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Original Article

Extracts of *Celastrus Orbiculatus* Inhibit Cancer Metastasis by Down-regulating Epithelial-Mesenchymal Transition in Hypoxia-Induced Human Hepatocellular Carcinoma Cells*

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ABSTRACT **Objective:** To evaluate the effects of *Celastrus Orbiculatus* extracts (COE) on metastasis in hypoxia-induced hepatocellular carcinoma cells (HepG2) and to explore the underlying molecular mechanisms. **Methods:** The effect of COE (160, 200 and 240 μg/mL) on cell viability, scratch-wound, invasion and migration were studied by 3-4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2-H-tetrazolium bromide (MTT), scratch-wound and transwell assays, respectively. $CoCl_2$ was used to establish a hypoxia model *in vitro*. Effects of COE on the expressions of E-cadherin, vimentin and N-cadherin were investigated with Western blot and immunofluorescence analysis, respectively. **Results:** COE inhibited proliferation and metastasis of hypoxia-induced hepatocellular carcinoma cells in a dose-dependent manner ($P<0.01$). Furthermore, the expression of epithelial-mesenchymal transition (EMT) related markers were also remarkably suppressed in a dose-dependent manner ($P<0.01$). In addition, the upstream signaling pathways, including the hypoxia-inducible factor 1α (Hif-1α) and Twist1 were suppressed by COE. Additionally, the Hif-1α inhibitor 3-5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), potently suppressed cell invasion and migration as well as expression of EMT in hypoxia-induced HepG2 cells. Similarly, the combined treatment with COE and YC-1 showed a synergistic effect ($P<0.01$) compared with the treatment with COE or YC-1 alone in hypoxia-induced HepG2 cells. **Conclusions:** COE significantly inhibited the tumor metastasis and EMT by suppressing Hif-1α/Twist1 signaling pathway in hypoxia-induced HepG2 cell. Thus, COE might have potential effect to inhibit the progression of HepG2 in the context of tumor hypoxia.

KEYWORDS *Celastrus Orbiculatus*, hepatocellular carcinoma, antimetastasis, epithelial-mesenchymal transition, Hif-1α/Twist1 signaling pathway

Hepatocellular carcinoma (HCC) is a common invasive malignancy of the liver. It is estimated that 30%–78% of HCC showed metastases at autopsy.[1] High metastasitic potential is not only a sign of deterioration, but also the major obstacle to improve long-term survival of HCC.[2] Therefore, it is very important to explore effective therapeutic agent targeting the cancer metastasis for HCC. Epithelial-mesenchymal transition (EMT) is a key event of metastatic in cancer cells.[3] It is a process in which the cells losing their epithelial phenotype and acquiring a migratory mesenchymal phenotype. Hypoxia microenvironment is one of the basic characteristics of a variety of solid tumors including HCC, and it is also the important factor that affects tumor metastasis.[4] Hypoxia-inducible factor 1α (Hif-1α) is the key regulator[5] of the hypoxia response, which is overexpressed in HCC.[6] Twist1, a critical regulator of EMT,[7] regulates EMT by repressing the expression of the E-cadherin gene.[8] Therefore, Hif-1α/Twist1 signaling pathway is involved in the acquisition of EMT in HCC cells.

Celastrus Orbiculatus, a Chinese medicine from

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natural herbs has been reported to have multiple effects on arthritis and other inflammatory diseases. Previous studies indicated that the ethyl acetate extract of *Celastrus Orbiculatus* (COE) showed significant anti-inflammation activities,[9] effects on suppression of tumor angiogenesis,[10] inhibition of the proliferation of various human tumor cell lines and inducing tumor cells apoptosis.[11,12] Moreover, preliminary experiments have shown that COE effectively inhibited EMT through inhibiting heat shock proteins (HSP) 27 and nuclear factor-kappa B (NF-κB)/Snail signaling pathway in human gastric adenocarcinoma.[13] Despite general exploration on COE reported in various biological effects under normoxic conditions, little is known about its action on hypoxic microenvironment. In this study, we investigated the effects of COE on cobalt chloride ($CoCl_2$)-induced EMT *in vitro* and explored the potential molecular mechanisms.

