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Original Article

Extract of cochinchina momordica seeds regulates angiogenesis of rheumatoid arthritis through glycolysis inhibition

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Abstract: Objective: To observe the effects of extract of cochinchina momordica seeds (ECMS) on glucose metabolism, proliferation, migration, and tube formation for human umbilical vein endothelial cells (HUVEC), and to explore the effect and mechanism of ECMS on angiogenesis in rheumatoid arthritis. Methods: Alcohol extraction for ECMS by Soxhlet extractor; ECMS and 3-bromopyruvate (3-BrPa) act on cells. Cell counting kit-8 was used to detect cell proliferation, and Hoechst 33258 staining was used to detect cells apoptosis. Detection of cell hexokinase activity, glucose, lactate and ATP content was done through kits. Transwell detected cell migration, and vitro matrigel tube formation experiments were used to detect the tube number. Results: A volume of 200 μg/mL ECMS and 40 μg/mL 3-BrPa significantly inhibited the proliferation activity of HUVEC stimulated by TNF-α (10 nmol/L) (P<0.001), ECMS and 3-BrPa significantly inhibited TNF-α stimulation HUVEC hexokinase activity, reduced ATP and lactic acid content, as well as inhibited cell migration and tube formation ability. Conclusion: ECMS may regulate endothelial cell proliferation, migration and tube formation *in vitro* by inhibiting glycolysis, thereby inhibiting angiogenesis in rheumatoid arthritis.

Keywords: Extract of cochinchina momordica seeds, rheumatoid arthritis, glycolysis, angiogenesis

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by joint destruction, synovitis, pannus formation, and symmetrical joint disease. There are currently about 5 million patients wit RA in China, and the survival time of patients is generally shorter than that of normal people by 3 to 18 years, and after a 10-year span, the overall risk of mortality will increase by 1.3-2 fold [1]. Current studies have shown that the pathogenesis of RA is related to multiple mechanisms such as infection, heredity, immune mechanism disorders, and metabolic abnormalities [2-4]. However, it has not yet been definitively clarified, and there is still a lack of effective preventive and therapeutic drugs.

Cochinchina momordica seeds (CMS) are mature seeds of the Cochinchina momordica plant. Studies shown that extract of cochinchina momordica seeds (ECMS) contain saponins, cochinchin, fatty acids, volatile oils, alkaloids and other components [5, 6], which have antitumor properties, anti-inflammatory, analgesic, antioxidant, antibacterial and other pharmacological effects, which are often used to treat muscle spasms, hemorrhoids, hemangioma and rheumatic pain [7-10]. At present, ECMS has been studied in many diseases. Meng [11] reported that ECMS induced cell cycle G2/M arrest and apoptosis in MDA-MB-231 cells by decreasing PI3K/Akt pathway activation, and propose that ECMS has potential as a breast cancer chemotherapeutic agent. Shen [12] demonstrated that ECMS inhibited the proliferation of A549 cells by inducing apoptosis, at least partly through the activation of p53 and inactivation of PI-3K/Akt signaling. STAT-3 and MMP-2 pathways may be partly involved in anti-metastasis activities of ECMS. Hence, ECMS might be a promising candidate for the therapy of non-small cell lung cancer by regulat-

ing multiple molecular targets. In stomach disease, Liu demonstrated that ECMS exerted cytotoxic activities via PARP and p53 signal pathways in human gastric cancer cells [13]. Furthermore, the antiulcer effect of an extract from cochinchina momordica seeds (SK-MS10) treatment accelerates the healing of gastric ulcers via upregulation of VEGF and angiogenesis in an acetic acid rat model [14].

In addition, Pan's study shown that ECMS has inhibitory glycolysis activity and its component MBZA has been screened and isolated [15]. However, for RA, the role of ECMS is rarely reported. Based on the regulation of glycometabolism activity of ECMS, this study intend to use ECMS to treat HUVEC cells to explore its effect and the mechanism, with aim to provide a theoretical basis for in-depth understanding of the pathogenesis of RA and the discovery of new therapeutic drugs.

