Int J Clin Exp Med 2020;13(4):2871-2878
www.ijcem.com /ISSN:1940-5901/IJCEM0087479

Original Article

Bear bile powder inhibits angiogenesis induced by hypoxia in hepatocellular carcinoma through targeting interleukin-8 expression

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Received October 28, 2018; Accepted February 12, 2019; Epub April 15, 2020; Published April 30, 2020

Abstract: Introduction: Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. Angiogenesis, under hypoxia conditions, plays an important role in patient prognosis. Bear bile powder (BBP) has been clinically used in China, traditionally. The current study aimed to evaluate whether BBP may inhibit angiogenesis induced by hypoxia in HCC. Methods: Cell proliferation was measured with MTT assay. Cobalt chloride ($CoCl_2$) was used to induce hypoxia-like conditions and RT-PCR was applied to evaluate transcriptional levels of HIF-1α, VEGF, and IL-8 in hypoxia conditions. Enzyme-linked immunosorbent assay was used to detect secreted IL-8 proteins. Anti-angiogenesis effects were assessed by capillary-like tube formation assay. *In vivo*, Matrigel plug angiogenesis assay was applied. Also, hemoglobin content, HE, and IHC staining were used to confirm the effects of BBP. Results: BBP can reduce Huh 7 cell-viability, significantly, in a dose-dependent manner. Under hypoxia conditions, transcriptional levels of HIF-1α, VEGF, and IL-8 are significantly upregulated, but reversed by BBP. ELISA confirmed the effects of BBP on IL-8, which were mostly realized as overexpression in hypoxia. IL-8 may promote capillary-like tube formation, but this is reversed by BBP. *In vivo,* Matrigel plugs of mice treated with IL-8 showed more hemoglobin content and positive expression of CD 31. VEGF and BBP may inhibit such effects. Conclusion: In conclusion, IL-8 plays an important role in angiogenesis under hypoxia conditions. Effects are reversed by BBP, in a dose-dependent manner. BBP might be a potential therapeutic candidate for HCC and IL-8 may be the potential target.

Keywords: Bear bile powder, angiogenesis, hepatocellular carcinoma, interleukin-8, hypoxia

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, ranking as the sixth most common cancer and the second leading cause of cancer-related deaths, worldwide. Reports have indicated that approximately 850,000 new cases of HCC are diagnosed each year, worldwide [1-3]. Although surgical resection and liver transplantation are available curative therapies, only 15% of patients are eligible for these therapies, since most patients are diagnosed at advance stages [4, 5]. Moreover, many patients may relapse even after receiving these therapies. To date, therapeutic approaches for advanced or relapsed HCC patients are limited, increasing the necessity for development of novel approaches.

Angiogenesis is the formation of new vessels from pre-existing vasculature. It contains a complex process involving multiple steps, such as the degradation of the basement membrane near the original vessels, as well as endothelial cell proliferation, migration, and aggregation [6, 7]. Dysregulation of angiogenesis has been associated with tumor growth, invasion, and metastasis in solid tumors, especially in HCC, considered a typical angiogenic tumor in moderately to poorly differentiated HCC [8, 9]. Transarterial chemoembolization (TACE) is effective for unresectable HCC and provides mod-

est survival benefits for patients [10]. However, it is well known that a presumed angiogenic reaction due to hypoxic conditions induced by TACE may potentially interfere with its effectiveness [11]. Although it was reported that sorafenib, a tyrosine kinase inhibitor that has anti-proliferative and antiangiogenic effects, may significantly increase HCC survival (7.9-10.7 months), whether it may inhibit angiogenesis induced by hypoxia after TACE remains unknown [12]. Therefore, development of novel drugs inhibiting angiogenesis maybe a useful strategy in treating HCC patients.

Currently, sufficient evidence has demonstrated that Traditional Chinese Medicine (TCM) and natural compounds with various types of medicinal ingredients may significantly alleviate symptoms, stabilize tumor size, obviously reduce incidence rates of adverse events, and prolong patient survival durations for unresectable HCC [13-15]. Therefore, exploring potential effective natural compounds of TCM is beneficial for HCC patients.

