Carbohydrate Polymers 82 (2010) 927–932

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Chitooligosaccharides inhibit nitric oxide mediated migration of endothelial cells *in vitro* and tumor angiogenesis *in vivo*

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ARTICLE INFO

Article history:
Received 19 November 2009
Received in revised form 2 June 2010
Accepted 11 June 2010
Available online 19 June 2010

Keywords:
Chitooligosaccharides
Endothelial cell
Migration
Angiogenesis
NO

ABSTRACT

Chitooligosaccharides (COS), with a polymerization degree of 2–8, were prepared by the enzymic hydrolysis of chitosan. The anti-angiogenic activity of COS in subcutaneous xenografts in mice has been studied for the first time. As nitric oxide (NO) plays a critical role in angiogenesis, we investigated the relationship between COS and the NO-mediated migration of endothelial cells. The results demonstrated that COS could suppress tumor angiogenesis and exhibit antioxidant activity by increasing the SOD activity in Kunming mice that were implanted with human breast cancer cells, dose-dependently. COS was able to inhibit the migration of endothelial cells induced by NO. In addition, COS altered the polymerization of actin and antagonized the formation of membrane extensions that were triggered by NO in endothelial cells. Together, these results indicated that COS had anti-angiogenic activity *in vivo* and *in vitro*, and the inhibitory activity of COS on endothelial cell migration may be due to interference with the NO signal transduction pathway.

1. Introduction

Angiogenesis, the development of new blood vessels from pre-existing vessels, is a multi-step process that includes the degradation of basal membranes and the proliferation, migration and formation of tubes by endothelial cells (Folkman, 1985; Fox, Gatter, & Harris, 1996). The directional migration of endothelial cells is a crucial step in this process, and it is regulated by a variety of pro- and anti-angiogenic molecules (Coultas, Chawengsaksophak, & Rossant, 2005). Endothelial-derived NO, catalytically produced by eNOS (endothelial nitric oxide synthase), is a critical promoter of the migration of endothelial cells and angiogenesis. In the VEGF signal transduction pathways, PI3 kinase (PI3K), Akt/PKB and eNOS play important roles in the NO-induced migration of endothelial cells (Hood, Meininger, Ziche, & Granger, 1998; Van der Zee et al., 1997). Similarly, pro-angiogenic molecules, including TGFβ and bFGF, stimulate endothelial cells to produce NO also (Inoue et al., 1995; Tiefenbacher & Chilian, 1997; Wu, Yuan, McCarthy, & Granger, 1996). On the other hand, the NOS antagonist L-NAME can abolish the migration and tube formation of endothelial cells that is induced by VEGF or bFGF (Babaei et al., 1998; Papapetropoulos, Garcia-Cardena, Madri, & Sessa, 1997). Therefore, the NO signal transduction pathway may be a useful target in the inhibition of tumor angiogenesis.

Hypoxia is one of the most important causes of tumor angiogenesis, and leads to the production of ROS (reactive oxygen species). These ROS, acting as signal molecules, can induce angiogenesis *in vivo* and promote the proliferation, migration and tube formation of endothelial cells *in vitro* (Maulik, 2002). The anti-angiogenic activity of many antioxidants has been tested, including catechin (EGCG) from green tea (Cao & Cao, 1999) and the antioxidant EGB-761 from ginkgo leaf (Monte, Davel, & De Lustig, 1994).

Chitosan, derived from chitin, is composed of β-(1-4)-linked-2-amino-2-deoxy-D-glucopyranose (GlcN, D-unit) and 2-acetamido-2-deoxy-D-glucopyranose (GlcAc, A-unit). Chitooligosaccharides (COS), the degradation products of chitosan, are more soluble in water and are absorbed more easily than chitosan. Therefore, COS is applied more widely than chitosan in health-care, food, medicine, pesticides and feedstuffs. The anti-tumor activity of COS has been known since 1986 (Suzuki et al., 1986), and several mechanisms have been proposed. These include the regulation of immunity (Yu, Zhao, & Ke, 2004), the direct killing of tumor cells, or causing tumor cell apoptosis and inhibiting tumor angiogenesis (Prashanth & Tharanathan, 2005). We have reported the anti-angiogenic activity of COS previously (Wu, Yao, Bai, Du, & Lin, 2008), but the mechanism of its anti-angiogenic activity was not fully understood. As NO and ROS play critical roles in angiogenesis, we investigated the relationship between COS and the NO-mediated migration of endothelial cells. We also studied the anti-angiogenic and antiox-

