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Anti-angiogenic activities of chitooligosaccharides

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Abstract

Chitooligosaccharides (COS) was obtained from chitosan by depolymerization with enzyme and analyzed by HPLC and TOF-MS, and the results indicated that the polymerization degree of COS was 2–18. In order to explore the anti-angiogenic activities of COS, the effect of COS on chicken chorioallantoic membrane (CAM) angiogenesis and on proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs) induced by human hepatoma carcinoma cells (HCC) culture fluid was measured. The results showed COS had no toxicity to normal HUVECs, but could inhibit the CAM angiogenesis and the proliferation, migration and tube formation of induced HUVECs. All of these results indicate that COS have potential anti-angiogenic activities and can counteract the stimulation of HCC-culture-fluid on endothelial cells at a certain level.

Keywords: Chitoologosaccharides; Endothelial cell; CAM; Angiogenesis

1. Introduction

Angiogenesis is the process of growing new blood vessels from pre-existing vessels. During adulthood, angiogenesis occurs only in such normal physiology as ovary cycling and wound healing. Angiogenesis also plays a critical role in some pathological states, for example, tumor growth, rheumatoid arthritis, diabetic retinopathy and inflammatory disorders (Carmeliet, 2003; Carmeliet & Jain, 2000; Milkiewicz & Ispanovic, 2006; Zetter, 1998). Especially in cancer, the growth and metastasis of tumor are dependent on angiogenesis (Sophie, Michel, & Andreas, 2005). Because the cancer cells are genetically unstable, heterogeneous, and have a high mutational rate, drug resistance is a major problem in cancer chemotherapy. Conversely endothelial cells are genetically stable, homogeneous, and have a low mutational rate, so in anti-angiogenic therapy, there is no or little drug resistance (Folkman, 1985). Thus interrupting the process of angiogenesis has been identified as an attractive way for the treatment of cancer. It is now widely accepted that the 'angiogenic switch' is 'on' when the balance between pro- and anti-angiogenic factors is broken (Bouck, Stellmach, & Hsu, 1996). Cancer cells can generate various pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF). These factors can promote the migration, proliferation and tube formation of endothelial cells and these activities are essential steps of angiogenesis (Ferrara, 2000).

In biomedicine field, chitooligosaccharides (COS) are more applicable than chitosan because of their water solubility and favorable properties of biodegradability, biocompatibility and other bioactivities, including the abilities of triggering cancer cells apoptosis (Prashanth & Tharanathan, 2005), inhibiting the growth of tumor (Suzuki et al., 1986), immuno-stimulation (Yu, Zhao, & Ke, 2004) and anti-oxidation (Yang, Shu, Shao, Xu, & Gu, 2006) in animals. In order to investigate the anti-angiogenic activities of COS, we used tumor cell culture fluid as an inducing factor and analyzed the effect of COS on the

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behavior of induced human umbilical vein endothelial cells (HUVECs).

2. Experiment

2.1. Materials

2.1.1. Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord by 0.25% trypsinase as described previously (Kim et al., 2002) and maintained in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in 5% CO_2 atmosphere. Bel-7402 human hepatoma carcinoma cells (HCC) were maintained in RPMI 1640 medium with 10% FCS and penicillin/streptomycin.

2.1.2. Chemicals

Chitosan (minimum 95% deacetylated, molecular weight (MW): 300–500 kDa) was purchased from Jinan Haidebei Marine Bioengineering Co., Ltd. (Shandong, China). Fertilized chicken eggs were purchased from the breeding bird farm of Dalian Hanwei Industry Association (Liaoning, China). All through the experiment 5% Bel-7402 human hepatoma carcinoma cells (HCC) culture fluid was used as an inducing factor, and equal volume of HUVECs culture fluid was used as a control. All other reagents were of analytical grade.