Materials and methods

Materials

Cochinchina momordica seeds were purchased from Feng Shengtang, Duyun City, Guizhou Province, and was authenticated by the deputy chief pharmacist; HUVEC cells were gifted by the Key Laboratory of Biomedical Engineering, Guizhou Medical University; Cell Counting Kit-8 (Beijing Soleibao), Transwell 24 Hole (Beijing Soleibao); Base Glue (Beijing Soleibao); Crystal Violet Saturated Methanol Solution (Beijing Soleibao); Hoechst 33258 (Beijing Soleibao); Human TNF-α (Peprotech); Fetal bovine serum (Sciencell); RPMI 1640 medium (Gibco); 3-bromopyruvate (3-BrPa) (Beijing Soleibao); Glucose content detection kit (Beijing Soleibao); ATP content detection kit (Beijing Soleibao) Leybold); Lactic acid content detection kit (Beijing Soleibao); Hexokinase activity detection kit (Beijing Soleibao).

Methods

Cell culture: Human umbilical vein endothelial cells (HUVEC) were cultured in RPMI 1640 medium containing 10% FBS and 1% Penicillin-streptomycin in incubator with 37°C, 5% CO_2 and saturated humidity. The medium was changed every other day, and the density reached 80-90% for generations.

Momordica chinensis extract: A weight of 150 g of the crushed mussel seeds, was added to 500 mL of 62% ethanol to cold soak for 24 h, with heat reflux for 2 h, we then collected the filtrate, added 160 mL of 62% ethanol to the residue, repeated the steps for reflux extraction, and poured the filtrate into the wet macropore in the resin column, after being adsorbed three times, the eluate was mixed and freeze-dried, the Momordica charantia extract (ECMS) was obtained finally. ECMS was dissolved in PBS, diluted RPMI 1640 medium to the required concentration, sterilized through a sterile filter, and stored at 4°C.

CCK-8 detects cell viability: The cells in the logarithmic growth phase were digested and collected by centrifugation, counted, and diluted with RPMI 1640 complete medium to 1×10^4/mL; 100 μL cells per well were added to a 96-well plate, grown overnight, and the drug concentration gradient was diluted by 1640 medium. We included TNF-α (0, 2.5, 5, 10, 20, 40, 80 nmol/L), 3-BrPa (0, 5, 10, 20, 40, 80, 160 μg/mL) and ECMS (0, 25, 50, 100, 200, 400, 600, 800 μg/mL). Cells were cultivated with the determined concentration of TNF-α with ECMS and 3-BrPa for 24 hours, added 10 μL of CCK-8, and incubated for 1-4 hours. The absorbance at 490 nm was detected by a microplate reader.

Hoechst 33258 staining solution to detect cell apoptosis: We digested and collected the cells in the logarithmic growth phase, spread them in a 24-well plate to culture overnight, added drugs for 24 hours, added Hoechst 33258 staining solution, incubated for 20-30 minutes, aspirated the staining solution, and washed 2-3 times with PBS, we observed and filmed by a fluorescence inverted microscope.

Hexokinase (HK) activity detection: We collected the cells by centrifugation. We added 1 mL of extraction solution to 1×10^7 cells, and ultrasonically disrupted the cells at 200 W for 3 S at an interval of 10 S under ice bath conditions. This was repeated 30 times. We centrifuged the mixture at 8000 g for 10 min at 4°C. The supernatant was removed. The sample was mixed well, and we recorded the initial absorbance value A1 at 340 nm wavelength when measuring the sample for 20 seconds, we quickly reacted the cuvete together with the reaction solution in a 37°C constant temperature water bath for 5 minutes, then it was quickly taken out and wiped dry, with a 340 nm

colorimetric reading, that was recorded for 5 minutes at 20 for the absorbance A2 in seconds, and we calculated ΔA = A2-A1. HK activity was calculated by the number of cells, HK (U/10^4 cells) = 2.226 × ΔA.