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doi:10.1016/j.carbpol.2010.06.015

idant activities of COS *in vivo* using the subcutaneous xenograft tumor model in mice.

2. Materials and methods

2.1. Cells and animals

Human umbilical vein endothelial cells (HUVECs) were purchased from the China Center for Type Culture Collection, and Human breast cancer cells (MCF-7) were a generous gift from Professor Changqian Zeng (Dalian University, Dalian, China). Both of these were maintained in RPMI 1640 medium, which was supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C under a 5% CO_2 atmosphere.

We purchased 5-week-old female mice from the Experimental Animal Center of Dalian Medical University and maintained them in a standard animal room for one week before the experiment began.

2.2. Chemicals

Chitosan (minimum 95% deacetylated, molecular weight (MW): 300–500 kDa) was purchased from the Jinan Haidebei Marine Bioengineering Co., Ltd. (Shandong, China). Cell culture reagents were obtained from Invitrogen. The kits for determining SOD (superoxide dismutase), GSH-Px (glutathione peroxidase) and MDA (malondialdehyde) were obtained from the Jiancheng Bioengineering Research Institute (Nanjing, China). All other reagents were of analytical grade.

2.3. Preparation of the chitooligosaccharides (COS)

COS was prepared by the enzymic hydrolysis of chitosan and analyzed with TOF-MS (Time of Flight Mass Spectrometry) method, according to the method we reported previously (Wu et al., 2008; Zhang, Du, Yu, Mitsutomi, & Aiba, 1999).

2.4. Cell scrape migration assay

To measure the ability of HUVECs to migrate after treatment with COS, we used a wound healing method (Sato & Rifkin, 1988). HUVECs were seeded (2×10^5 per well) in 6-well culture plates and incubated for 24 h, to allow the formation of a complete monolayer. The HUVECs monolayer was interrupted using a 0.5 mm cell scraper and washed twice with PBS. Then, it was incubated for 12 h in RPMI1640 medium, either with or without 20 μg/ml sodium nitroprusside (SNP) (Sigma), a NO donor, and with different concentrations of COS. The migration of the cells was photographed under an inverted microscope (Leica, German).

2.5. Phalloidin staining

The HUVECs were cultured on cover slips in 6-well culture plates, and the experiment was begun when the cells reach 40% confluence. The HUVECs were incubated with 20 μg/mL SNP for 15 min, and then 50 μg/mL COS was added to the cells, which were then incubated for a further 15 min at 37 °C. The cover slips were washed three times with PBS, then the cells were fixed in 2% paraformaldehyde for 7 min, permeabilized with 0.1% Triton X-100 for 2 min, and finally incubated with 0.5 μM (final concentration) phalloidin-FITC (Sigma) for 1 h. The fluorescence of phalloidin bound to F-actin was viewed using laser scanning confocal microscopy (Leica, TCS SP2) at 560 nm (emission wavelength).

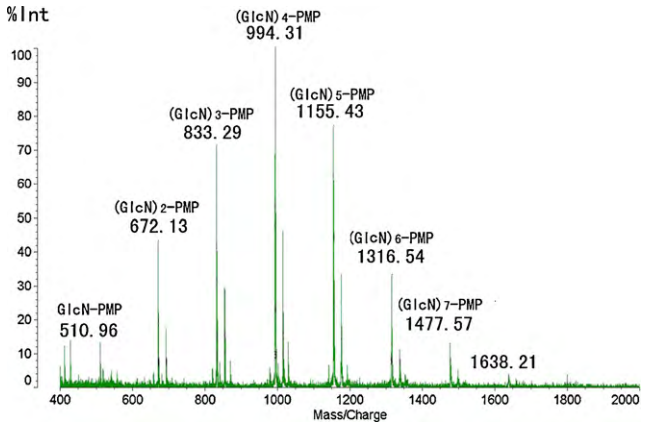

Fig. 1. TOF-MS of COS sample (Daltons).

