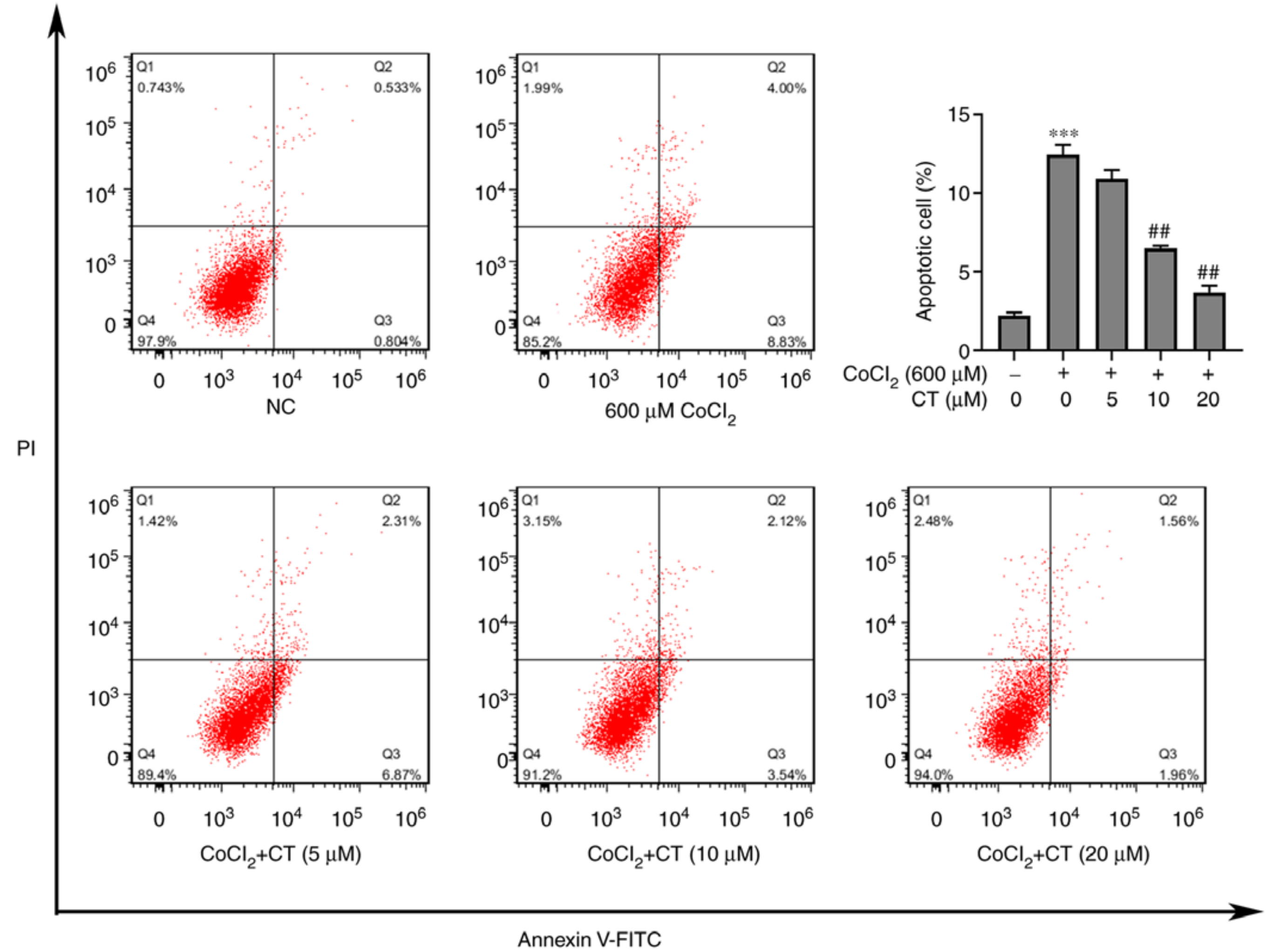

Figure 2. CT inhibits CoCl2-induced apoptosis of ARPE-19 cells. ARPE-19 cells were cultured with different concentrations of CT (5, 10 or 20 μM) and $CoCl_2$ (600 μM) for 24 h. Cell apoptosis was analyzed using flow cytometry. Data are presented as the mean ± the standard error of the mean. ##P<0.01 vs. 600 μM $CoCl_2$ alone; ***P<0.001 vs. NC. NC, negative control cells treated in serum-free DMEM/F12; CT, cryptotanshinone; $CoCl_2$, cobalt chloride.