Bear bile powder (BBP), considered as a cold medicine according to the theory of TCM, has been considered to have properties of heat-clearing, detoxifying, and cholagogic. It has been clinically used to treat high fevers and pyocutaneous diseases, traditionally [16]. More recently, BBP has been reported to be effective as an anti-cancer agent that may inhibit the growth of HCC through mitochondrion-mediated apoptosis [16]. It also has been demonstrated that BBP could inhibit angiogenesis *in vivo* and *in vitro* [17]. One previous study found that BBP suppresses the increase of IL-8 and improves liver function post-TACE in hepatocellular carcinoma patients [18]. However, the precise mechanisms of such activities remain unclear. Therefore, the aim of the present study was to evaluate the effects of BBP on angiogenesis of HCC *in vitro* and *in vivo*, examining the underlying molecular mechanisms.

Materials and methods

Cells and reagents

Human HCC cell line Huh 7 and human immortalized endothelial cells, EA.hy 926, were obtained from Cell Bank of the Chinese Academy of Sciences Committee Type Culture Collection (Shanghai, China). They were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Thermo, Shanghai, China) with 10% fetal bovine serum (FBS, Gibco Life Technologies, Carlsbad, CA, USA). The cells were cultured in an incubator at 37°C in a humidified atmosphere of 5% CO_2. They were sub-cultured when cell density reached 80% confluence. Cells in the logarithmic growth phase were used in the experiment.

BBP was provided by Fujian Guizhentang Pharmaceutical Co., Ltd., China, with China Food and Drug Administration approval No. Z10980024. Bear bile was extracted using the free-dripping fistula technique, which could be completed in 10 seconds and made no obvious signs of pain or other uncomfortable manifestations [16]. As research progresses, synthesis of BBP, with its irreplaceable effects, may finally come true. BBP was dissolved in distilled water to prepare stock solutions of 200 mg/mL and diluted in high glucose (DMEM) to indicated concentrations before each assay. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Cobalt chloride ($CoCl_2$) were purchased from Sigma-Aldrich Company, USA.

MTT assay for cell proliferation

Cell proliferation was measured with MTT assay, according to manufacturer instructions. Briefly, Huh-7 cells were seeded onto a 96-well plate at a density of 5×10^3 cells per well. The culture medium was aspirated after overnight incubation. Different concentrations of BBP (0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mg/mL) were added and cultured for 24 hours or 48 hours. Next, 20 μL MTT solution (5 mg/mL) was added to each well and incubated for an additional 4 hours at 37°C. The medium was carefully removed and 150 μl DMSO was added. Absorbance was detected at a wavelength of 570 nm using a multi-scan spectrum microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative RT-PCR

Total RNA was isolated from huh 7 cells with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), as described previously [19], and reverse-transcribed into cDNA using the Prime Script RT-PCR kit (Takara Bio Dalian, China). A CFX96 Real-Time PCR system (Bio-Rad, CA, USA) and commercial SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan) were used to detect

expression of specific genes. Specific primers: β-actin, forward: 5'-AGCGGGAAATCGTGCGTG-3', reverse: 5'-CAGGGTACATGGTGGTGCC-3'; HIF-1α, forward: 5'-TTCCCGACTAGGCCCATTC-3', reverse: 5'-CAGGTATTCAAGGTCCCATTTCA-3'; VEGF, forward: 5'-GCCTCGGGCTTGTCACATTTT-3', reverse: 5'-CCCTGATGAGATCGAGTACATCT-3'; IL-8, forward: 5'-TCTTGGCAGCCTTCCTGATT-3', reverse: 5'-TGGTCCACTCTCAATCACTCTCAGT-3'. Cycling conditions for qRT-PCR were as follows: 95°C for 3 minutes for denaturation and subjected to 40 cycles of 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 25 seconds.

Relative expression levels of mRNA of each sample were calculated by the $2^{-\Delta\Delta Ct}$ method and β-actin was used for normalization.

Enzyme-linked immunosorbent assay

ELISA assay of cell culture supernatants of IL8 collected was used to determine protein levels. Huh 7 cells were treated for 24 hours with $CoCl_2$ and 0.2, 0.4, and 0.8 mg/mL BBP. Cell culture media was then harvested through centrifuging at 1,500 × g for 5 minutes. Supernatants were collected and IL-8 levels were measured using human IL-8 ELISA kits (R&D Systems, Minneapolis, MN, USA), according to manufacturer instructions.