2.6. NO estimation

HUVECs were cultured in 24-well culture plates for 6 h, to allow the cell adhere, and then different concentrations of COS were added, either with or without 20 μg/mL SNP. The NO content was measured using the Griess assay protocol, as described elsewhere (Nims et al., 1996).

2.7. The subcutaneous xenograft model in mice

The experimental protocol was approved by the China Institutional Ethics Review Committee for Animal Experimentation. The MCF-7 human breast cancer cells were harvested sterilely (the concentration was adjusted to 1×10^7 ml^{-1}) and implanted subcutaneously in the front flank region of female 6-week-old Kunming mice, using a dose of 200 μl/mouse, as described by Lu et al. (2003). The mice were divided randomly into four groups (15 mice per group), five days after the tumor transplantation. They were then treated with PBS or with different concentrations of COS by intragastric administration for 15 days. The mice that were treated with PBS were used as the negative control group, and the mice that were treated with 50, 200 and 500 mg/kg body weight/day of COS were used as the treated groups. Then the mice were killed, and the tumor masses were fixed with formalin and embedded in paraffin. Sections of the local primary tumors were stained using H&E.

2.8. The antioxidant properties of COS in xenograft tumor mice

Before killing, the eyeballs of the mice were removed, and blood was collected in anticoagulation tubes. The activities of SOD and GSH-Px, and the MDA content in the blood, were determined in the xenograft tumor mice according manufacturer's instructions (Jiancheng, Nanjing, China).

2.9. Statistical analysis

The experiments were performed in triplicate ($n = 3$) unless otherwise specified. The data were analyzed using paired and unpaired SPSS as appropriate. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Preparation of COS

The TOF-MS spectrum of the COS sample is shown in Fig. 1. The result indicates that the polymerization degree of the COS sample was 2–8.

