Luteolin inhibits angiogenesis of the M2-like TAMs via the downregulation of hypoxia inducible factor-1α and the STAT3 signalling pathway under hypoxia

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Abstract. The imbalance between angiogenic inducers and inhibitors appears to be a critical factor in tumour pathogenesis. Angiogenesis serves a key role in the occurrence, invasion and metastasis of tumours. Macrophages are a major cellular component of human and rodent tumours, where they are usually termed tumour-associated macrophages (TAMs). In malignant tumours, TAMs tend to resemble alternatively activated macrophages (M2-like), promote TA angiogenesis, strengthen tumour migration and invasive abilities, and simultaneously inhibit antitumor immune responses. In our previous study, luteolin, commonly found in a wide variety of plants, had a strong antitumor effect under normoxia; however, it is unknown whether luteolin serves a similar role under hypoxia. In the present study, cobalt chloride ($CoCl_2$) was used to simulate hypoxia. Hypoxia-inducible factor-1α (HIF-1α), which is difficult to detect under normoxic conditions, was significantly increased. Additionally, vascular endothelial growth factor (VEGF) was also significantly increased in response to $CoCl_2$ treatment. Subsequently, luteolin was applied with $CoCl_2$ to examine the effects of luteolin. Luteolin decreased the expression of VEGF and matrix metalloproteinase-9, which promote angiogenesis. In addition, luteolin also suppressed the activation of HIF-1 and phosphorylated-signal transducer and activator of transcription 3 (STAT3) signalling, particularly within the M2-like TAMs. The results of the present study provide novel evidence that luteolin, under hypoxic conditions, has a strong anticancer effect via the HIF-1α and STAT3 signalling pathways.

Introduction

Neighbouring normal cells are recruited by cancer cells to perform tumour processes. Thus, the study of cancer requires not only the focus on tumour cell activity, but to also evaluate the activity of the neighbouring normal cells (1). Macrophages are the most abundant non-tumour cell in cancers (2). Plasticity and flexibility are key features of macrophages because their cytokine production responds to specific environmental cues (3,4). According to their functional properties, previous studies proposed the concept of macrophage polarization (1,5). There are two phenotypes of polarized macrophages: M1 (classical) and M2 (alternative) macrophages. M1 macrophages, also termed classically-activated macrophages, which exhibit a strong proinflammatory and pathogen-killing effect in tissues. They are characterized by the production of destructive free radicals and inflammatory cytokines (6). M2 macrophages, which are also alternatively called activated macrophages, serve a role in immunoregulation, tissue remodelling and angiogenesis. It is now generally accepted that tumour-associated macrophages (TAMs) have an M2 phenotype and have the ability to promote tumour growth, migration, invasion and metastasis in various cancers (7-9). Therefore, the examination of TAMs may be a valuable addition to standard of care therapies.

Without vascular proliferation, tumour growth is restricted. Therefore, the process of angiogenesis is important for the growth and metastasis of tumors (10). Endothelial cell proliferation, migration and extracellular matrix decomposition are the three main stages of new blood vessel formation. This process is regulated by angiogenic factors, which are released from host cells and tumour cells, and is controlled by positive and negative regulatory factors. These vascular factors interact with receptors in the vicinity of blood vessels and induce endothelial cell activation, proliferation and migration towards the tumour. Inhibiting angiogenesis is an effective method for treating cancer.

The tumour microenvironment is a special environment that is formed by the interaction of tumour cells, stromal cells and extracellular matrix. During the progression of numerous malignant tumours, hypoxia is one of the most common microenvironmental conditions and it may stimulate the expression of vascular endothelial growth factor (VEGF) (11). Hypoxia-inducible factor-1α (HIF-1α) is a key protein upregulated by hypoxia. HIF-1α regulates the expression of VEGF at the gene level and influences tumour development. Signal transducer and activator of transcription 3 (STAT3) is an important component of the signal transducer and activator of transcription family of proteins. STAT3 was initially identified as a component of the interleukin (IL)-6/Janus kinase signalling pathway and was confirmed to participate in numerous physiological processes, including cell proliferation, apoptosis and differentiation (12). Its role in the development of cancer should not be overlooked.

Numerous medicinal plants or chemical substances extracted from plants are potential anti-vascular drugs. Flavonoids have been reported to affect the initiation and development of tumours. Luteolin, a common dietary flavonoid, is found in fruits, vegetables and herbs. This compound has a variety of functions that induce anti-tumour effects, including the inhibition of tumour cell proliferation and the promotion of tumour cell apoptosis (13). However, the mechanisms by which luteolin exerts its effects on angiogenesis and on M2-like TAMs, under hypoxic conditions, are unclear. In the present study, a chemical hypoxic model was established using $CoCl_2$ (14) to investigate whether hypoxia promotes the expression of HIF-1α and VEGF in M2-like TAMs. Furthermore, by culturing cells in the presence of $CoCl_2$ and luteolin, under hypoxic conditions, the present study investigated whether luteolin has an anti-cancer role via M2-like TAMs.