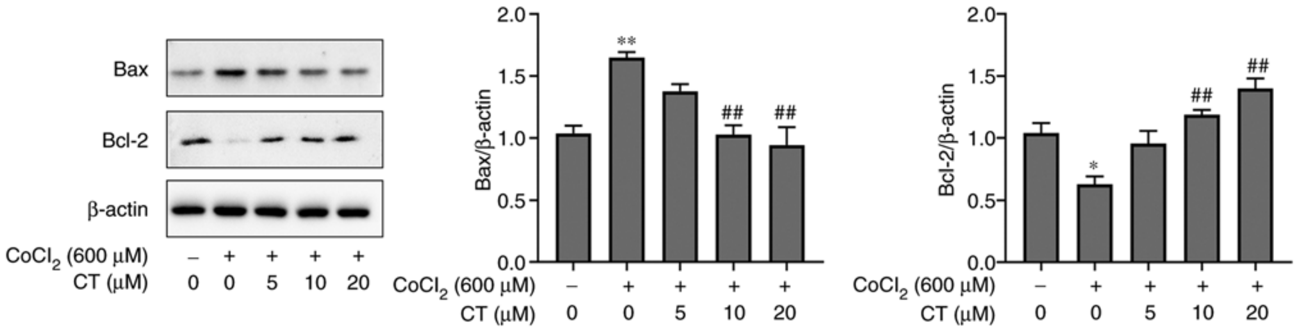

Figure 3. CT inhibits $CoCl_2$-induced apoptosis of ARPE-19 cells by regulating Bax and Bcl-2 expression. Bax expression was decreased, whereas Bcl-2 expression was increased after culturing with different concentrations of CT (5, 10 or 20 μM) and $CoCl_2$ (600 μM) for 24 h. Data are presented as the mean ± the standard error of the mean. ##P<0.01 vs. 600 μM $CoCl_2$ alone; *P<0.05, **P<0.01 vs. NC. NC, negative control cells treated in serum-free DMEM/F12. CT, cryptotanshinone; $CoCl_2$, cobalt chloride.

is altered (43,44). However, Semenza (45) found an increase in HIF-1α mRNA expression levels when they exposed animals to prolonged or intermittent hypoxic conditions. In fact, there are a variety of factors caused by hypoxia that could affect the mRNA levels of HIF-1α (43). Hypoxia-induced activation of NF-κB can bind to the HIF-1α promoter and lead to a rapid increase in HIF-1α transcription (46). The elevated expression of the inflammatory factors IL-1 and TNF-α induced by hypoxia also increases the mRNA expression levels of HIF-1α (47). Additionally, it has been previously shown that $CoCl_2$-induced hypoxia increases the mRNA expression levels of HIF-1α (48). For example, Oh *et al* (49) found that HIF-1α