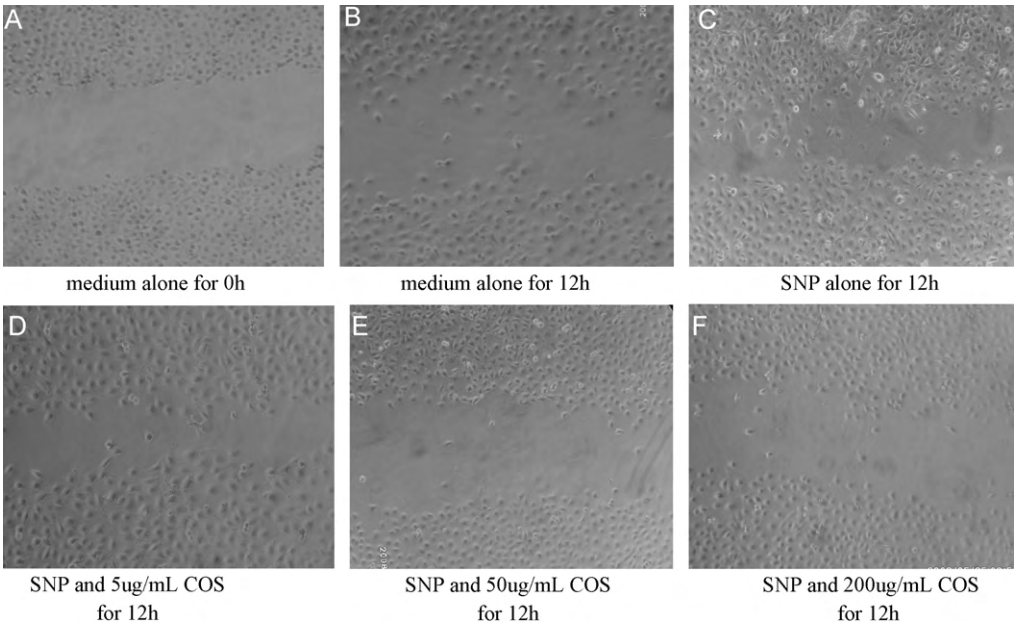

Fig. 2. Effect of COS on NO-induced HUVECs migration. HUVECs monolayer interrupted by cell scraper were incubated with (A) medium alone for 0 h or (B) medium alone for 12 h or (C) SNP alone for 12 h or (D) SNP and 5 μg/ml COS for 12 h or (E) SNP and 50 μg/ml COS for 12 h or (F) SNP and 200 μg/ml COS for 12 h. The migration of the cells was photographed under an inverted microscope.

3.2. COS inhibits the NO-induced migration of HUVECs

The wound healing method was used to measure the NO-induced migration of HUVECs, and the result is shown in Fig. 2. SNP, as a NO donor, promoted the migration of HUVECs significantly, and a large number of HUVECs migrated to the cleared area. COS suppressed the migration of the NO-induced cells in a concentration-dependent manner, and the greatest effect was

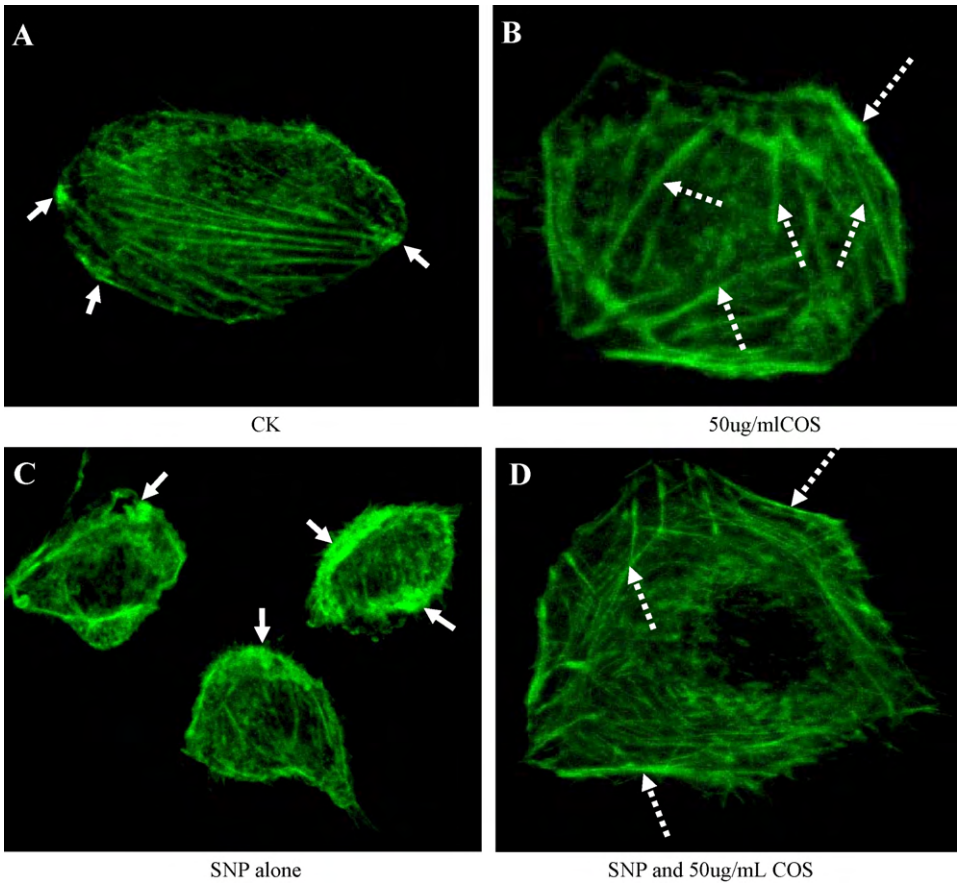

Fig. 3. Effect of COS on the cytoskeleton rearrangement of HUVECs induced with or without SNP. HUVECs were incubated with 20 μg/mL SNP (C and D) or not (A and B) for 15 min, then 50 μg/mL COS was added to the cells (B and D) and further incubated for 15 min at 37 °C, finally incubated with 0.5 μM final concentration phalloidin-FITC for 1 h. The fluorescence of phalloidin bound to F-actin was viewed under laser scanning confocal microscopy at 560 nm emission.