Materials and methods

Reagents. Luteolin (Sichuan Chengdu Mansite Biotechnology Co. Ltd., Sichuan China) was stored at -4˚C and dissolved in dimethyl sulfoxide prior to use. Cobalt chloride ($CoCl_2$) was obtained from Sigma-Aldrich (Merck-KGaA, Darmstadt, Germany) and IL-4 was from PeproTech EC Ltd (London, UK). Foetal bovine serum (FBS) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Trypsin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Anti-HIF-1α (cat. no. ab113642), anti-VEGF (cat. no. ab69479), anti-MMP9 (cat. no. ab38898) and anti-tissue inhibitor of metalloproteinase 1 (TIMP1) antibodies (cat. no. ab38978) were purchased from Abcam (Cambridge, MA, USA). The anti-β-actin antibody (cat. no. ap0731) was purchased from Bioworld Technology Inc. (St. Louis Park, MN, USA). Antibodies against STAT3 (cat. no. 12640) and phosphorylated (p)-STAT3 (cat. no. 9145) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. The mouse macrophage cell line RAW264.7 was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (KCB200603YJ, Shanghai, China). Cells were cultured in DMEM with 10% FBS,

100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37˚C. When the cells were ~80% confluent, trypsin containing 0.25% EDTA was used to detach the cells from the plates to proceed with the specific experimental treatments.

Induced polarization. To induce the polarization of macrophages into M2-like TAMs, the cells were treated with IL-4 (10 ng/ml) for 2 h at 37˚C. Macrophages and M2-like TAMs were used for the following experiments.

Cell viability assay. A Cell Counting Kit-8 (CCK8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to detect whether varying concentrations of $CoCl_2$ (25, 50, 100, 150, 200, 250, 300 or 600 μM) and luteolin (5, 10, 20, 30, 40, 60, 80 or 160 μM) affected the survival rate of mouse macrophages. Cells ($1x10^4$) were plated in 96-well plates, and at ~70% confluence, the medium was replaced with serum-free DMEM for 12 h. A total of 10 μl of CCK8 solution was added to each well following incubation with various concentrations of $CoCl_2$/luteolin for 24 h at 37˚C. After 2 h, a microplate reader was used to measure the absorbance of each well at 450 nm.

Cell invasion and migration assays. First, to polarize macrophages to M2-like TAMs, when cells were ~80-90% confluent, the cells were treated with IL-4 for 2 h at 37˚C. Then, the macrophages and TAMs were used for the following experiments. To test cell invasion and migration, Transwell filter chambers (Corning, Inc., Corning, NY, USA) were used with an 8-μm pore size, which were coated with or without Matrigel basement membrane matrix (Corning, Inc.). In the invasion assays, 100 μl serum-free DMEM with $5x10^4$ cells was added to the upper chamber, and 600 μl DMEM with 10% FBS was added to the lower Transwell chamber. Conversely, for the migration assays, 100 μl serum-free DMEM with $5x10^3$ cells was added to the upper chamber, and 600 μl DMEM with 10% FBS was placed in the lower chamber. Following the addition of $CoCl_2$ (100 μM) or luteolin (20 μM), the cells were placed in in a 5% CO_2 incubator for 24 h at 37˚C. Subsequently, the cells were washed three times with PBS, and the chambers were soaked in 4% paraformaldehyde for 30 min at room temperature. Following three washes with PBS, the cells were stained with haematoxylin for 20 min at room temperature. Finally, an inverted microscope at magnification of x200 was used to observe and count the stained cells by randomly selecting 10 fields of view.

Reverse transcription-quantitative polymerase chain reaction (qPCR). First, cells were treated with IL-4 for 2 h at 37˚C to polarize macrophages into M2-like TAMs. When cells were 80-90% confluent, the cells were then cultured with $CoCl_2$ (100 μM) for 2 h and with luteolin (20 μM) for 24 h. TRIzol reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA according to the manufacturer's protocols. The quantitative analysis of β-actin, MMP9, VEGF and TIMP1 expression was performed using a Fast Start Universal SYBR Green Master kit (ROX; Roche Diagnostics, Basel, Switzerland) on a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reactions were then subjected to the following temperature protocol: Stage 1, 95˚C for 3 min;

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Sense (5'-3')	Antisense (5'-3')
VEGF	ACTTTCTGCTCTCTTGGGT	GACTTCTGCTCTCCTTCTGT
MMP9	TACGATAAGGACGGCAAA	CAAAGATGAACGGGAACA
TIMP1	TCTGGCATCCTCTTGTTG	GGTGGTCTCGTTGATTTCT
β-actin	GGGAAATCGTGCGTGAC	AGGCTGGAAAAGAGCCT

MMP9, matrix metalloproteinase 9; TIMP1, tissue inhibitor of metalloproteinase 1; VEGF, vascular endothelial growth factor.

stage 2, 95°C for 15 sec and 60°C for 1 min for 40 cycles, and stage 3, 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. Finally, the $2^{-\Delta\Delta Cq}$ method (15) was to calculate the relative expression of the target gene for the analysis. The details: $\Delta\Delta Cq=(Cq_{experimental\ group\ target\ gene}-Cq_{experimental\ group\ \beta\text{-actin}\ gene})-(Cq_{control\ group\ target\ gene}-Cq_{control\ group\ \beta\text{-actin}\ gene})$. The qPCR primers were purchased from Synbio-Tech (Jiangsu, China), and their sequences are listed in Table I.