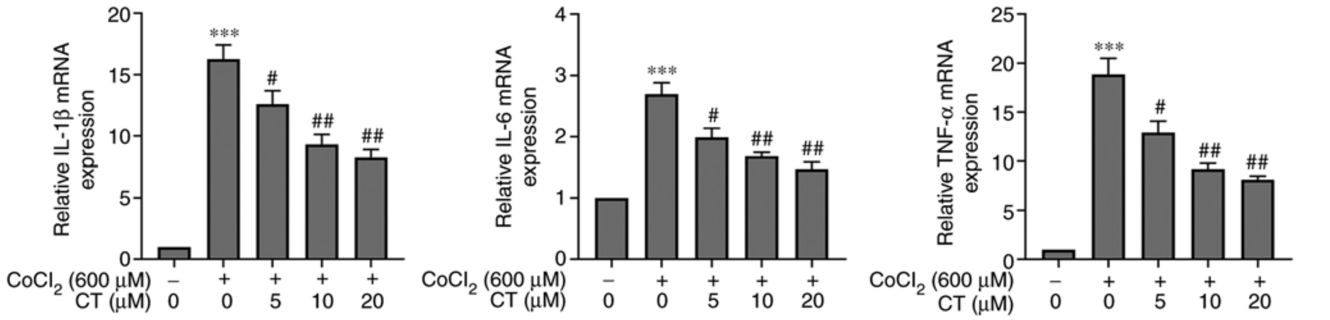

Figure 4. CT regulates expression of inflammatory factors in hypoxic ARPE-19 cells. ARPE-19 cells were cultured with different concentrations of CT (5, 10 or 20 μM) and $CoCl_2$ (600 μM) for 12 h. The mRNA expression levels of IL-1β, IL-6 and TNF-α were increased following treatment with $CoCl_2$ compared with the control group, whereas CT treatment reversed this phenomenon. Data are presented as the mean ± the standard error of the mean. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. 600 μM $CoCl_2$ alone; ***P<0.001 vs. NC. NC, negative control cells treated in serum-free DMEM/F12; CT, cryptotanshinone; $CoCl_2$, cobalt chloride.