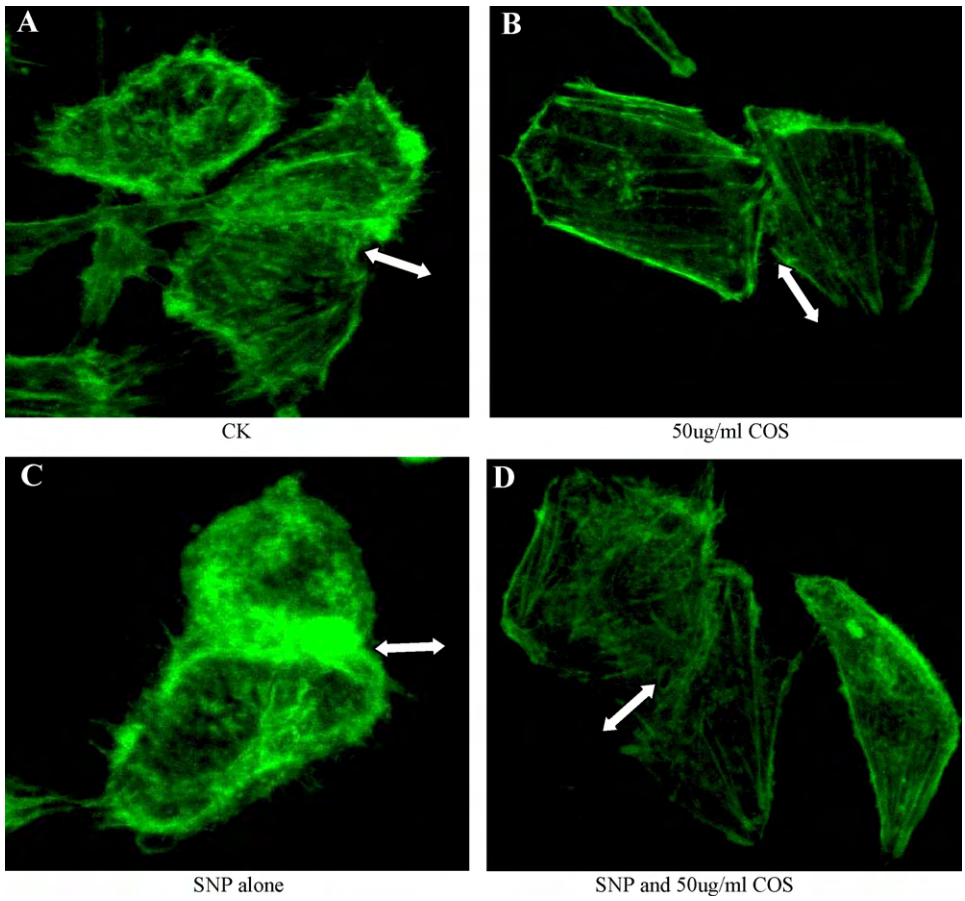

Fig. 4. COS changed the actin polymerization pattern at the cell-cell interface in HUVECs with or without SNP. HUVECs were incubated with 20 μg/mL SNP (C and D) or not (A and B) for 15 min, then 50 μg/mL COS was added to the cells (B and D) and further incubated for 15 min at 37 °C, finally incubated with 0.5 μM final concentration phalloidin-FITC for 1 h. The fluorescence of phalloidin bound to F-actin was viewed under laser scanning confocal microscopy at 560 nm emission.

observed when the COS concentration was more than 50 μg/ml. As COS had no effect on the proliferation of HUVECS (Wu et al., 2008), we propose that COS could inhibit the NO-induced migration of HUVECs.