Western blot analysis. Following the aforementioned treatments, at 80-90% confluence, total proteins were extracted from the cells. Radioimmunoprecipitation assay lysate buffer (Beyotime Institute of Biotechnology, Shanghai, China) with 10% phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) and 1% PMSF (Beyotime Institute of Biotechnology). A Bicinchoninic Acid kit (Beyotime Institute of Biotechnology) was used to determine the protein concentration. Then, 50 μg protein was dissolved in loading buffer containing SDS and was heated for 5 min at 100°C. Following denaturation, 10% SDS-PAGE was used to isolate the proteins, which were transferred onto polyvinylidene fluoride (PVDF) membranes via wet blotting transfer. The PVDF membranes were incubated with tris-buffered saline and Tween-20 (TBST) with 5% skim milk for 1.5 h at room temperature. Various antibodies at different dilutions (HIF-1α, VEGF, MMP9, TIMP1, STAT3 and p-STAT3 all 1:1,000 and β-actin 1:10,000) were incubated with the membranes overnight at 4°C. Following three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit immunoglobulinG (H+L)-horseradish peroxidase; 1:10,000; cat. no. BS13278, Bioworld Technology, Inc.) for 1.5 h at room temperature. Enhanced chemiluminescence was used to detect and image the bands with a Bio-Rad gel imaging system (version 5.1, Bio-Rad Laboratories, Inc., Hercules, CA, USA) or with X-ray film.

Immunofluorescence staining. Following the aforementioned treatments, cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min at 4°C Then, the cells were permeabilized with 0.5% Triton X-100 for 10 min and blocked with PBS containing 10% goat serum (Beyotime Institute of Biotechnology) for 1 h at room temperature. Subsequently, the cells were incubated with anti-VEGF antibody (1:200) at 4°C overnight. Following three washes with PBS, the cells were incubated with a secondary antibody conjugated with Alexa Fluor 488 (1:200; cat. no. A-11034; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Following three washes with

PBS, DAPI was used to stain the cell nuclei for 5 min at room temperature. Finally, the glass slides were photographed using an automated upright microscope system with anti-fluorescence quenching (magnification, x400).

Statistical analysis. All analyses were performed using SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). The results are expressed as the means ± standard deviation of multiple experiments or representative images. One-way analysis of variance followed by Tukey's Multiple Comparison test) was used to assess significance when evaluating the statistical correlation of data between groups. $P<0.05$ was considered to indicate a statistically significant difference; all experiments were repeated three times.

Results

Effect of luteolin or $CoCl_2$ on cell survival rate and the impact of $CoCl_2$ on the expression of HIF-1α and VEGF. A CCK8 assay was used to investigate the effect of various concentrations of luteolin and $CoCl_2$ on cell survival rate. In the CCK8 experimental results with luteolin treatment only, the cell survival rate was not significantly reduced following treatment with 5,10 or 20 μM of luteolin (Fig. 1A). However, upon treatment with >20 μM luteolin, the cell survival rate was significantly decreased. The CCK8 experimental results with $CoCl_2$ treatment confirmed that 100 μM $CoCl_2$ did not significantly decrease the cell survival rate (Fig. 1B). However, concentrations of $CoCl_2$ ≥150 μM caused a significant decline in the cell survival rate. At these concentrations, $CoCl_2$ led to a substantial increase in macrophage death. Therefore, the expression of VEGF and HIF-1α was used to evaluate the efficacy of the hypoxic environment induced by $CoCl_2$. The western blotting results demonstrated that HIF-1α expression was low in the normal group. However, when the concentration of $CoCl_2$ was increased, the expression of HIF-1α also increased; the same result was observed for the protein and mRNA expression of VEGF (Fig. 1C and D). Treatment with 200 μM $CoCl_2$ did not markedly increase the expression of these proteins. These results indicated that $CoCl_2$ (100 μM) effectively induced a hypoxic environment and provided appropriate conditions for the following experiments. A total of 20 μM luteolin and 100 μM $CoCl_2$ were used for subsequent experiments.