Determination of glucose content: We collected the cells and then added 1 mL of distilled water to 1 × 10^7 cells. Under ice-bath conditions, 200 W ultrasound was used for 3 S, at 10 S intervals, and the ultrasound was used to break apart the cells, and this was repeated 30 times. We boiled the samples in boiling water for 10 minutes, and centrifuge at 8000 g for 10 minutes at room temperature. The supernatant was removed. We added the samples and mixed well, and keep them at 37°C for 15 minutes. We read the absorbance value at 505 nm with a spectrophotometer, and then calculated by the number of cells. Glucose content (μmol/10^4 cells) = C × (A measuring tube-A blank tube)/(A standard tube-A blank tube) × V sample/(500/V sample total × V sample) = 0.002 × (A determination Tube-A blank tube)/(A standard tube-A blank tube).

ATP content detection: We collected the cells, and then added 1 mL of extraction solution to 1 × 10^7 cells, under ice bath conditions, 200 W ultrasonic for 2 S, at an interval of 1 S, to sonicate the cells for 1 min. Samples were centrifuged at 10000 g for 10 min at 4°C, the supernatant was removed to another EP tube, then we added 500 μL of chloroform, and the samples were shaken and mixed thoroughly, centrifuged at 10000 g for 3 min at 4°C, and the supernatant was removed. Next we added the sample and mixed well, recorded the initial absorbance value A1 at 340 nm wavelength when measuring the sample for 10 seconds, we quickly reacted the cuvette together with the reaction solution in a 37°C constant temperature water bath for 3 minutes, and wiped it dry, with 340 nm colorimetric reading that was recorded for 3 minutes. The second absorbance was A2. We calculated the ΔA measurement = A2 measuring tube-A1 measuring tube, and ΔA standard = A2 standard-A1 standard. ATP content calculation, ATP (umol/10^6 cell) = 0.125 × ΔA determination/ΔA standard.

Lactic acid (LA) content detection: We centrifuged and collected the cells. Next we added 1 mL of solution 1 to 1 × 10^7 cells, and ultrasonicated the cells for 3 minutes at 300 W in an ice bath with an interval of 7 seconds. The samples were centrifuged at 12000 g for 10 min at 4°C. A volume of 0.8 mL of the supernatant was removed, then we added 0.15 mL of solution 2, centrifuged it again and removed the supernatant. Next, we added the samples and mixed to dissolve the precipitate, measured the absorbance at 570 nm. We drew a standard curve. LA content is calculated based on the number of cells, LA (umol/10^6 cells) = 0.2375 × X.

Transwell detects cell migration ability: We spread the cells in a 24-well plate cultured overnight, added drugs for 24 h, digested and collected the cells. We prepared a cell suspension (1 × 10^5) with 10% FBS, added 100 μL to the upper chamber of the Transwell chamber. Then, 20% FBS 1640 medium was used to culture overnight, and a cotton swab was used to wipe away the cells that did not migrated on the inner surface, the cells that migrated in the lower chamber were stained with crystal violet made of 20% methanol at room temperature for 10 minutes, and the cells were inspected by an upright microscope imaging system. We counted the number of cells under a 4 × microscope.

Vitro gelatin test cell formation tube: After dissolving the matrigel, we took 60 μL and coated 96-well plates, equilibrated it at room temperature for 30 minutes, letting it gel at 37°C for 60 minutes, the drugs were used to treat the cells for 24 hours, then we digested and use 2% FBS 1640 medium to make a 1 × 10^5 cells suspension. We took 2 × 10^4/50 μL of cells and spread them in a 96-well plate containing base glue and cultured then for 6 hours. An inverted microscopic imaging system was used for microscopic examination and filming.

Statistical methods

Data were expressed as means ± standard errors of the means (SD). Differences were analyzed with one-way analysis of variance, followed by SPSS 12.0 and GraphPad Prism 5.0 statistical software analysis. Differences were considered significant at $P<0.05$.