3.3. Immunofluorescence

Phalloidin, as the specific antibody of F-actin, selectively binds to F-actin at nanomolar concentrations (Tamilarasan et al., 2006). As shown in Fig. 3, COS induces the formation of central microfilaments (dashed arrows) and reduces the number of lamellipodia (plain arrows), relative to the control group. Treatment with SNP (as the NO donor) increased the number of filopodia and lamellipodia, whereas COS inhibited the formation of filopodia and lamellipodia significantly.

The connection between two cells is essential, not only for tube formation, but also for communication between cells, and cell–cell communication is required for the proliferation, migration and tube formation of endothelial cells. To investigate the effect of COS on the polymerization of actin at the cell–cell interface, we treated the cell doublet (where two HUVECs are attached at the cell–cell interface) with COS, SNP, and SNP + COS respectively. The result is shown in Fig. 4. The degree of actin polymerization at the cell–cell interface increased visibly in the SNP group (double arrow), and COS lowered the degree of actin polymerization in both normal and SNP-induced HUVECs (double arrow).

3.4. Estimation of NO

The NO produced by HUVECs was determined using the Griess assay. The result is shown in Fig. 5, COS could inhibit the production of NO in HUVECs, whether treated with SNP or not. At 50 μg/ml, the inhibitory effect of COS was greatest.

3.5. Microvessel density in xenograft tumors

The microvessel density of xenograft tumors was examined using histological methods and a typical result is shown in Fig. 6. In the control group, there were much more microvessels (white arrows) around every glandular tube (black arrows) than in the COS-treated groups. COS decreased the microvessel density in a dose-dependent manner, and when the dose of COS was increased to 200 mg/kg body weight/day, almost no microvessels were observed around the glandular tube (black arrows). Moreover, the density and size of the glandular tubes were decreased greatly, relative to the control group.

3.6. Antioxidant properties of COS in xenograft tumor mice

The antioxidant properties of COS in mice that had been transplanted with human breast cancer cells were evaluated by determining the activities of SOD and GSH-Px, and by the contents of peroxide production MDA in the blood. The results are shown in Fig. 7. COS clearly increased the activity of SOD and reduced

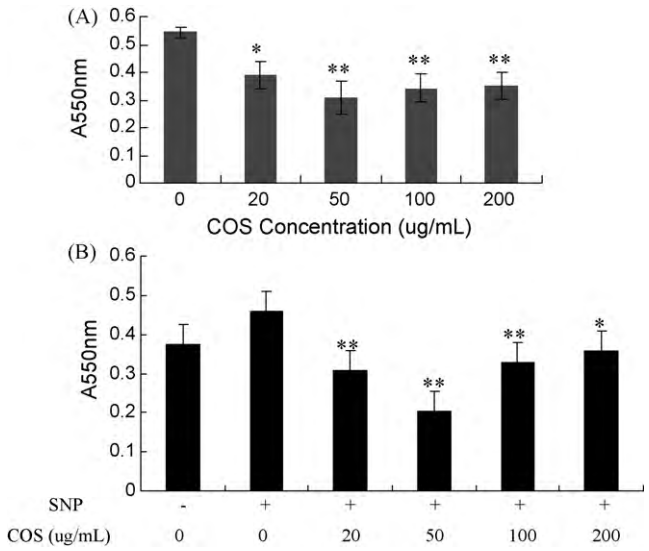

Fig. 5. Effect of COS on NO generation of HUVECs induced with (B) or without (A) SNP. Different concentrations of COS were applied to normal and SNP induced HUVECs for 24 h and NO concentration was assayed by Griess assay. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control respectively.