Effects of luteolin on invasion and migration. Transwell invasion and migration assays were used to detect the effect of
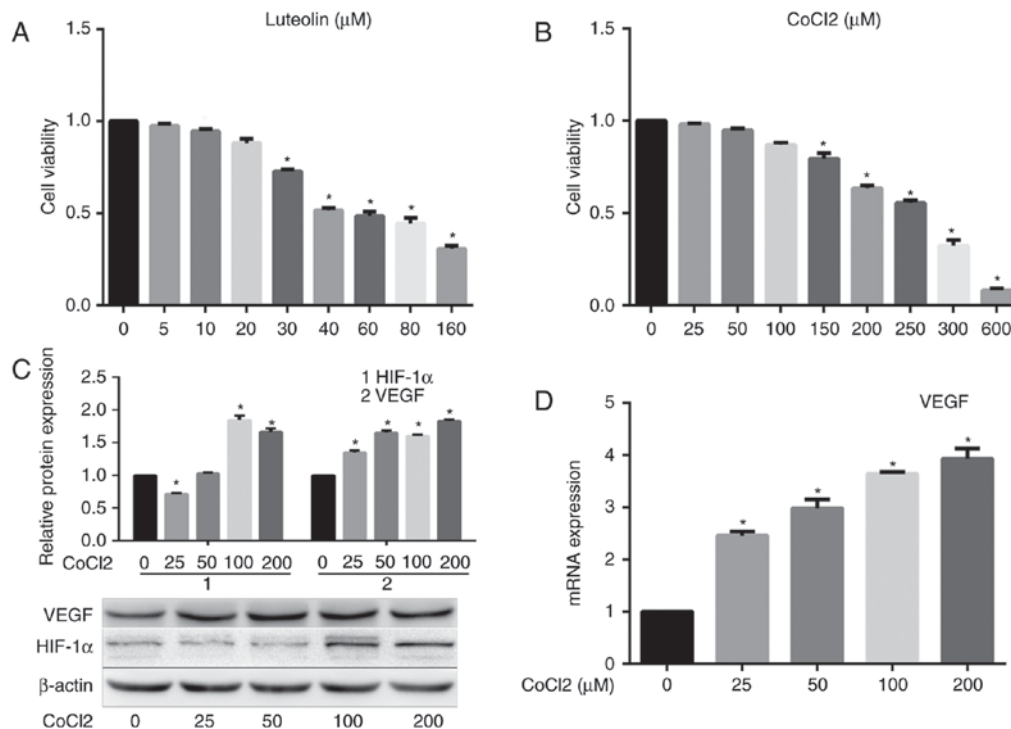
Figure 1. Cell Counting kit-8 toxicity assay and the effect of CoCl₂ on HIF-1α and VEGF expression. (A) RAW264.7 cells were treated with various concentrations of luteolin as indicated. (B) RAW264.7 cells were treated with various concentrations of CoCl₂ as indicated. (C) Expression of HIF-1α protein was analysed by western blotting. (D) VEGF mRNA expression was determined using reverse transcription-quantitative polymerase chain reaction. *P<0.05, compared with the untreated control group. HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

luteolin on cell invasion and migration. Following the addition of IL-4, the invasive ability of the cells was increased. Under the hypoxic environment, the invasion ability of M2-like TAMs was greater than that of the macrophages, which was consistent with the MMP9 expression results from western blotting described below. However, following treatment with luteolin, the invasive capability of these two cell types was significantly weakened, particularly that of the M2-like TAMs (Fig. 2A and C). In addition, in the migration experiment, the number of M2-like TAMs was significantly lower than the number of macrophages untreated with IL-4. The migration ability of the cells was also significantly inhibited following treatment with luteolin, particularly in the M2-like TAMs treated with IL-4 (Fig. 2B and D). Therefore, the results of the present study indicated that luteolin may inhibit M2-like TAM and macrophage invasion and migration, particularly in the M2-like TAMs.

Under hypoxic conditions, luteolin regulates angiogenesis. As hypoxic environments promote angiogenesis, the ability of M2-like TAMs to promote angiogenesis was increased compared with that of macrophages in a hypoxic environment. VEGF and MMP9 are the main factors that promote angiogenesis (16-18). The expression levels of VEGF and MMP9 were higher within M2-like TAMs than in macrophages, as presented by western blotting and PCR experiments (Fig. 3A-D). However, following treatment with luteolin (20 μM), the expression levels of VEGF and MMP9 were significantly decreased. In addition, the expression of TIMP1, a biological inhibitor of MMP9, was increased when cells were treated with luteolin (20 μM; Fig. 3E and F). Immunofluorescence also revealed that the expression of VEGF was lower in macrophages than in

M2-like TAMs (Fig. 4A). The expression of VEGF was inhibited following the addition of luteolin, which was consistent with the PCR and western blotting results. Therefore, luteolin may inhibit the ability of macrophages and M2-like TAMs in particular, to promote angiogenesis.