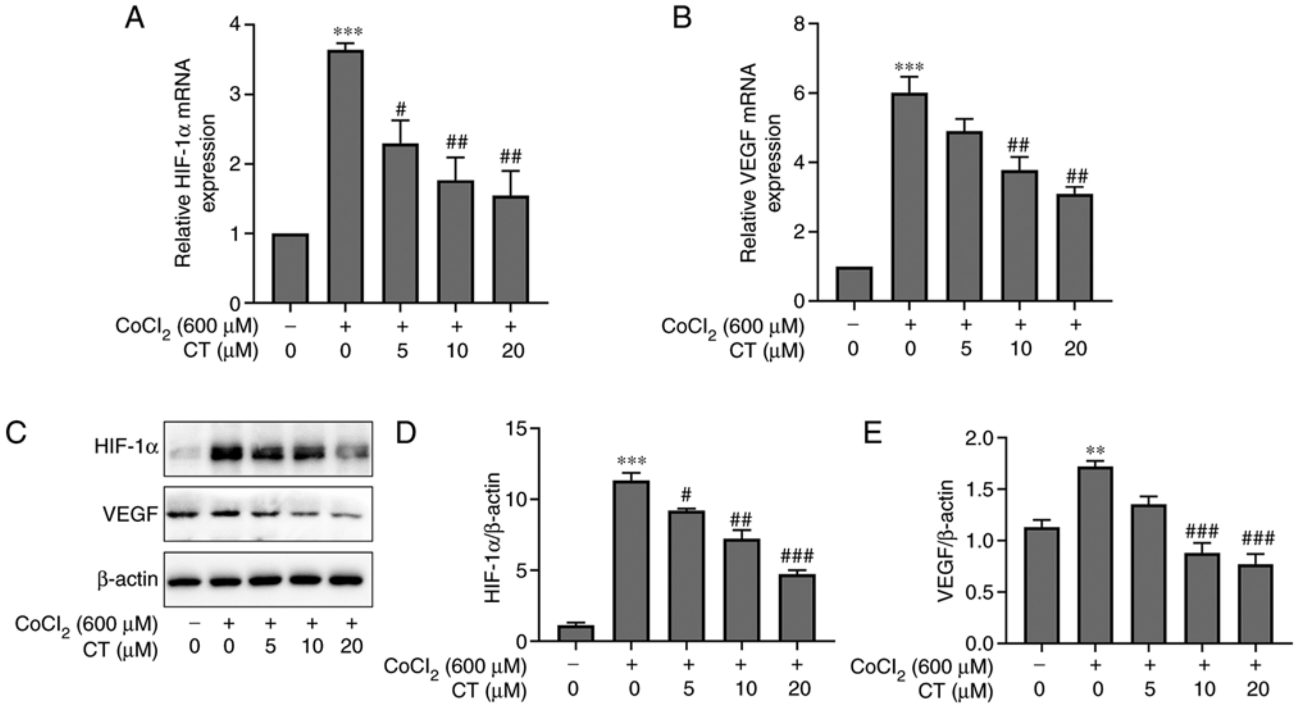

Figure 5. CT reduces VEGF expression in ARPE-19 cells following $CoCl_2$-induced hypoxia. The mRNA expression levels of (A) HIF-1α and (B) VEGF in ARPE-19 cells were detected after treatment with different concentrations of CT (5, 10, 20 M) and $CoCl_2$ (600 M) for 12 h, and the (C-E) protein expression levels were detected after 24 h. Data are presented as the mean ± the standard error of the mean. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. 600 μM $CoCl_2$ alone; **P<0.01, ***P<0.001 vs. NC. NC, negative control cells treated in serum-free DMEM/F12; CT, cryptotanshinone; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible transcription factor-1α; $CoCl_2$, cobalt chloride.

mRNA levels were increased in $CoCl_2$-induced hypoxic RPE cells.

The results of the present study showed that the mRNA and protein expression levels of HIF-1α were increased in the $CoCl_2$ treated groups, suggesting that hypoxia promoted HIF-1α expression. Further experiments showed that CT treatment protected RPE cells against the $CoCl_2$-induced hypoxia by reducing HIF-1α mRNA and protein expression levels, suggesting that CT could inhibit HIF-1α protein accumu2lation and transcriptional activity in hypoxic RPE cells. Thus, the effects of CT on HIF-1α protein stability may be related to the inhibition of nuclear translocation of HIF-1α. It is well established that HIF-1α primarily exerts its effects through nuclear translocation (50). Under hypoxic conditions, HIF-1α is translocated to the nucleus and further activates transcription of several factors (27). Zhang *et al* (38) reported that CT could reduce the nuclear levels and increase the cytosolic levels of HIF-1α, which may have an effect on the stability of the HIF-1α protein.

In the present study, the decreased expression of VEGF following CT treatment may partially be due to the inhibition of HIF-1α expression, limiting binding of HIF-1α to the VEGF promoter region (51). This may partially reduce the formation of new blood vessels caused by hypoxia, thus playing a therapeutic role in wet age-related macular degeneration, which is characterized by aberrant angiogenesis (52). CT was also shown to exert a similar anti-angiogenic effect in bovine aortic endothelial cells (53) and human umbilical vein endothelial cells (54).

The present study has some limitations. Although CT exerted a protective effect on cytotoxicity, apoptosis and inflammation of RPE cells induced by $CoCl_2$, the underlying molecular mechanisms require further study. Another limitation of this study was the lack of *in vivo* experiments.

In conclusion, hypoxia is closely associated with a variety of ophthalmic diseases. Neurodegenerative glaucoma is associated with fluctuations in oxygen levels, and hypoxia has been used as a model for studying multiple neurodegenerative diseases in animals (55). Diabetic retinopathy and retinopathy of prematurity are proliferative retinopathies that are characterized by retinal blood vessel ischemia, resulting in hypoxia (56). HIF-1α may directly increase angiogenesis and inflammation, both of which are involved in the progression of age-related macular degeneration, and studies have shown that specific targeting of HIF has emerged as an attractive strategy for the treatment of neovascular age-related macular degeneration (57,58).

To the best of our knowledge, the present study is the first study to show that CT can protect RPE cells against hypoxia through its anti-inflammatory, anti-apoptotic and anti-VEGF effects, and CT did not exert any notable cytotoxic effects on the RPE cells at the doses used. The beneficial pharmacological effects and the apparent lack of notable side effects of CT highlight it as a novel therapeutic option for the treatment of hypoxic eye diseases.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

PL and GL conceived the study and revised the manuscript. YG and WL performed the experiments and were responsible for the draft manuscript. YG and XL analyzed data and organized the figures. YG, WL and XL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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