METHODS

Plant Material

The stems of *Celastrus Orbiculatus* plants were obtained from Guangzhou Zhixin Pharmaceutical Co., Ltd. (China) in 2007. The COE was prepared at the Department of Chinese Materia Medica Analysis, China Pharmaceutical University (China). The preparation procedure has been described previously.[10] Briefly, the stems of *Celastrus Orbiculatus* were cut off and ground into powder, followed by drying (15 kg). Then, 95% ethanol was utilized for extraction for 3 h, which was repeated 3 times; subsequently, the extract was collected and the ethanol was retrieved. After the extractum (900 g) dispersed with water, extraction using petroleum ether was performed 3 times, extraction applying ethyl acetate was conducted 3 times, followed by collecting ethyl acetate layer which was washed 3 times by water, decompressed and concentrated, followed by vacuum freeze-drying. Finally, the ethyl acetate extract was condensed and lyophilized into powder and stored at 4 ℃. The chemical constituents from the stems of *Celastrus Orbiculatus* were investigated and compounds were isolated as previously described. 23-hydroxybetulonic acid, oleanolic acid, 23-hydroxyl-3-oxoolean-12-en-28-oic acid, 3-oxo-24-norolean-12-en-28-oic acid and wilforlide B were confirmed to be included in the extract by high performance liquid chromatography (HPLC) assay.

Cell Culture

The human hepatocellular carcinoma cell line HepG2 was purchased from the Cell Bank of Chinese Academy of Sciences Shanghai Institute of Cell Biology (China). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) containing 10% fetal bovine serum (GIBCO, USA) and maintained at 37 ℃ in a humidified incubator in an atmosphere of 5% CO_2. Alternatively, to mimic hypoxia, 100 μmol/L $CoCl_2$ was added to the culture medium, and the cells were cultured for the indicated hours.

Cell Viability Assays

The cell viability was analyzed using the 3-(4, 5-dimethyl-2-thiazyl)- 2, 5-diphenyl-2H-tetrazolium bromide assay (MTT, Sigma, USA). Totally, 5,000 cells/well of HepG2 were seeded in 96-well plates and treated with $CoCl_2$ and COE at different concentrations (80, 160, 240, 320 and 400 μg/mL). After 24 and 48 h of incubation, 20 μL MTT (0.5 mg/mL) was added to each well, and the cells were cultured for another 4 h. Then, added 100 μL DMSO after the medium was removed. The absorbance at 490 nm was determined by a microplate reader and presented as relative cell viability. The tests were performed at least 3 independent times.

Cell Scratch-Wound Assays

Cells were seeded in 6-well plates and starved for 24 h immediately after cells had reached full confluency. Then, a cellular area was created using a 10 μL pipette tube. The cells were washed twice to remove the floating cells with phosphate buffer saline (PBS), after that a serum-free medium was added with or without various concentrations (160, 200, 240 μg/mL) of COE and $CoCl_2$ for 24 h. The spread of wound closure was observed in 10 random fields and photographed under a microscope (Olympus, Japan) at 100× magnification.

Cell Invasion and Migration Assays

The invasion assays were performed using Millicell inserts (Millipore, Billerica, MA, USA) coated with matrigel (BD Biosciences, USA). After respectively treated with various concentrations (160, 200 and 240 μg/mL) of COE and $CoCl_2$ for 24 h, 2.5×10^4 cells were seeded per upper chambers in serum-free DMEM whereas the lower chambers were loaded with DMEM containing 10% fetal bovine serum.

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After 24 h, a cotton swab was used to remove the cells which on the upper chambers, and cells invaded through the matrigel layer to the underside of the membrane were stained by crystal violet. The average number of invasive cells selected on each membrane was calculated using a microscope (Olympus, Japan) in 10 random fields. Cell migration assays were performed using Millicell chamberwithout putting matrigel.