Capillary-like tube formation assay

The current study performed capillary-like tube formation assays, as previously described [20]. Briefly, Matrigel (BD Biosciences, USA) was diluted with serum-free DMEM to 5 mg/mL and 50 μL gel was plated into each well of a 96-well plate, allowed to polymerize at 37°C for one hour. EA.hy 926 cells (2×10^4) suspended in 100 μL serum-free DMEM with 100 ng/mL IL-8 and BBP were plated into each well. After 12 hours of incubation, capillary-like structures were calculated through a microscope system (Leica, Germany). Number of junctions, total segments length, and mean mesh size were calculated with the open source software ImageJ (version 1.51) to quantify tube formation.

Matrigel plug angiogenesis assay in vivo

Matrigel plug angiogenesis assays were performed, as described previously [21]. Briefly, 2×10^6 EA.hy 926 cells premixed with Corning Matrigel Matrix phenol red-free (Cat. No. 356-237) and 100 ng/mL IL-8 and 0.4 mg/mL BBP were immediately injected, subcutaneously, into the right flank of nude mice (BALB/c nu/nu, 5 weeks old). Fifteen nude mice were divided into 3 groups (Control group, IL-8 Group, and IL-8+BBP Group; $n = 5$). After 10 days, the animals were killed and the Matrigel plugs were removed. The hemoglobin content of Matrigel plugs was determined using the BestBio reagent kit (Bestbio; China), according to manufacturer instructions. Plugs were also embedded in Optimum Cutting Temperature cryostat sections and stained by hematoxylin and eosin (HE). Paraffin-embedded tissue sections were used for immunohistochemistry (IHC) staining using CD31 and VEGF antibodies (Santa Cruz Biotechnology, CA). All animal experimental procedures were in accordance with Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were approved by the Committee on the Ethics of Animal Experiments of the Second Military Medical University.

Statistical analysis

Statistical analyses were conducted using SPSS 19.0 software (SPSS, Chicago, IL, USA). Data are expressed as mean ± SD. One-way analysis of variance was used for multiple comparisons of differences between groups. *P*-value < 0.05 indicates statistical significance.

Results

Effects of BBP on cell viability

In this study, MTT assay was conducted to evaluate the antiproliferative effects of BBP on Huh 7 cells, which were treated with BBP at different concentrations (0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mg/mL) for 24 and 48 hours. Results are shown in **Figure 1**. Results demonstrated that BBP can reduce Huh 7 cell-viability, significantly, at concentrations of 0.8 and 1.6 mg/mL both for 24 hours and 48 hours. However, the viability of cells changed very little when treatment concentrations were less than 0.4 mg/mL.

BBP inhibits transcriptional levels of HIF-1α, VEGF, and IL-8 in hypoxia conditions

The hypoxic environment of HCC, due to exceeding growth of functional blood vessels or side

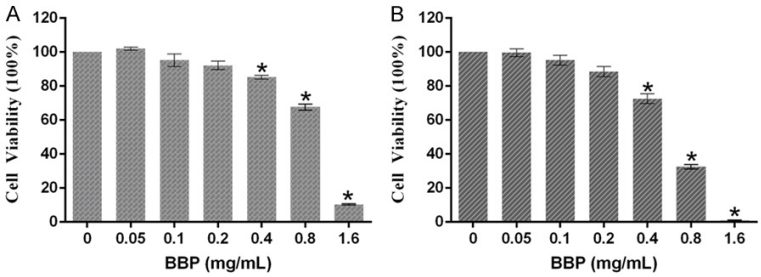

Figure 1. Effects of BBP on the cell viability of Huh 7 cells. Cell proliferation was determined by an MTT assay. Cells were treated with 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/mL BBP for 24 h (A) and 48 h (B). Data are expressed as mean ± standard deviation and all experiments were repeated three times. **P* < 0.05 vs. control group.