Under hypoxia, luteolin regulates the HIF-1α and STAT3 signalling pathways. There is a close association between angiogenesis and the STAT3 and HIF-1α signalling pathways: Both pathways have been previously reported to contribute to the formation of blood vessels (16,19). In the hypoxic environment of the present study, the ability of M2-like TAMs to promote angiogenesis was confirmed by the activation of the HIF-1α and p-STAT3 signalling pathways (Fig. 4B and C). The present study demonstrated via western blotting that STAT3 expression was not significantly different among the groups. This indicated that within M2-like TAMs, the STAT3 signalling pathway may have served an angiogenic role via the phosphorylation of STAT3 rather than by an increase in STAT3 expression. Following treatment with luteolin (20 μM), the expression of HIF-1α and p-STAT3 was significantly reduced, possibly inhibiting the expression of angiogenic factors, such as VEGF and MMP9. These data indicated that under hypoxic environments, luteolin may mainly have an anti-angiogenic role via the inhibition of the HIF-1α and p-STAT3 signalling pathways, particularly within the M2-like TAMs.

Discussion

Angiogenesis occurs due to the proliferation of tumour cells, which causes an increased demand for nutrients and
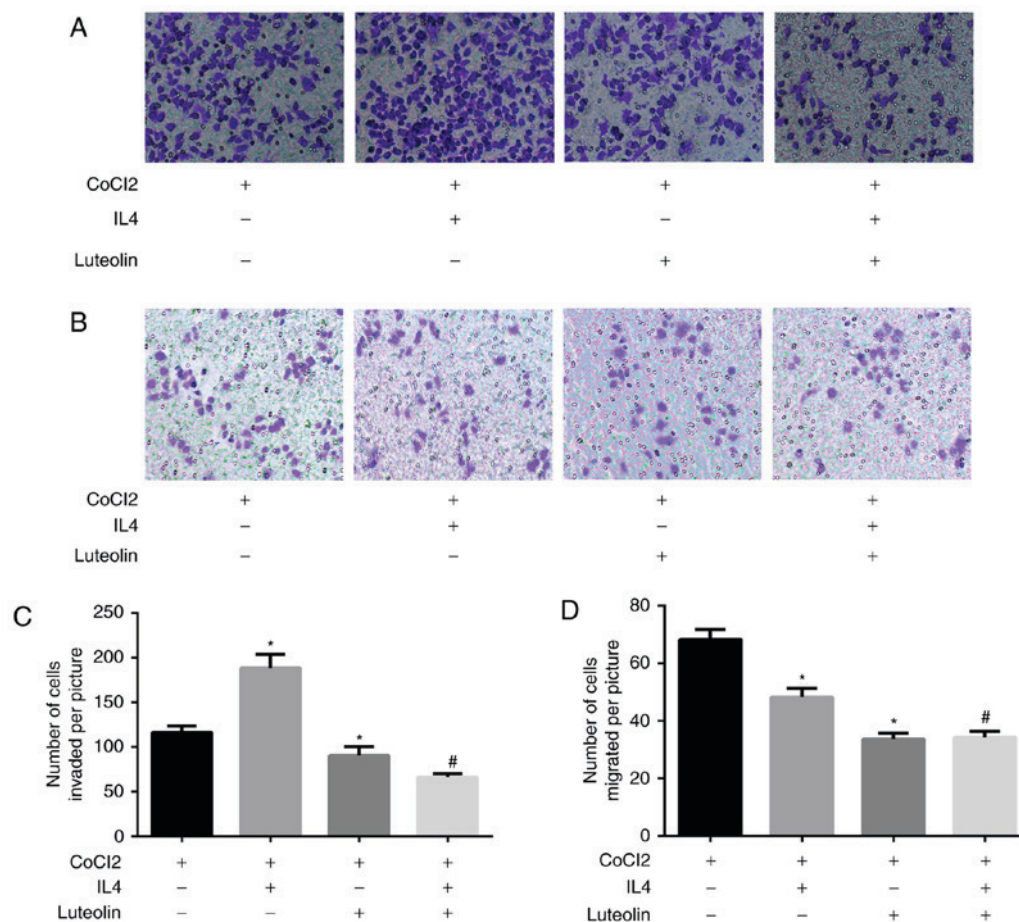
Figure 2. Effects of luteolin on invasion and migration. (A) Representative images of RAW264.7 cells in invasion experiments. Magnification, x200. (B) Representative images of RAW264.7 cells in migration experiments. Magnification, x200. (C and D) Cells counted in the Transwell image fields. *P<0.05, vs. the hypoxia group; $^#$P<0.05, compared with the group induced with IL-4 and not with luteolin. IL-4, interleukin-4.