2.2. Preparation of chitoosaccharides (COS)

COS was prepared from enzymic hydrolysis chitosan according to our previous method (Zhang, Du, Yu, Mitsutomi, & Aiba, 1999). In brief, chitosan (0.5 g) was dissolved in 2% AcOH (10 ml), and then the pH of the solution was adjusted to 5.6. Enzyme mixture (5 mg) in 0.05 mol/l acetate buffer was added and the mixture was incubated for 40 min at 40 °C. The reaction was stopped by boiling for 10 min. The hydrolyzates were filtered on a hollow-fiber membrane. Excess crude COS was added to ethanol and the mixture was stirred forming an supersaturated solution and stored at 4 °C overnight. The insoluble precipitate was filtered under vacuum through Whatman-#1 filter paper. The obtained COS solution with a concentration of ~20% (v/v, COS/ethanol) was concentrated to dryness with a rotary evaporator under diminished pressure. An aliquot of COS was derived by 3-amido-9-ethyl-carbazoled (AEC) and analyzed with a high performance liquid chromatography (HPLC) and a matrix-assisted laser desorption–ionization time-of-flight mass spectrometer (TOF-MS). The reaction principle of COS and AEC was shown in Fig. 1.

2.3. Chicken chorioallantoic membrane (CAM) assay

The CAM assay was carried according to the method described by Zhao, et al. (Zhao, Miao, Zhao, Zhang, & Yin, 2005). Fertilized eggs were incubated at 37 °C in a humidified atmosphere for 6 days. On the seventh day, the air space end of the egg was cleaned with 75% ethanol, and a 1 cm^2 window was opened to expose a part of the CAM. Sterilized gelatin sponges saturated with different concentration of COS or PBS were placed on the CAM. The windows were covered, and the eggs were incubated for additional 48 h. The neovascular zones of the CAM under the sponges were photographed under an inverted microscope (Leica, German) and the neovascular area was analyzed by the Microsoft of Auto CAD (Autodesk, America).

2.4. Determination of the effect of COS on HUVECs and induced HUVECs viability by MTT assay

Effect of COS on the viability of HUVECs and induced HUVECs was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. HUVECs were seeded (5×10^3 per well) in 96-well culture plates. After 24 h, the medium was renewed, with different concentrations of COS, or with 5% HCC culture fluid supplement. After the cells were treated for 48 h,

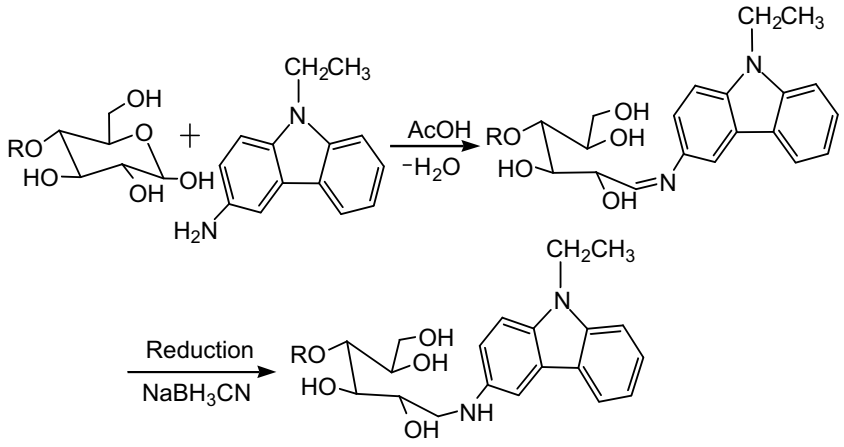

Fig. 1. Reaction principle of COS and AEC.

20 μl MTT (5 mg/ml) solution was added to each well, and plates were incubated for another 4 h. After the medium was removed, 100 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the blue crystals and the absorbance was measured by using an ELISA microplate reader (Rayto instrument) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

2.5. Determination of the effect of COS on induced HUVECs migration

To measure the migrating ability of induced HUVECs after treated with COS, a wound healing method was used (Sato & Rifkin, 1988). HUVECs were seeded (2×10^5 per well) in 6-well culture plate and incubated for 24 h. ECs monolayer was interrupted by using a 0.5 mm cell scraper, washed twice with PBS, and then further incubated for 24 h in RPMI1640 medium with or without 5% HCC culture fluid, and with different concentration of COS. The migration of the cells was photographed under an inverted microscope.