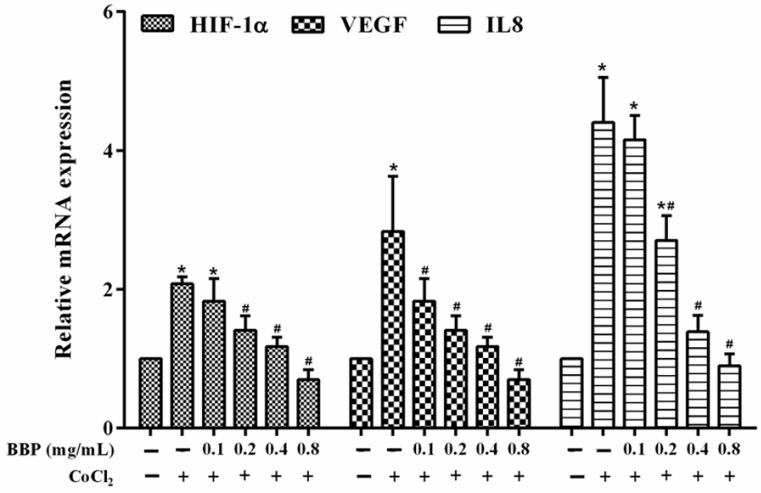

Figure 2. Effects of BBP on transcriptional levels of HIF-1α, VEGF, and IL-8 in hypoxia conditions. Quantitative RT-PCR was used to detect transcriptional levels of HIF-1α, VEGF, and IL-8. Cells were treated with 200 μM $CoCl_2$ or co-incubate with different concentrations of BBP (0.1, 0.2, 0.4, and 0.8 mg/mL) for 24 h. Data are expressed as mean ± standard deviation and all experiments were repeated three times. **P* < 0.05, vs. control group and #*P* < 0.05 vs. $CoCl_2$ group.

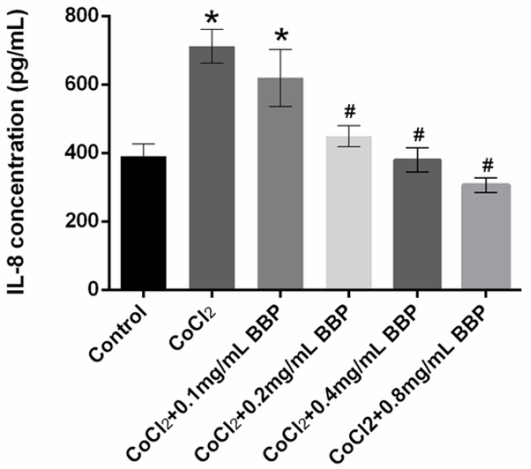

Figure 3. Effects of BBP on IL-8 protein secretion in hypoxia conditions. ELISA assays of cell culture supernatants of IL8 were used to determine protein secretion levels. Huh 7 cells were treated with $CoCl_2$ and 0.1, 0.2, 0.4, and 0.8 mg/mL BBP for 24 h. **P* < 0.05 vs. control group and #*P* < 0.05 vs. $CoCl_2$ group.

effects of treatments, such as TACE, may correlate with tumor progression. Therefore, in the present study, Cobalt chloride ($CoCl_2$) was used to induce hypoxia-like conditions [22]. Results indicated that it may significantly raise transcriptional levels of HIF-1α, VEGF, and IL-8. It also showed that IL-8 mRNA expression, following treatment with $CoCl_2$, changed the most folds when compared to controls. However, overexpression of HIF-1α, VEGF, and IL-8, stimulated by $CoCl_2$, was decreased when treated with BBP for 24 hours. Effects were significant when BBP was more than 0.2 mg/mL (**Figure 2**).

BBP inhibits secreted IL-8 proteins in hypoxia conditions

Present results indicate that IL-8 mRNA expression increases the most in hypoxia conditions. Therefore, to examine whether an increase in IL-8 transcription leads to an increase in IL-8 protein secreted from cells, ELISA assay was employed to determine protein levels of IL-8. As shown in **Figure 3**, $CoCl_2$ may significantly stimulate the secretion of IL-8, while BBP inhibits such effects. This is in accord with the above results of Quantitative RT-PCR.