oxygen (20). Angiogenesis has an essential role in tumour processes, including migration, metastasis and growth and is regulated by pro-angiogenic and anti-angiogenic factors (21). The process of angiogenesis includes the activation, proliferation and migration of endothelial cells. In most malignant tumours, the factors that induce angiogenesis are increased; among these, VEGF is the most important (22). VEGF is a highly specific vascular endothelial cell mitogen that has an important role in physiological and pathological processes (22). VEGF exerts its biological effects by binding to the tyrosine kinase receptors expressed on endothelial cells. The VEGF receptor belongs to the tyrosine kinase family, and based on structure and function, this receptor is divided into three types: VEGFR1 (Flt21), VEGFR2 (KDR/Flk21) and VEGFR3 (Flk24) (23). VEGFR2 and VEGFR1 are mainly expressed on vascular endothelial cells and have an important role in the formation of blood vessels (24). Conversely, VEGFR3 is mainly expressed within lymphatic endothelial cells. VEGF achieves its effect by primarily interacting with VEGFR2 (25). VEGF binding to its receptor directly promotes endothelial cell mitosis, induces plasminogen degradation and leads to increased vascular permeability, inducing the exudation of plasma proteins, and thereby promoting the proliferation and migration of tumour vascular endothelial cells (22). A previous study demonstrated that higher levels of VEGF were associated with a greater probability of micrometastasis in liver cancer (26). VEGF activation

leads to the activation of a series of signal transduction proteins, including extracellular signal-regulated kinase, protein kinase B and mechanistic target of rapamycin (mTOR) (27-29). These signal transduction proteins may promote the growth, migration, differentiation and proliferation of vascular endothelial cells. Previous studies have suggested that TAMs are involved in processes of a variety of different types of cancers (30). Thus, cancer progression may be suppressed by inhibiting M2-like TAMs (31). Previous epidemiological studies have indicated an association between increased macrophage infiltration and poor prognoses of ovarian, cervical, thyroid, lung and hepatocellular cancers (32-34). In addition, an increasing evidence suggests that M2-like TAMs have an important role in cancer progression and metastasis, establishing TAMs, M2-like TAMs in particular, as an appealing target for therapeutic interventions. Therefore, specifically affecting M2-like TAMs and reducing macrophage infiltration may effectively interfere with tumour progression. A previous study demonstrated that luteolin promotes apoptosis and inhibits cancer cell metastasis, thereby interfering with the progression of cancer (35). In the present study, mouse macrophages were used to investigate the potential anticancer effect of luteolin in hypoxic environments.

Due to the lack of hypoxia incubators, $CoCl_2$ was used to mimic hypoxic conditions in the experiment design. A low-oxygen environment was simulated with $CoCl_2$ treatment and the results revealed that in the presence of $CoCl_2$, the
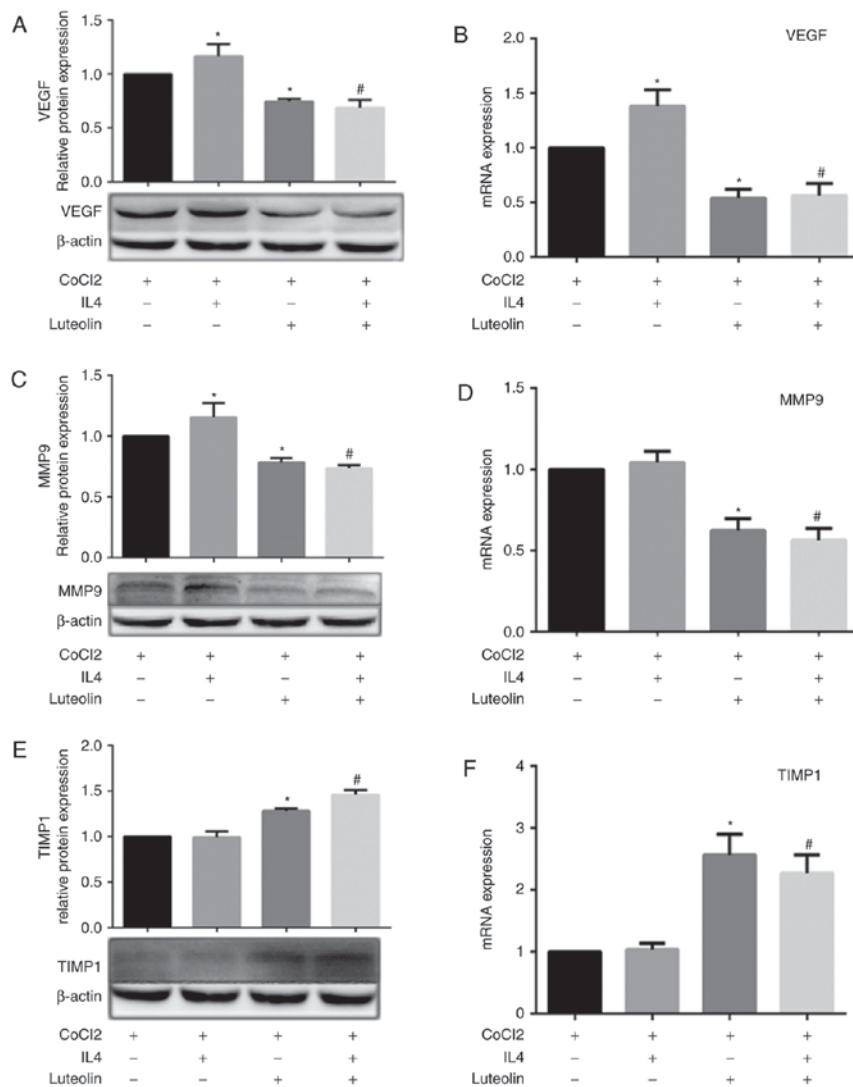
Figure 3. Effects of luteolin on angiogenesis in hypoxia. Expression levels of the VEGF protein and mRNA were analysed by (A) western blotting and (B) RT-qPCR, respectively. Expression levels of (C) MMP9 protein and (D) mRNA were analysed by western blotting and RT-qPCR, respectively. Expression of TIMP 1 (E) protein and (F) mRNA was analysed by western blotting and RT-qPCR. *P<0.05, vs. the hypoxia group induced by CoCl₂. #P<0.05 vs. the group induced with IL-4 and not with luteolin. IL-4, interleukin-4; MMP9, matrix metalloproteinase 9; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TIMP1, tissue inhibitor of metalloproteinase 1; VEGF, vascular endothelial growth factor.