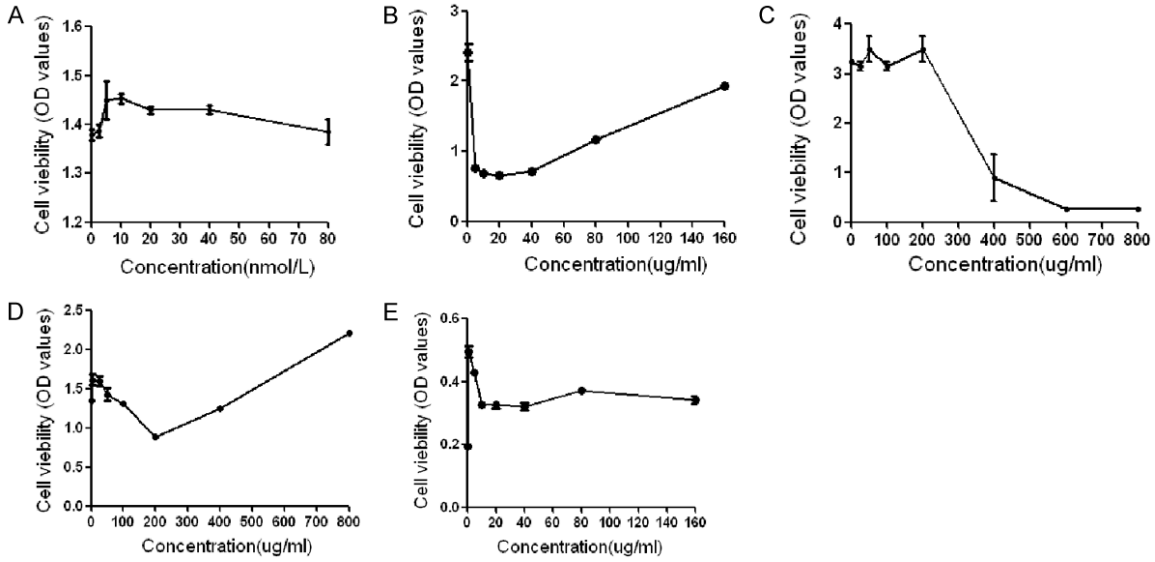

Figure 1. The effect of TNF-α, 3-BrPa and ECMS on HUVEC activity. A: The effect of TNF-α on the proliferation of HUVEC, the optimal concentration is 10 nmol/L ($P<0.001$); B: The effect of 3-BrPa on the proliferation of HUVEC, the optimal concentration is 20 μg/mL ($P<0.001$); C: The effect of ECMS on the activity of HUVEC, 400 μg/mL is the optimal concentration ($P<0.001$); D and E: TNF-α (10 nmol/L) + different concentrations of ECMS and TNF, respectively-α (10 nmol/L) + different concentrations of 3-BrPa on the activity of HUVEC 200 μg/mL, 40 μg/mL ($n = 3$, $P<0.001$).

Results

CCK-8 detects cell viability

According to literature reports [16], HUVEC is used to study the effect of drugs on angiogenesis in RA. In order to determine the optimal conditions for TNF-α to stimulate HUVEC, TNF-α (0, 2.5, 5, 10, 20, 40, 80 nmol/L) was used to induce proliferation of human HUVEC for 24 hours *in vitro*, and the cell proliferation level was detected by Cell Counting Kit-8. The results showed that TNF-α (10 nmol/L) acting on HUVEC for 24 h can be used as the optimal experimental condition for subsequent experiments (**Figure 1A**). Twenty μg/mL 3-BrPa and 400 μg/mL ECMS significantly inhibited the proliferation activity of HUVEC respectively (**Figure 1B**, **1C**), 200 μg/mL ECMS and 40 μg/mL 3-BrPa significantly inhibit the proliferation activity of HUVEC stimulated by TNF-α (10 nmol/L) (**Figure 1D**, **1E**).