Western Blot Analysis

Total protein contents from the COE-treated HepG2 cells and vehicle control for 24 h were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking the membranes with non-fat milk in PBS with Tween-20 (PBST) for 2 h at room temperature, the membranes were incubated with primary antibodies overnight at 4 ℃, and then washed in TBST. Blots were then incubated for 2 h at 37 ℃ with goat anti-rabbit secondary antibody. The enhanced chemoluminescence reagent was used to visualize the positive bands on the membrane (Amersham, USA). Densitometry was determined using Quantity One Analysis Software (Bio-Rad). All antibodies (anti-E-cadherin, anti-vimentin, anti-N-cadherin, anti-Hif-1α, and anti-β-actin) were purchased from Cell Signaling Technology, Inc. (USA). Anti-Twist1 was purchased from Abcam (USA). The secondary anti-rabbit antibody was purchased from Huaan Biotechnology Company (China).

Immunofluorescence

HepG2 cells grown on coverslips were fixed with 4% formaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton X-100 for 10 min, and blocked with 3% bovine serum albumin (BSA) for 30 min. The cells were incubated with primary antibodies at 1:50 dilution at 4 ℃ for overnight, and then washed with PBS. Slides were washed with PBS and counter-stained with 4',6-diamidino-2-phenylindole (DAPI, GenMed, USA). Fluorescent images were acquired with confocal microscopy (TCS SP8 STED, Leica, Wetzlar, Germany), and exported for quantification analysis. The fluorescence density was measured by Image-Pro Plus 6.0 (Leica, Wetzlar, Germany).

Inhibitor Treatment

To further investigate the effect of Hif-1α inhibitor on cell invasion, migration and EMT of $CoCl_2$-induced HepG2 cells, confluent cell cultures were pretreated with 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1, 100 μmol/L, Sigma, USA) and then incubated in the presence or absence of COE (200 μg/mL) for 24 h. The cells were subjected to the invasion or migration, Western blot and the immunofluorescence assay.

Statistical Analysis

All experiments were repeated at least 3 times. All values were presented as means ± standard deviation ($\bar{x} \pm s$). The differences between groups were evaluated by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

$CoCl_2$-Induced EMT in HepG2 Cells

The tumor microenvironment is featured by hypoxia, which initiates EMT in diverse human solid malignancies.[14] $CoCl_2$-induced EMT-related morphological changes. As shown in Figure 1, the simple cuboidal epithelial tumor cells transformed into spindle mesenchymal cells characterized with long-protuberances. Meanwhile, cells incubated with $CoCl_2$ acquired the ability to move through the extracellular matrix. The Western blot assays showed that the E-cadherin protein expression was suppressed, whereas the protein levels of Hif-1α, vimentin and N-cadherin were significantly increased after $CoCl_2$ treatment ($P<0.01$, Figure 2A). Moreover, immunofluorescence data also demonstrated $CoCl_2$ treatment down-regulated the expression of E-cadherin and increased expression of vimentin ($P<0.01$, Figure 2B).

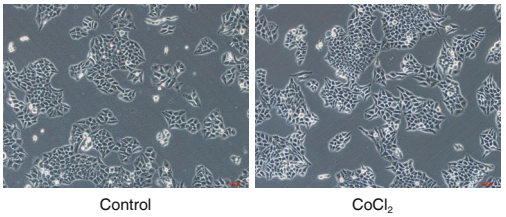

Figure 1. Morphological Changs between HepG2 Cells and $CoCl_2$-Induced HepG2 Cells

COE Inhibits Proliferation and Metastasis in $CoCl_2$-Induced HepG2 Cells

As shown in Figure 3A, COE inhibited the proliferation of $CoCl_2$-induced HepG2 cells in both time- and dose-dependent manners ($P<0.01$).