expression levels of VEGF and HIF-1α were increased. The results of the present study indicated that the treatment with CoCl₂ successfully simulated low oxygen conditions. VEGF may promote the migration and invasion of tumour cells and the formation of tumour blood vessels, resulting in an increase in the degree of tumour malignancy (36). The results of western blotting, RT-qPCR and immunofluorescence experiments revealed that the expression of VEGF was increased in M2-like TAMs; however, the expression of VEGF was significantly inhibited following treatment with luteolin, particularly within the M2-like TAMs. VEGF has an important role in tumour angiogenesis, vascular permeability, tumour stem cell function and the occurrence and development of tumours (18). The finding that luteolin reduces the expression of VEGF of the present study, indicated that luteolin may have an anti-angiogenic role and inhibits tumour progression under hypoxic conditions.

In the formation of blood vessels, the first step is to degrade the extracellular matrix and basement membrane, conducted

by MMPs, which belong to a family of endopeptidases (37). Degradation of the vascular basement membrane is an indispensable step for the invasion of endothelial cells. An increase in MMPs is associated with tumour invasion, metastasis and angiogenesis (38). Among the proteolytic enzymes, MMP9 has been reported both *in vivo* and *in vitro*, to have an important role in angiogenesis. In the present study, western blotting and RT-qPCR confirmed the increased expression of MMP9 within M2-like TAMs, which is conducive to tumour angiogenesis. However, following treatment with luteolin, the expression of MMP9 was significantly decreased; the expression of its inhibitor, TIMP1, was increased. This result suggested that the anti-angiogenic effect of luteolin may be achieved via the inhibition of VEGF and MMP9 expression.

The hypoxic environment simulated by CoCl₂ significantly promoted the expression of HIF-1α, which was confirmed by western blotting. The protein expression level of HIF-1α was increased in hypoxic conditions, mimicked by CoCl₂, but no alterations in its mRNA expression level was observed (39).
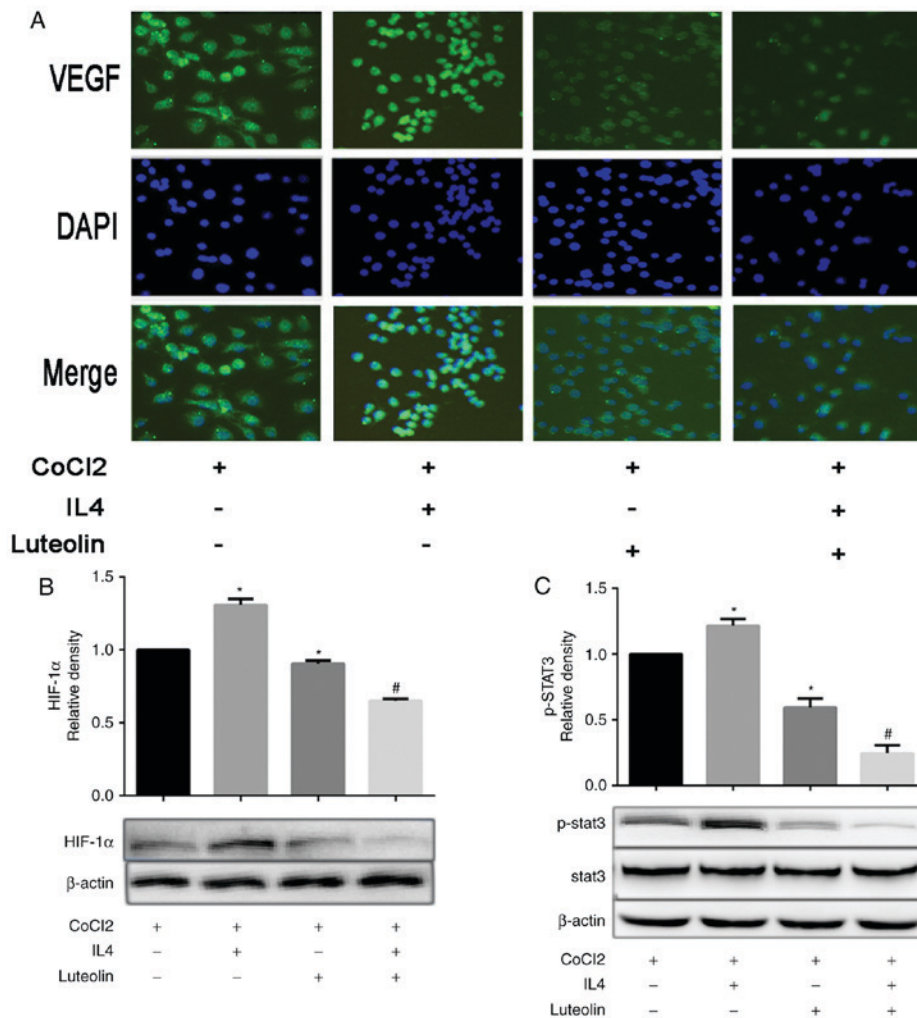
Figure 4. Effects of luteolin on VEGF expression under hypoxic conditions and the effects of luteolin on the HIF-1α and STAT3 signalling pathways. (A) Expression of VEGF protein was analysed by immunofluorescence. (B) Expression of HIF-1α, (C) p-STAT3 and STAT3 protein was analysed by western blotting. *P<0.05 vs. the hypoxia group induced by CoCl$_{2}$; $^{#}$P<0.05 vs. the group induced with IL-4 and not with luteolin. HIF-1α, hypoxia-inducible factor-1α; IL-4, interleukin-4; p-STAT3, phosphorylated signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.