Hoechst 33258 staining solution to detect cell apoptosis

The cells were treated with drugs for 24 hours, and then stained to detect cell apoptosis. The results showed that compared with the control group (**Figure 2A**), apoptosis of the TNF-α group was reduced (**Figure 2B**), while the apoptosis of the 3-BrPa and ECMS groups was significantly increased (**Figure 2C**, **2D**).

Hexokinase (HK) activity detection

To detect the effect of ECMS on HUVEC cells stimulated by TNF-α, the effect of the drug on the activity of hexokinase (HK) was first measured. The results showed that the activity of HUVEC hexokinase stimulated by TNF-α was significantly enhanced, and 3-BrPa and ECMS were significantly weakened (**Figure 3**).

Determination of glucose, lactic acid and ATP content

After the cells were treated with drugs, the glucose content of the cells stimulated by TNF-α decreased, the lactic acid content was significantly increased, and the 3-BrPa and ECMS groups were significantly decreased (**Figure 4A-C**).

Transwell test cell migration ability and in vitro base glue test cell formation

TNF-α (10 nmol/L) significantly increased the migration and tube formation ability of HUVEC,

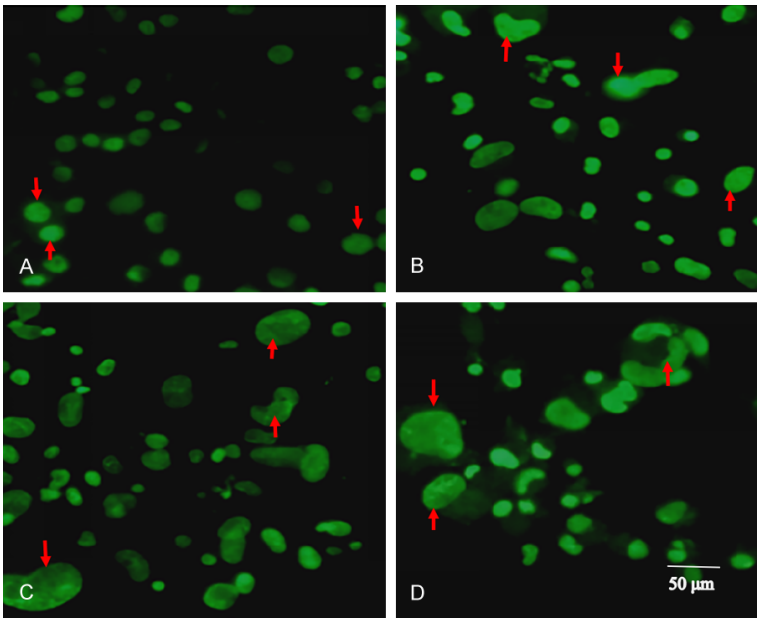

Figure 2. Hoechst 33258 staining to detect cell apoptosis after drug treatment. A: HUVEC; B: HUVEC + TNF-α (10 nmol/L); C: HUVEC + TNF-α (10 nmol/L) + 3-BrPa (40 μg/mL); D: HUVEC + TNF-α (10 nmol/L) + ECMS (200 μg/mL).

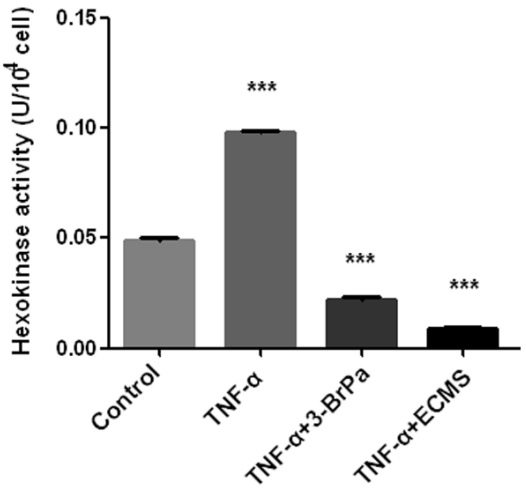

Figure 3. Hoechst 33258 staining to detect cell apoptosis after drug treatment. A: HUVEC; B: HUVEC + TNF-α (10 nmol/L); C: HUVEC + TNF-α (10 nmol/L) + 3-BrPa (40 μg/mL); D: HUVEC + TNF-α (10 nmol/L) + ECMS (200 μg/mL).