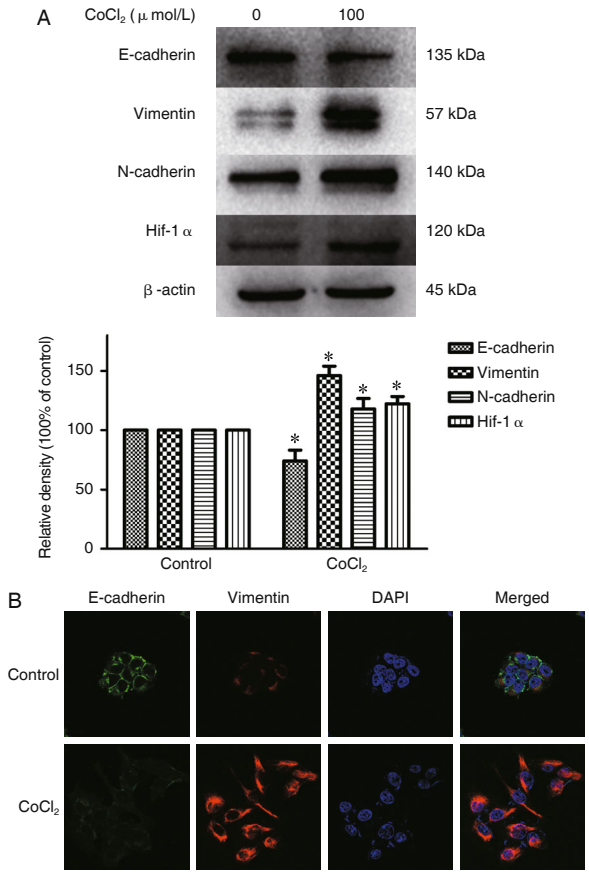

Figure 2. Protein Expression of EMT Markers in $CoCl_2$-Induced HepG2 Cells (n=3, $\bar{x} \pm s$)

Notes: A: The proteins were analyzed by Western blot; B: Direct immunofluorescence staining (630×). $^{*}P$<0.01 *vs*. control

There was no influence on cell viability when the concentration of COE was less than 80 μg/mL, while 160 μg/mL COE significantly inhibited the HepG2 cells viability. Therefore, the low toxicity concentrations (160, 200, 240 μg/mL) of COE were used to perform the following experiments.

The effects of COE on the anti-metastatic potential were further investigated using the wound healing assay and the transwell assay. Compared with the control, HepG2 cells treated with $CoCl_2$ alone exhibited increased migration into the wound area after 24 h wounding occurred (Figure 3B). Meanwhile, COE obviously decreased $CoCl_2$-induced cell migration in a dose-dependent manner (P<0.01). In addition, after treatment of COE, the number of cells invaded to the lower chamber was significantly reduced in a dose-dependent manner after COE treatment (P<0.01, Figure 4). COE (160 to 240 μg/mL) inhibited the invasion and migration of $CoCl_2$-induced HepG2 cells. The rates of invasion inhibition were 12.0%±0.5%, 27.0%±0.4% and 34.0%±0.4%. And the rates of migration inhibition were 12.0%±0.5%, 27.0±0.3% and 34.0%±0.6%, respectively.

COE Inhibits the Expression of EMT-Related Proteins

EMT plays a critical role in promoting metastasis in HCC.[15] The Western blot analysis showed that COE increased the E-cadherin protein level, but decreased N-cadherin and vimentin protein levels in $CoCl_2$-induced HepG2 cells (Figure 5A). Furthermore,