2.6. Determination of the effect of COS on induced HUVECs tube formation

Endothelial cell tube formation assay was modified from a method previously described (Jones et al., 1999). Type I collagen (5 mg/ml) was added to a 96-well culture plate

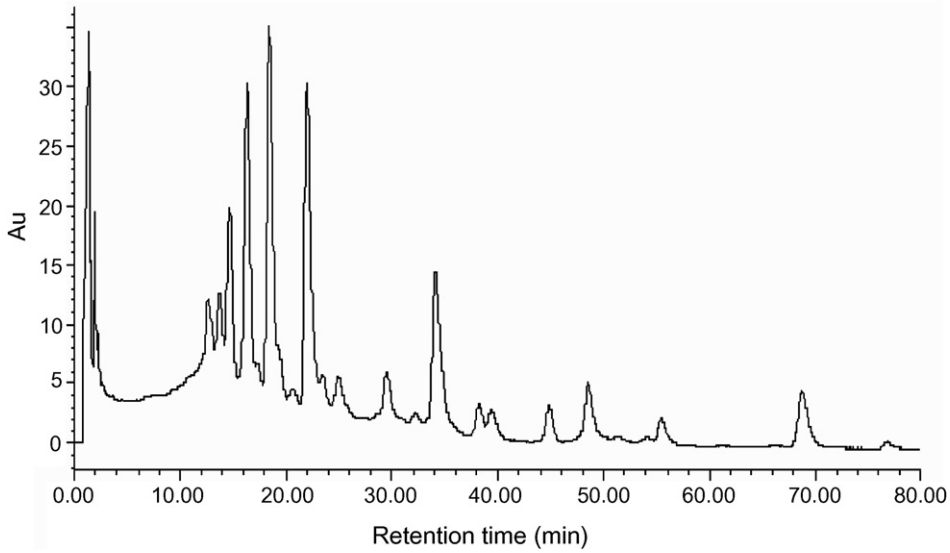

Fig. 2. HPLC of COS sample.

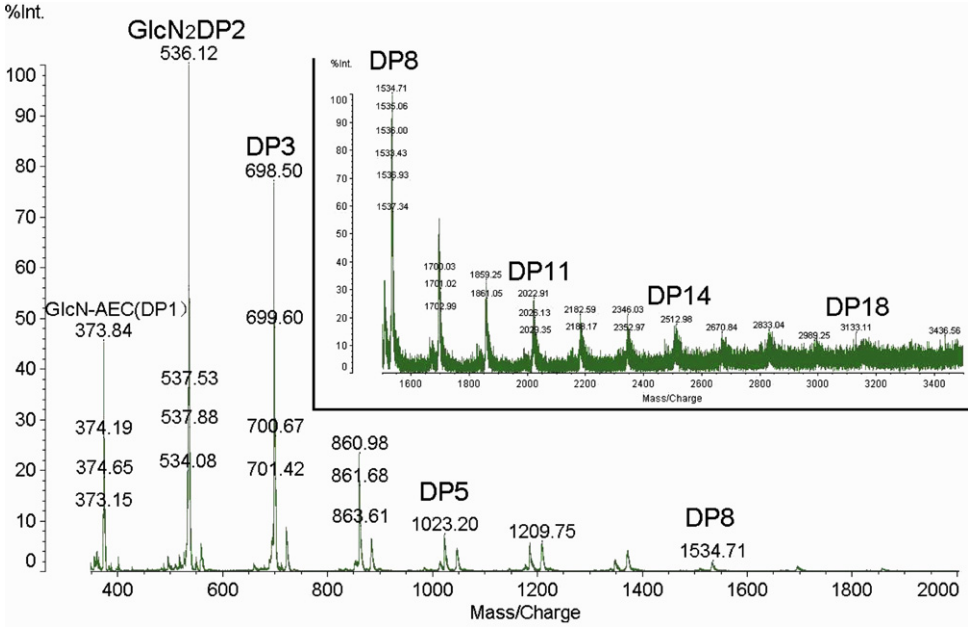

Fig. 3. TOF-MS of COS sample.

(50 μl per well) and allowed to solidify for 20 min at 37 °C. The HUVECs were seeded (2×10^4 per well) on the surface of the gel and incubated for another 24 h in RPMI1640 medium with or without 5% HCC culture fluid, and with different concentration of COS. The tube formation of the cell was photographed under an inverted microscope.

3. Results

3.1. Preparation of COS

The HPLC and TOF-MS of COS sample are shown in Figs. 2 and 3, respectively. The result indicates that the polymerization degree of COS sample is 2–18.