BBP inhibits capillary-like tube formation induced by IL-8

Capillary-like tube formation assay was performed to investigate the effects of BBP on the angiogenic capacity of EA.hy 926 cells. Results showed that IL-8 significantly increased

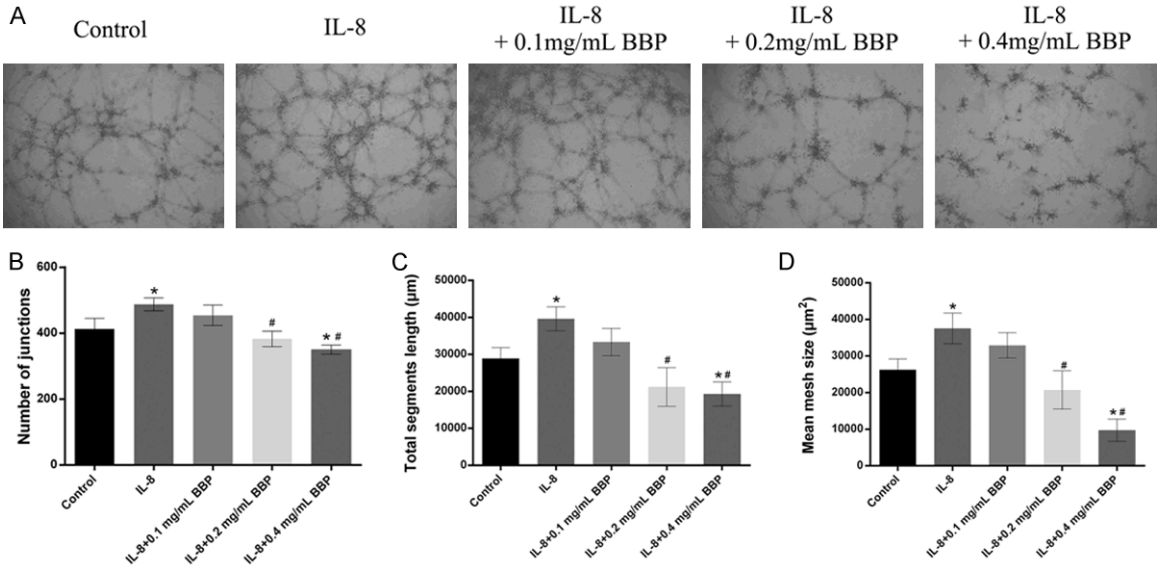

Figure 4. Effects of BBP on the capillary-like tube formation induced by IL-8. EA.hy 926 cells were treated with 100 ng/mL IL-8 and different doses of BBP (0.1, 0.2 and 0.4 mg/mL) for 12 h. A. Inverted phase contrast microscopy images of capillary-like tube formation (magnification of 100 ×). B. Number of junctions of the capillary-like tube. C. Total segments length of the capillary-like tube. D. Mean mesh size of the capillary-like tube. Data are expressed as mean ± standard deviation and all experiments were performed in triplicate. *P < 0.05 vs. control group and #P < 0.05 vs. IL-8 group.