A previous study reported that luteolin significantly inhibited the expression of ubiquitin E2S ligase, which regulates the expression of HIF-1α (40). Another study demonstrated that luteolin may inhibit the activation of HIF-1, which may contribute to the inhibition of the mitogen-activated protein kinase pathway (41). In addition, the other major regulator in hypoxia is mTOR, which promotes HIF-1α protein activation when hyperactivated (42,43). A previous study indicated that luteolin may inhibit the activation of Mtor (44). Therefore, the negative effect of luteolin and HIF-1α may contribute to the hyperactivation of mTOR. HIF-1α, a key regulator of hypoxia, initiates the gene expression of associated factors that contribute to angiogenesis, cell survival, invasion and migration (45). Research on effective HIF-1α inhibitors has gained attention. It has been reported that HIF-1α inhibitors may have potential novel anticancer drugs (46,47). HIF-1α may regulate the expression of VEGF and MMP9 and thereby contribute to the occurrence and development of tumours (48). However, the CoCl$_{2}$-induced increase in HIF-1α expression was inhibited by luteolin in the present study. The ability of luteolin to inhibit VEGF and MMP9 expression may be achieved via the regulation of HIF-1α. Therefore, luteolin may exert its anti-angiogenic effects via the HIF-1α-VEGF/MMP9 signalling pathway.

The STAT3 protein in the cytoplasm of normal cells has an important role in regulating cell growth and differentiation. STAT3 appears to be a nexus for numerous oncogenic signalling pathways. The STAT3 gene, located at 17q21.2, is considered a proto-oncogene, and p-STAT3 enters the nucleus and directly binds to DNA, thereby inducing downstream gene expression. Recently, many studies have shown that activated STAT3 upregulates the expression of VEGF and induces tumour angiogenesis (26,36). Furthermore, previous studies have described STAT3 as a potential modulator of HIF-1α-induced VEGF signalling in cancer cells (39,49). P-STAT3 is additional promoter of angiogenesis (50,51). The activation of p-STAT3 was significantly inhibited by luteolin in M2-like TAMs. The STAT3 signalling pathway may be another mechanism luteolin uses to contribute to angiogenesis. The expression of HIF-1α and VEGF was reduced concomitantly with the expression of p-STAT3 in the present study. This suggested that the STAT3

signalling pathway may also be involved with the expression of HIF-1α, VEGF and MMP9 under hypoxic conditions. In addition, the reduced expression of VEGF and MMP9 due to luteolin may be the result of the combined action of HIF-1α and MMP9. Under hypoxic conditions, luteolin may have an anti-angiogenic role, which may be achieved via the HIF-1α and STAT3 signalling pathways; however, the association between STAT3 and HIF-1α requires further investigation (39).

In summary, luteolin may inhibit the abilities of M2-like TAMs, which are induced by IL-4, to induce angiogenesis, thereby inhibiting tumour growth. In combination with previous research, luteolin may have a potent anticancer role, either under normoxic or hypoxic conditions. The use of luteolin may be a novel therapeutic strategy for targeting tumour invasion, migration, apoptosis and vessel generation. In conclusion, the development of novel luteolin-based drugs may be a new direction for anticancer research.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BF, XC, MW, HK, GC, ZZ and CZ performed the experiments. BF and XC wrote the manuscript. BC, BF and XC designed the experiments and analysed all data.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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