while 3-BrPa (40 μg/mL) and ECMS (200 μg/mL) significantly inhibited HUVEC's migration and management capabilities (**Figure 5**).

Discussion

RA is a complex multi-system autoimmune disease. The entire course of the disease is accompanied by continuous proliferation of synovial cells, inflammatory cell infiltration, angiogenesis and pannus formation. The pathological process of RA is closely related to angiogenesis; where blocking angiogenesis can significantly inhibit synovial inflammation and the formation of pannus. The joint cavity of patients with RA secretes many pro-angiogenesis substances, such as TNF-α, which can activate endothelial cells, promote migration, and form new microvessels [17]. Effectively controlling the activation of blood vessels will become a new target for the control of rheumatoid arthritis.

Normal joint tissue cells obtain energy and satisfy the physiological activities of growth and metabolism through aerobic oxidation of glucose, and studies have shown that RA tissue converts glucose from oxidative phosphorylation to glycolysis in an hypoxic environment to maintain its energy supply [18]. There is evidence showing that the glycolytic activity of RA synovial tissue is enhanced, leading to an acidic microenvironment that further induces the transformation of normal synovial cells [19]. The enhancement of glycolytic activity is related to RA synovial hypoxia. Glucose phosphate isomerase, enolase and aldolase, as well as key enzymes of the glycolytic pathway, promote RA autoimmunity by acting as autoantigens. Lactic acid and pyruvate are the substrates of RA synovial metabolism, which can stimulate abnormal cell proliferation, angiogenesis and the formation of pannus. Study [15] has shown that MBZA which is one componet of ECMS has inhibitory glycolysis activity, but its role in RA is still unclear. Effective inhibition of glycolysis can alleviate the proliferation of synovial cells and regulate the function of immune cells; however, it is not clear whether angiogenesis can be inhibited by regulating the activation of vascular endothelial cells. Considering that the endothelial cells in the pannus are difficult to separate, we choose HUVEC as an alternative. Since TNF-α is closely related to the inflammatory microenvironment of RA joint tissues,

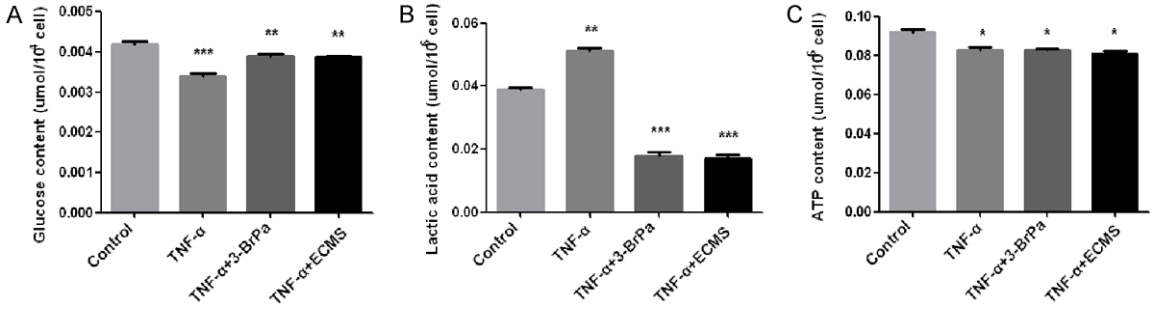

Figure 4. Glucose, lactate and ATP content of cells treated with drugs. A: Glucose content; B: Lactic acid content; C: ATP content. Control: HUVEC; TNF-α: HUVEC + TNF-α (10 nmol/L); TNF-α + 3-BrPa: HUVEC + TNF-α (10 nmol/L) + 3-BrPa (40 μg/mL); TNF-α + ECMS: HUVEC + TNF-α (10 nmol/L) + ECMS (200 μg/mL) (n = 3, **P*<0.05, ***P*<0.01, ****P*<0.001).