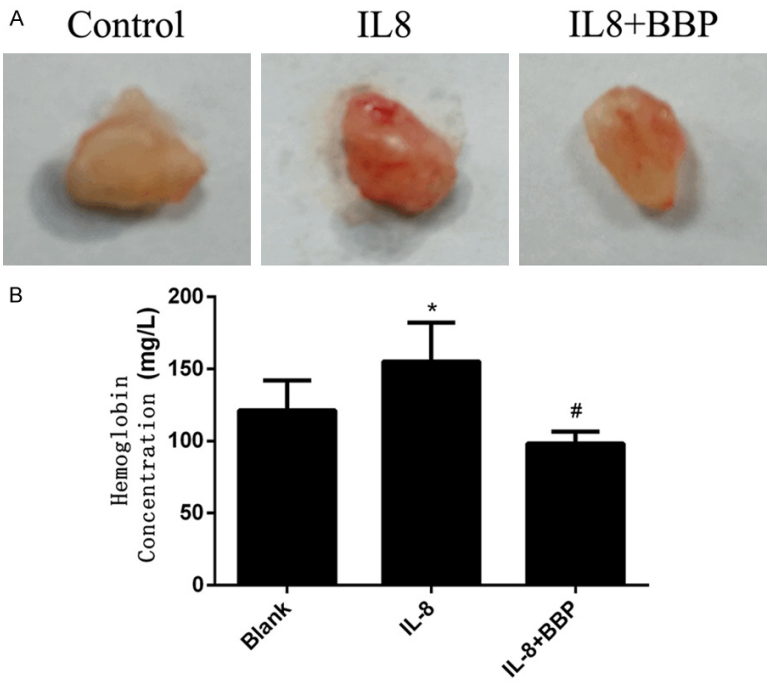

Figure 5. BBP inhibits angiogenesis using a Matrigel plug assay *in vivo*. Three groups of nude mice, including Control group, IL-8 Group, and IL-8+BBP Group, were injected subcutaneously with Corning Matrigel Matrix phenol red-free. After 10 days, the animals were killed and the Matrigel plugs were removed. A. Representative images of Matrigel plugs from each group. B. Hemoglobin content of the Matrigel plugs determined using the BestBio reagent kit. Data are expressed as mean ± standard deviation (n = 5). *P < 0.05 vs. control group and #P < 0.05 vs. IL-8 group.

the number of junctions, total segment length, and mean mesh size, indicating that it may have promoted angiogenesis significantly. Different concentrations of BBP may reverse such effects of IL-8, in a dose-dependent way. Also, 0.2 and 0.4 mg/mL BBP inhibited angiogenesis significantly (**Figure 4**).

BBP inhibits angiogenesis in vivo

In vivo, this study used a Matrigel plug assay to determine the anti-angiogenesis effects of BBP in nude mice. As shown in **Figure 5A**, angiogenesis was induced by IL-8. BBP may inhibit such effects, in accord with results of the hemoglobin content determined by the BestBio reagent kit (**Figure 5B**). HE staining results showed more newly formed blood vessels in the stained sections from IL-8 tre-