3.2. COS suppress CAM angiogenesis

The chicken chorioallantoic membrane (CAM) is a simple and commonly used model for the initial screening of potential inhibitors of angiogenesis. The ability of COS inhibiting the CAM angiogenesis is shown in Fig. 4. In control group, the blood vessels under the gelatin sponge grew productively, but in COS groups, the vessels under the sponge grew rarely and lacked branches and the vessel growth rate of CAM was 29.7% (20 μg/ml COS), 6.8% (50 μg/ml COS) and 7.3% (200 μg/ml COS), respectively. So this demonstrates that COS have the potential activity of anti-angiogenesis.

3.3. Influence of COS on the proliferation of HUVECs and HCC-culture-fluid-induced HUVECs

To evaluate the influence of COS on cell proliferation of both HUVECs and HCC-culture-fluid-induced HUVECs, the MTT assay was used. Cells were treated with different concentration of COS for 24 h and cellular viability was determined. As shown in Fig. 5, at first HCC-culture-fluid evidently could enhance the proliferation of HUVECs, and all concentrations of COS showed no effect on the proliferation of normal HUVECs. For HCC-culture-fluid-induced HUVECs, COS (5–50 μg/ml) could slightly inhibit the proliferation of cells in a concentration dependent manner, but the inhibitory action disappeared when the concentration was increased to 200 μg/ml. So, COS showed no toxicity towards HUVECs, but could counteract the enhancement of HCC-culture-fluid towards HUVECs proliferation at a certain level.

3.4. Suppression of COS on the migration of HCC-culture-fluid-induced HUVECs

The effect of COS on the induced cells migration is shown in Fig. 6. After being induced by HCC-culture-fluid, more HUVECs migrated to the clear area. COS suppressed the migration of induced cells in a concentration dependent manner and the best effect was observed when the COS concentration was up to 50 μg/ml and 200 μg/ml. Com-

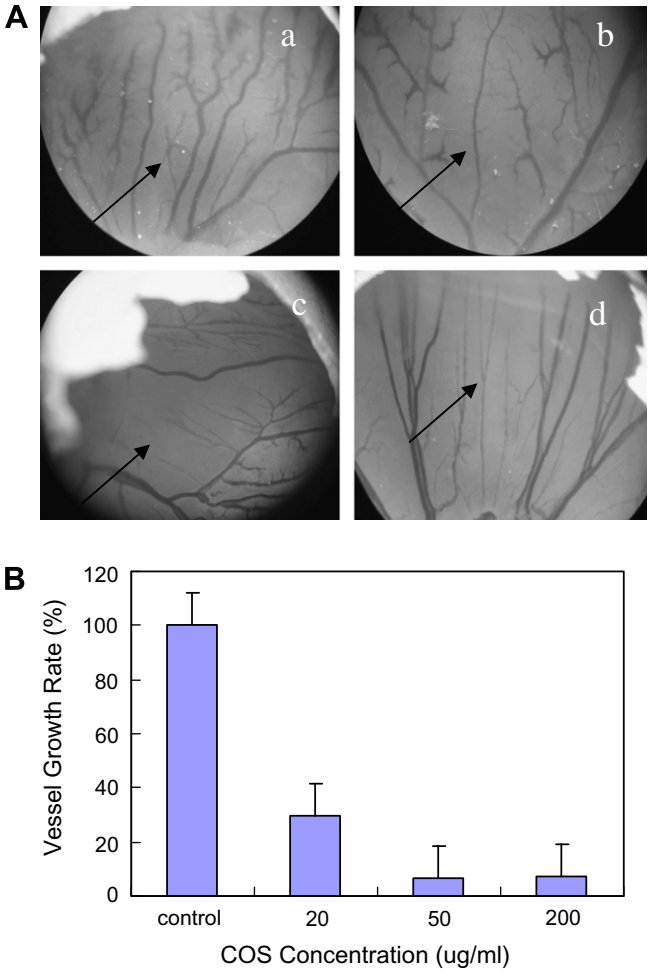

Fig. 4. Effect of COS on CAM angiogenesis. CAM were treated with different concentrations of COS for 48 h and photographed. (A) Morphological micrographs. (a) Control, CAM treated with PBS. (b) CAM treated with 20 μg/ml COS. (c) CAM treated with 50 μg/ml COS. (d) CAM treated with 200 μg/ml COS. (B) Vessel growth rate of CAM. The neovascular area of the CAM under the sponges was analyzed by the Microsoft of Auto CAD. The neovascular area of control CAM under the sponges was regarded as 100%.