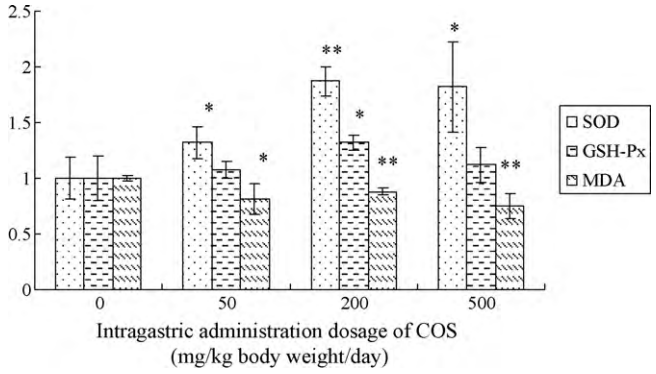

Fig. 7. Anti-oxygenic property of COS to xenograft tumor mice. Before sacrificed, the eyeballs of mice were removed and blood was collected in anticoagulation tubes. The activities of SOD, GSH-Px and content of MDA in xenograft tumor mice blood were tested according to the kit demonstration (JianCheng, NanJing). The activity of SOD, GSH-Px and content of MDA of control groups were regarded as 1, and the experimental groups were calculated compared to the control group respectively. The data were dealt with SPSS software and $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

the level of MDA in the blood, in a dose-dependent manner. However, the activity of GSH-Px increased only when 200 mg/kg body weight/day was used.

4. Discussion

Due to the importance of angiogenesis for tumor growth and metastasis, anti-angiogenesis has become a focus of cancer research, and several clinical drugs have been used with satisfactory effects (Morabito, Sarmiento, Bonginell, & Giampietro, 2004). The directional migration of endothelial cells is an early and crucial step in angiogenesis. NO, produced by NOS, is an important second messenger in many signal transduction processes and a potent vasodilator (Sessa, 2005). NO also plays a role in endothelial functions such as angiogenesis and migration (Lee et al., 2005). Recent studies have shown that endothelial-derived NO is required for Ang1-induced angiogenesis, and that PI3-kinase signaling mediates the activation of eNOS and NO release in response to Ang1 (Babaei et al., 2003). NO has been regarded as a critical mediator of angiogenesis, as it enhances the survival, proliferation, and migration of endothelial cells (John, 2003).

The inhibitory activity of COS on the migration and tube formation of HUVECs has been confirmed by our previous study (Wu