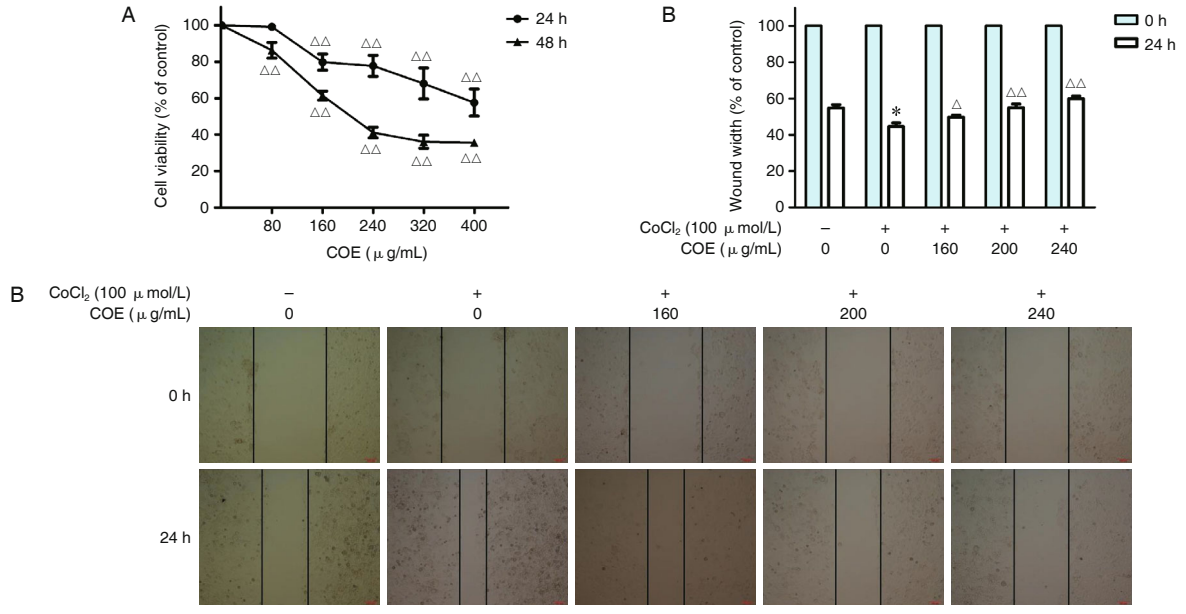

Figure 3. COE Inhibits Proliferation in $CoCl_2$-Induced HepG2 Cells (n=3, $\bar{x} \pm s$)

Notes: A: Effect of COE on cell viability in $CoCl_2$-induced HepG2 cells; B: Effect of COE on the migration of $CoCl_2$-induced cells (100×). $^{*}P$<0.05 *vs*. control; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. $CoCl_2$-induced group.

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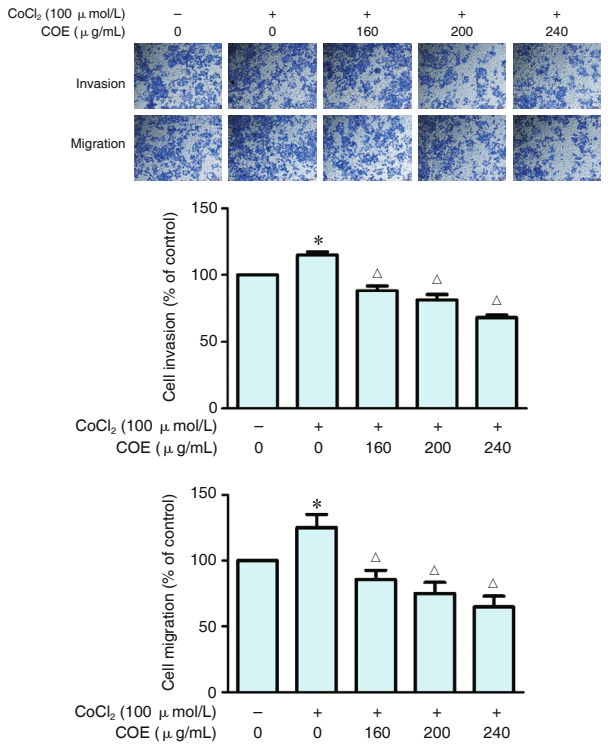

Figure 4. COE Inhibits Metastasis in $CoCl_2$-Induced HepG2 Cells (n=3, $\bar{x} \pm s$)

Notes: The invasion and migration ability of cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate membrane under microscope (100×); *P<0.05 *vs.* control; △P<0.01 *vs.* $CoCl_2$-induced group

the immunofluorescence staining showed consistent results with Western blot assay (Figure 5B).