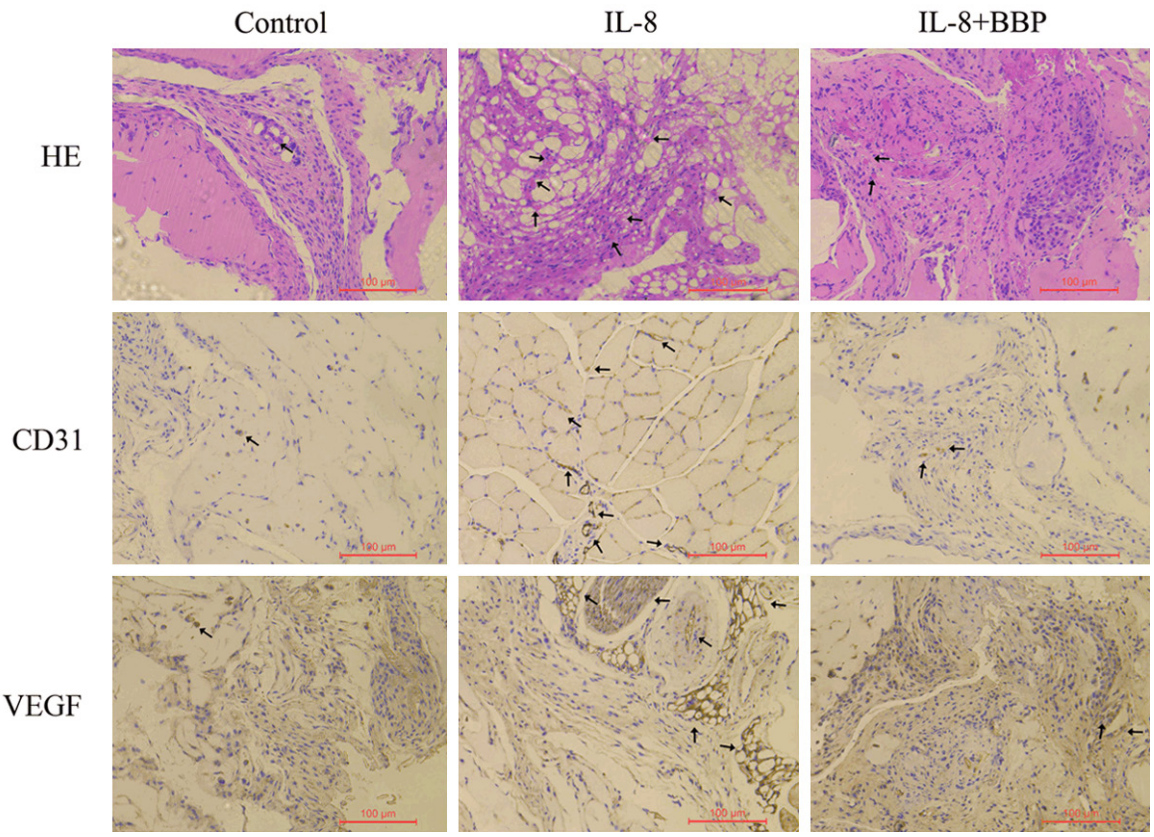

Figure 6. Hematoxylin & eosin and immunohistochemistry staining of the Matrigel plugs. Matrigel plugs were removed from the nude mice after 10 days. They were embedded in Optimum Cutting Temperature cryostat sections and stained by H&E and immunohistochemistry. The figure shows representative images of H&E staining and IHC staining using CD31 and VEGF antibody from each group.

atment plugs than in those from controls, while BBP treatment may decrease blood vessel formation (**Figure 6**). Importantly, IHC results also revealed that CD31 and VEGF expression was upregulated in the IL-8 Group but decreased when treated with BBP. This implies that angiogenesis, induced by IL-8, was obviously inhibited by BBP.

Discussion

Since the growth of HCC cells often exceeds the growth of functional blood vessels, insufficient O_2 supplement exists in the regions of HCC [23]. Therefore, the hypoxic environment is a fundamental characteristic in solid tumor microenvironments, especially in HCC [24]. A presumed angiogenic reaction due to hypoxic conditions induced by TACE may potentially interfere with its effectiveness. Therefore, understanding the underlying molecular mechanisms of angiogenesis induced by hypoxia and exploring the potential effective drugs are essential for treatment of this disease.

The present study found that BBP may inhibit the proliferation of HCC, especially in high doses (> 0.8 mg/mL), in accord with previous studies [16, 17]. This study also found that, under hypoxia conditions induced by $CoCl_2$, transcriptional levels of HIF-1α, VEGF, and IL-8 were significantly increased, while BBP may reverse such effects even in different doses (0.1, 0.2, 0.4, and 0.8 mg/mL). This did not inhibit HCC viability. Since expression of IL-8 changes the most under hypoxia conditions, this study further explored whether IL-8 may promote angiogenesis. Results indicate that IL-8 significantly induces the formation of capillary-like tubes. IL-8 is produced by macrophages, monocytes, neutrophils, endothelial cells, and fibroblasts. It is proinflammatory and increases chemotaxis, phagocytosis, promoting angiogenesis and the progression of tumors [25]. Hassanin et al. demonstrated that expression of IL8 was significantly higher in all enrolled Egyptian HCC patients, compared to healthy controls, and also higher in cirrhosis

patients with HCC than cirrhosis patients without HCC [26]. Another study found that miR-506 specifically target the 3' untranslated region (3'-UTR) of IL8 mRNA and acted as a tumor suppressor that may inhibit migration, invasiveness, and metastasis of HCC cells [27]. Chen et al. also found that IL-8 was increased by overexpressing CXCR7 and plays an important pro-angiogenic role in HCC [28]. In the present study, results indicate that BBP may inhibit angiogenesis through IL-8 expression.

Thus, the effects were also confirmed by the *in vivo* study. This study used phenol red-free Corning Matrigel Matrix for the Matrigel plug angiogenesis assay *in vivo*. Result indicate that hemoglobin content, H&E staining, and IHC staining of CD31 and VEGF all reveal that BBP may suppress angiogenesis induced by IL-8.

However, there were some limitations to the present study. Although it was found that hypoxia conditions may stimulate expression of HIF-1α, VEFG, and IL-8, the relationship among them requires further examination. Moreover, concerning IL-8, as a potential target of BBP for anti- angiogenesis, the exact molecular mechanisms or pathways should be further researched. Lastly, researchers should determine the exact and unique characteristics of BBP, discovering active ingredients, such as ursodeoxycholic acid and Tauro ursodesoxy cholic acid, necessary to research alternatives of BBP.

Conclusion

In conclusion, IL-8 plays an important role in angiogenesis under hypoxia conditions. These effects are reversed by BBP, in a dose-dependent manner. BBP might be a potential therapeutic candidate for treatment of hepatocellular carcinoma and IL-8 may be the potential target. However, the exact molecular mechanisms require further research.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 81774077, 81430101 and 81673655).

Disclosure of conflict of interest

None.

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