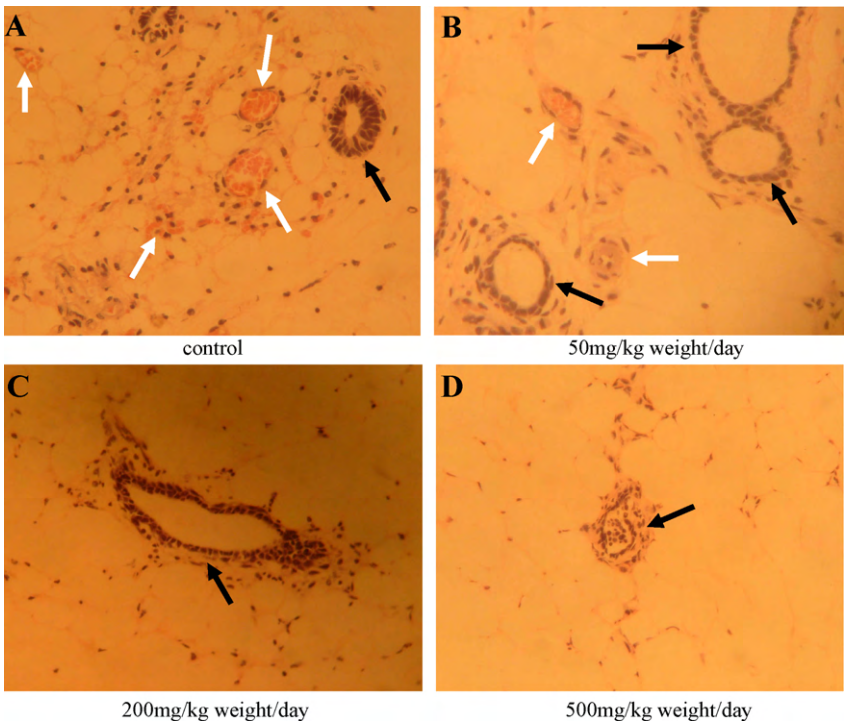

Fig. 6. Effect of COS on the angiogenesis of breast cancer cellular transplantation tumor. 5 days after the human breast cancer cell transplantation, the mice were treated with PBS (A) or different concentration of COS (50 mg/kg weight/day (B), 200 mg/kg weight/day (C) or 500 mg/kg weight/day (D) respectively) by intragastric administration for 15 days. Then the mice were sacrificed, and the tumor masses were fixed with formalin, embedded with paraffin and stained routinely by H&E in sections of local primary tumors. The sections were photographed under an inverted microscope. .

et al., 2008). However, the mechanism remains unclear. NO promotes the migration of HUVECs in angiogenesis as a downstream effector. Therefore, we induced HUVECs with NO and demonstrated that NO could promote the migration of HUVECs. In addition, we demonstrated that different concentrations of COS could suppress the inductive effect of NO in a concentration dependent manner, and that the greatest effect was observed when the COS concentration was 50 μg/ml and 200 μg/ml. This result accords with the inhibitory activity of COS on the migration of HUVECs induced by tumor cells culture fluid (Wu et al., 2008). Various growth factors, such as VEGF in the tumor cells culture fluid, can promote the migration of HUVECs by binding to its receptor, opening the signal conduction, and producing many signal molecules. NO is one of these important mediators, and it is possible that COS could inhibit the migration-promoting activity of tumor cells culture fluid by suppressing the NO activity.

During the angiogenesis, the signals from all angiogenesis stimulators will converge to the cytoskeleton that controls the cell migration state (Tamilarasan et al., 2006). Therefore, dynamic rearrangement of the cytoskeleton is the key to the migration of the cells (Lamalice, Le, & Huot, 2007). COS could reduce the formation of lamellipodia and induce the formation of the central microfilaments of the HUVECs in both the presence and absence of SNP, so we conclude that COS can affect the migration pattern of HUVECs by rearranging the cytoskeleton. At the same time, COS could inhibit the polymerization of actin at the cell–cell interface, and this is important for the tube formation of HUVECs. Our previous study has demonstrated the inhibitory effect of COS on tube formation of HUVECs. The effect of COS on the rearrangement of the cytoskeleton simply verifies the inhibitory effect of COS on the migration, and tube formation of HUVECs.

The use of animal models is a direct and objective method to evaluate the anti-angiogenic activity of a compound. As apparent in the animals, the color of the tumor mass in the control group was red, and that of the COS treatment groups was white (results not shown). Correspondingly, the density of microvessels in the COS treatment groups was much lower than that of the control group.

As ROS promote tumor angiogenesis, many anti-oxidants have proven anti-angiogenic activity (Polytarchou & Papadimitriou, 2005). The antioxidant properties of COS to mice that had been transplanted with human breast cancer cell were evident, and the most effective dose was 200 mg/kg body weight/day. The principal factor was the increase in SOD activity.

In conclusion, COS has anti-angiogenic activity *in vivo* and *in vitro*. In our previous study, we found that COS could counteract the ability of human hepatoma carcinoma cell culture fluid to induce the migration and tube formation of HUVECs (Wu et al., 2008). Here, we found that COS could counteract the ability of NO to induce the migration of HUVECs, and that COS could inhibit the generation of NO by HUVECs, with or without SNP present. In the animal tumor model, the anti-angiogenic and antioxidant activity of COS on the xenograft tumors was evident. During angiogenesis, VEGF promotes the generation of NO by binding to its receptor (Lamalice et al., 2007). Which step of VEGF signal pathway is influenced by COS to decrease the amount of NO? Answering this question may help us to understand the origin of the anti-angiogenic activity of COS. The relevant research is continuing in our laboratory.

Acknowledgement

This research was supported by grants from the National Natural Science Foundation of China (NSFC 30970645 and 30901875).

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