COE Inhibits EMT through Hif-1α/Twist 1 Signaling Pathway

As shown in Figure 6, the protein expression levels of Hif-1α and Twist1 were decreased in a dose-dependent manner in presence of COE treatment (P<0.01). In addition, Hif-1α/Twist1 signaling pathway was blocked by YC-1, a potential anticancer agent which reduces the protein stability of Hif-1α,[16] which synergized the effects of COE on the invasion and migration (Figure 7). Furthermore, the Western blot analysis showed that the Hif-1α and Twist1 protein expression levels were decreased in YC-1-treated cells (Figure 8). YC-1 and COE co-treatment could further increase the E-cadherin protein level, and decreased N-cadherin and vimentin protein levels (P<0.01). As shown in Figure 9, an increased expression of E-cadherin was observed by using confocal microscope, while decreased N-cadherin and vimentin after YC-1/COE combined treatment.

DISCUSSION

HCC is one of the main causes of cancer-related

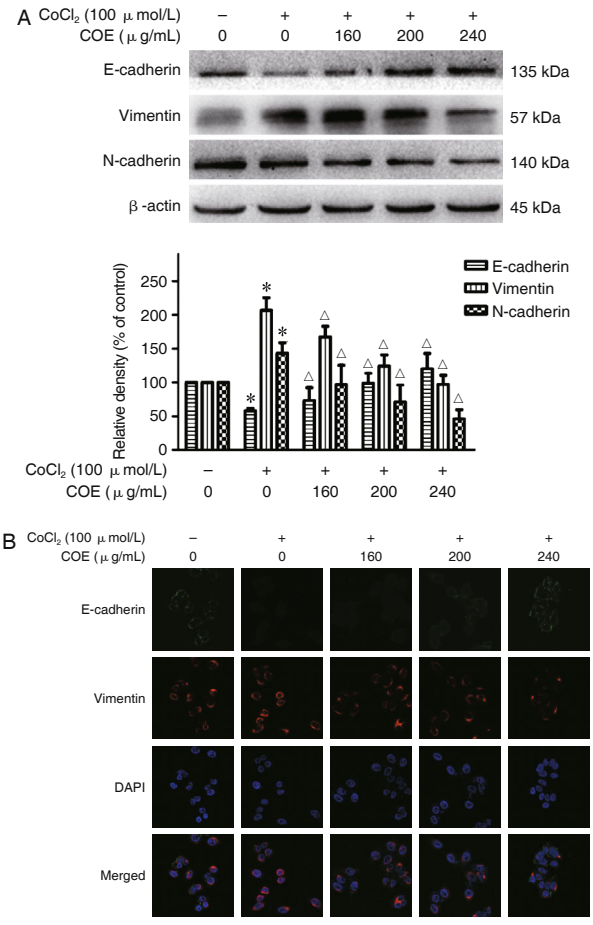

Figure 5. COE Inhibits the Expression of EMT-Related Markers in $CoCl_2$-Induced HepG2 (n=3, $\bar{x} \pm s$)

Notes: A: Effects of COE on the protein expression of EMT markers in $CoCl_2$-induced HepG2 cells, Western blot analysis was performed to detect the levels of E-cadherin, vimentin and N-cadherin; *P<0.01 *vs.* control; △P<0.01 *vs.* $CoCl_2$-induced group. B: Expression of E-cadherin and vimentin after COE treatment by direct immunofluorescence staining in $CoCl_2$-induced HepG2 cells (630×).

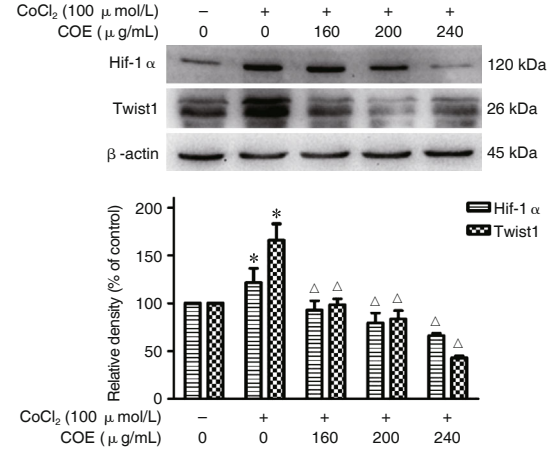

Figure 6. COE Inhibits Hif-1α/Twist1 Signaling Pathway in $CoCl_2$-Induced HepG2 cells (n=3, $\bar{x} \pm s$)

Notes: The protein expression levels of Hif-1α and Twist1 by using Western blot assay in $CoCl_2$-induced HepG2 cells. *P<0.01 *vs.* control; △P<0.01 *vs.* $CoCl_2$-induced group.