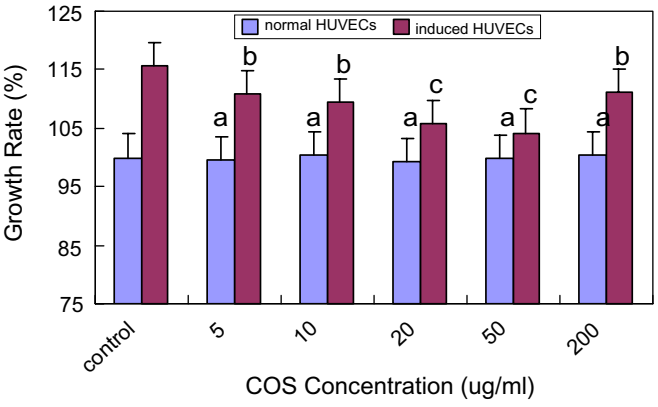

Fig. 5. Effect of COS on HUVECs and induced HUVECs viability by MTT assay (*t*-test). Different concentrations of COS were applied to normal and HCC culture fluid induced HUVECs for 24 h and cell viability was assayed by MTT assay. a, $p > 0.05$ vs. normal HUVECs control; b, $p < 0.05$ and c, $p < 0.01$ vs. induced HUVECs control.

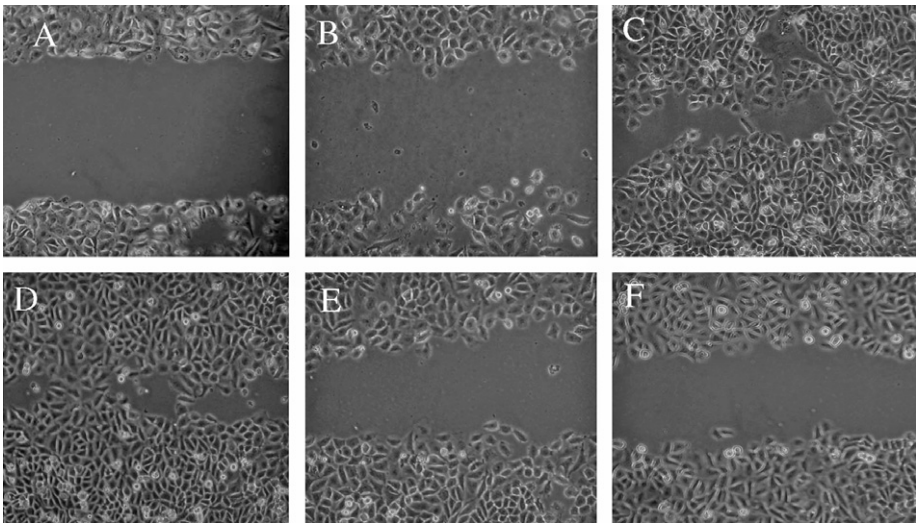

Fig. 6. Effect of COS on induced HUVECs migration. HUVECs monolayer interrupted by cell scraper were incubated with (A) medium alone for 0 h or (B) medium alone for 24 h or (C) HCC culture fluid alone for 24 h or (D) HCC culture fluid and 5 μg/ml COS for 24 h or (E) HCC culture fluid and 50 μg/ml COS for 24 h or (F) HCC culture fluid and 200 μg/ml COS for 24 h. The migration of the cells was photographed under an inverted microscope.

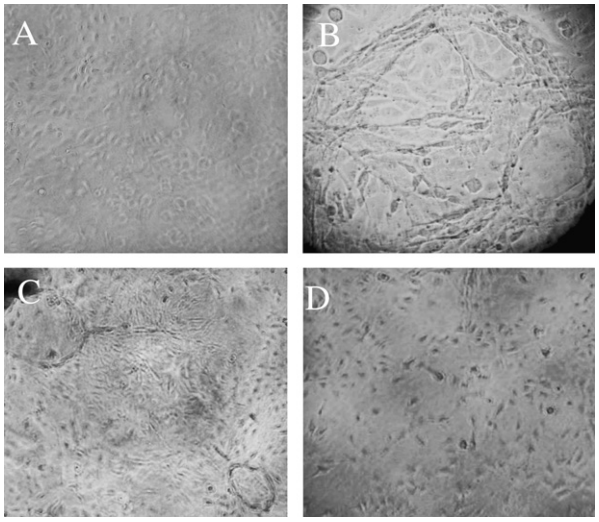

Fig. 7. Effect of COS on induced HUVECs tube formation. HUVECs seeded in collagen-coated 96-well plates were incubated for 24 h with (A) medium alone or (B) HCC culture fluid alone or (C) HCC culture fluid and 5 μg/ml COS or (D) HCC culture fluid and 50 μg/ml COS. The enclosed networks of tubes were photographed under an inverted microscope.

pared to the inhibition of cell growth, the inhibition on migration was more evident.