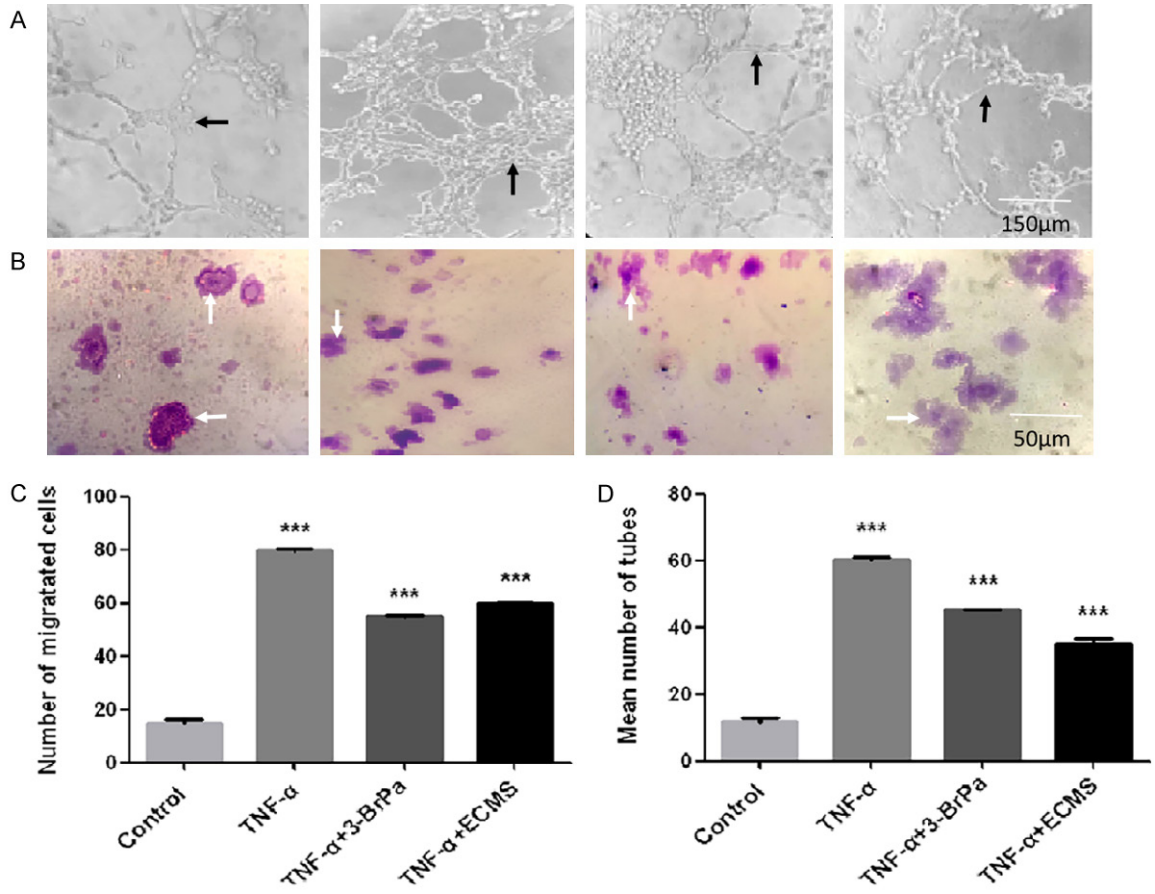

Figure 5. The effects of 3-BrPa and ECMS on cell formation and migration *in vitro*. A: Cell tube formation analysis. From the left to right are control group, TNF-α (10 nmol/L) group, TNF-α (10 nmol/L) + 3-BrPa (40 μg/mL) group, and TNF-α (10 nmol/L) + ECMS (200 μg/mL) group respectively (the same below). B: Cell migration; C: Cell migration measurement; D: Cell tube formation analysis (n = 3, ****P*<0.001).