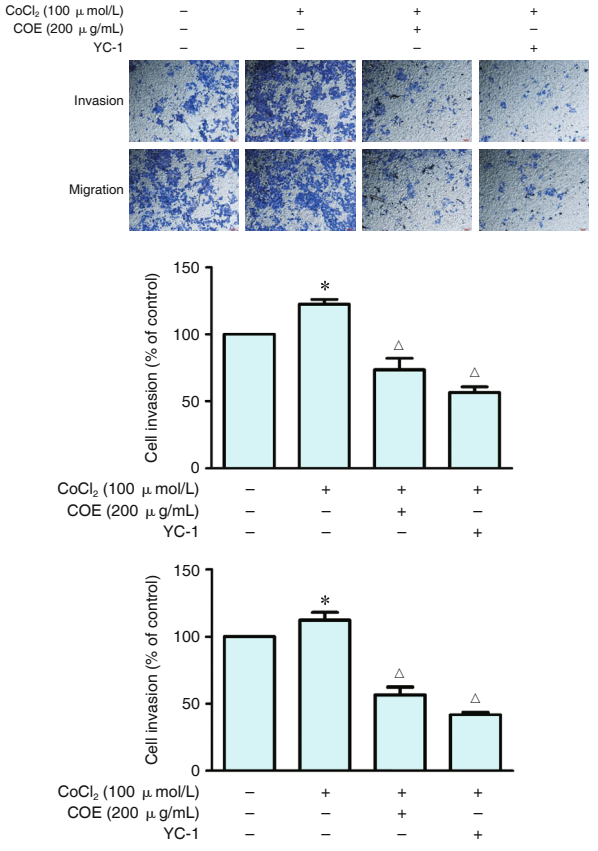

Figure 7. Effect of YC-1/COE on the Invasion in $CoCl_2$-Induced HepG2 Cells

Notes: Invading cells on the lower surface of filter were stained and quantified under a microscope (100 ×); $^{*}P<0.05$ *vs*. control; $^{\triangle}P<0.01$ *vs*. $CoCl_2$-induced group.

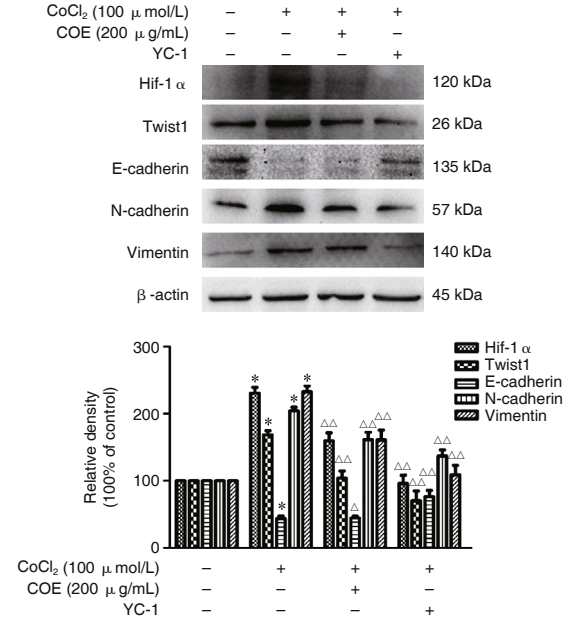

Figure 8. Effects of YC-1/COE on the EMT in $CoCl_2$-Induced HepG2 Cells

Notes: Western blot assay was performed to detect the levels of E-cadherin, vimentin and N-cadherin; $^{*}P<0.01$ *vs*. control; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. $CoCl_2$-induced group.