3.5. Suppression of COS on the tube formation of HCC-culture-fluid-induced HUVECs

The effect of COS on the tube formation of HCC-culture-fluid-induced HUVECs is shown in Fig. 7. In this experiment, normal HUVECs on the collagen gel did not form tubes. When treated with HCC-culture-fluid, the HUVECs shaped tube-like structure. COS effectively inhibited the tube formation in a concentration dependent manner, and the tube-like structure was not be observed when the COS concentration was up to 50 μg/ml.

4. Discussion

Since angiogenesis is indispensable for tumor growth and metastasis, early in 1971, Forlkman proposed that the growth and metastasis of tumor could be reduced by inhibiting angiogenesis (Folkman, 1971). Up to now, anti-angiogenesis has been a focus in cancer research, and several clinical drugs have been used with satisfactory effects (Morabito, Sarmiento, Bonginelli, & Giampietro, 2004). In recent years, knowledge of endothelial cells and the relationship between angiogenesis and endothelial cells indicates that the migration, proliferation and rearrangement of endothelial cells are essential for angiogenesis (Coultas, Chawengsaksophak, & Rossant, 2005). Thus endothelial cells provide a potential target for anti-angiogenic therapy. Anti-tumor activity of COS has been demonstrated by many researches (Prashanth & Tharanathan, 2005; Yang et al., 2006; Yu et al., 2004) and potential anti-angiogenic activities of COS had been reported by Prashanth in 2005 (Prashanth & Tharanathan, 2005).

CAM is perhaps the most widely used in vivo vessel development model. The result of CAM assay showed that COS had the potential anti-angiogenic function.

Activation of endothelial cells is initiated by the binding of pro-angiogenic factors and their receptors on the endothelial cells, and then the angiogenic signals are transmitted in the cells (Diane, Kusumanto, Meijer, Mulder, & Hospers, 2006). Cancer cells can secrete these pro-angiogenic factors. So in this study, we use Bel-7402 human hepatoma carcinoma cells (HCC) culture fluid as an inducing factor, and an equal volume of HUVECs culture fluid was used as a control. The results indicated that the HCC-culture-fluid

certainly promoted the proliferation, migration and tube formation of endothelial cells. Next, we investigated whether COS could counteract the HCC-culture-fluid stimulation of the endothelial cells.

In MTT assay, we found that COS had no influence on the proliferation of endothelial cells, or in other words COS had no toxicity on endothelial cells, but could slightly offset the promotion of HCC-culture-fluid on the proliferation of endothelial cells in a concentration dependent manner.

The exciting result was the effect on the migration and tube formation experiment, COS could significantly offset the promotion of HCC-culture-fluid on the migration and tube formation of endothelial cells in a concentration dependent manner.

In conclusion, COS have a potential function of anti-angiogenesis. The role of COS in inhibiting the migration and tube formation of endothelial cells is greater than their effect in inhibiting endothelial cells proliferation. Thus COS could counteract the stimulation of HCC-culture-fluid on endothelial cells. According to the angiogenic mechanism, COS possibly inhibit angiogenesis by interfering with the interaction of pro-angiogenic factors and their receptors, thereby blocking the signal conduction or by influencing the expression and production of anti-angiogenic factors in the endothelial cells. The research of Stamenkovic demonstrated that COS can inhibit the activation and expression of matrix metalloproteinase-2 (MMP2) in human dermal fibroblasts (Kim & Kim, 2006). In the angiogenesis process, MMPs play a primary role in the invasion and migration of endothelial cells (Matrisian, 1992; Stamenkovic, 2003). In order to reveal the mechanism of COS inhibition of angiogenesis, the receptor binding experiments and other molecular biology experiments are underway in our laboratory.

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