HUVEC was stimulated *in vitro* with TNF-α to simulate the environment of the RA joint cavity. Hexokinase (HK) is the first key enzyme of glycolysis, there are four isoforms. HK1 is universally expressed. HK2 is considered to be an inducible form of HK, it is only found in a few adult tissues such as fat, bone, lung and myocardial tissues where it is highly expressed [20]. 3-BrPa is a specific inhibitor of HK2 [21]. Therefore, this study stimulated HUVEC cells

by TNF-α, and simultaneously administered glycolysis inhibitors 3-BrPa and ECMS to the cells to observe the effects of 3-BrPa and ECMS on the proliferation and migration of HUVEC and tube formation *in vitro*. The results showed that TNF-α (10 nmol/L) acts on HUVEC for 24 h which can be used as the optimal concentration and time for subsequent experiments. 3-BrPa (40 μg/mL) and ECMS (200 μg/mL) can significantly inhibit proliferation, migration and tube formation of HUVEC *in vitro* and the regulation of glucose, lactic acid and ATP content through the glycolytic pathway. This indicates that ECMS can affect the angiogenesis of RA through glycolytic pathway.

Glucose metabolism plays an important role in the pathogenesis of RA. The glycolytic activity in RA synovial tissue increases, which easily causes hypoxia in the synovium and forms an acidic microenvironment [22]. The proliferation of RA synovial cells and the infiltration of a large number of inflammatory cells leads to increased local capillary oxygen diffusion distance and increased oxygen consumption, resulting in hypoxia in the RA joint cavity [23]. Hypoxia-inducible factor 1 (HIF-1) acts as a nuclear transcription factor that regulates cell oxygen balance and hypoxia response gene expression. Its expression increases under hypoxic conditions, improves sugar transport and glycolytic enzyme gene expression, and promotes glycolysis to adapt to the lack of energy in cells under hypoxia [24]. Cell proliferation and activation in inflammatory joints converts glucose metabolism into high-energy glycolysis to maintain energy homeostasis [25, 26]. Intracellular adaptation to hypoxia is mainly the conversion from oxidative phosphorylation to glycolysis to produce ATP. When the cell is hypoxic, the expression of the protein encoded by the glycolytic enzyme gene increases, or the transcriptional expression of part of the glycolytic enzyme gene increases. In hypoxic cells, the binding site of HIF-1 is in aldolase, lactate dehydrogenase, phosphoglycerate kinase 1, pyruvate kinase, phosphofructokinase, enolase, etc. The promoters of these enzymes are transcriptionally activated by exposure, HIF-1 activates enzyme genes by activating these sites, thereby inducing the synthesis of glycolytic enzymes, promoting glycolysis, and producing ATP through glycolysis to meet the body's energy metabolism needs [27, 28]. This study further shows that glucose metabolism in HUVEC stimulated by TNF-α is abnormal, glycolytic enzyme activity and its product lactic acid increases, while glucose decreases, turning to glycolysis to support its own high proliferation, invasion and blood vessel formation. After treatment, glucose in the cells is increased, and the content of lactic acid and ATP are decreased.

Conclusion

In summary, ECMS can be used as a glycolysis inhibitor to inhibit the proliferation, migration and angiogenesis of endothelial cells. This effect may be closely related to its regulation of glucose metabolism, lactate and ATP production, but the specific mechanism needs to be further confirmed. This study provides an important basis for ECMS to participate in the pathological process of RA and new clinical treatment drugs for RA.

Acknowledgements

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Disclosure of conflict of interest

None.

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