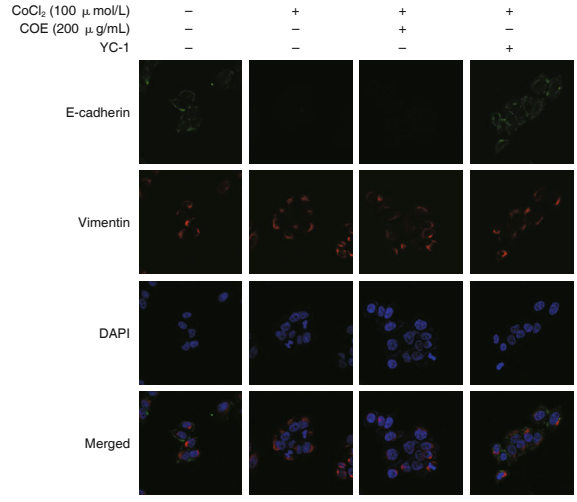

Figure 9. Expression of E-cadherin and Vimentin in $CoCl_2$-Induced HepG2 Cells after YC-1/COE Treatment by Direct Immunofluorescence Staining (630 ×)

death.[17] Hepatic resection is a potentially curative and popular therapy for HCC patients. However, the postoperative outcome remains unsatisfactory, with a 5-year post hepatic resection recurrence rate of approximately 80%.[18] The metastasis of cancer is a pathological process that is the major reason to the morbidity and mortality of cancer patients.[19] Therefore, it is of great clinical importance to study the mechanisms of HCC metastasis and promising treatment. COE has been previously found effective on suppressing proliferation, invasion and migration in HCC. In the present study, we found the anti-metastatic effects of COE in $CoCl_2$-induced HepG2 cells.

EMT triggers the metastasis potential of cancer cells.[20] Cells obtain the mesenchymal phenotypes, thus have less cell adhesion capacity, leading to increased cell invasion and migration, and tumor aggressiveness.[21] Therefore the effective drugs that can block or reverse EMT will potentially become new chemotherapeutic drugs used for anti-invasive tumor treatment.

Recently, evidence from a number of studies has suggested that EMT occurrence is associated with specific protein molecules,[22] microenvironment[23] and microRNAs,[24] which are involved in numerous signaling pathways[25] and complex molecular mechanisms. Hypoxia is one of the most basic biological phenomenons that are tightly associated with the aggressiveness and development in a few types of tumors.[26] Hifs play primary transcription

regulators, which regulate hypoxia responsive genes and have been recognized to play critical roles in tumor invasion, metastasis, angiogenesis, and it is an important initial factor of EMT.[27] A large number of clinical evidence suggests that Hifs and their downstream targets are considered as key markers of EMT in solid tumors.[28] Twist1 has been recognized as a new tumor associated gene and plays an important role in tumor formation, invasion, metastasis, drug resistance and other processes. Described as a pro-metastatic factor, Twist1 was found to promote cell invasiveness and motility through EMT.[29] In this study, we found that the mechanism of COE affecting EMT process involved the Hif-1 α /Twist1 signaling pathway.

The Celastraceae plant *Celastrus Orbiculatus*, which is widely distributed in China, has been used as a Chinese medicine for the treatment of many diseases, including arthritis and other inflammatory diseases.[30] In our previous study, we had found that COE displays anti-cancer effects *in vitro* and *in vivo* through the inhibition of angiogenesis, proliferation, invasion and metastasis ability.[31] Furthermore, COE has been well documented on anti-EMT effects in several different human cancers.[32] In this study, the results indicated that COE might also inhibit HCC cell growth, migration and invasion under hypoxic environment induced by $CoCl_2$. Nevertheless, all of these studies were carried out just by the experiments *in vitro*, and *in vivo* studies are required for further investigation.

Taken together, our present studies provide evidence that COE inhibits invasion and metastasis in $CoCl_2$-induced HCC cell, and the mechanisms may involve the suppression of EMT by targeting Hif-1 α / Twist1 signal pathway. COE may be a new effective antitumor medicine in the future.

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

All authors edited or commented on the manuscript. All authors read and approved the final manuscript.

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