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Enzymatic preparation of κ-carrageenan oligosaccharides and their anti-angiogenic activity

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ABSTRACT

The mixture of κ-neocarrabiose-sulfate, κ-neocarrahexaose-sulfate and κ-neocarraoctaose-sulfate were prepared from κ-carrageenan with enzyme. The anti-tumor and anti-angiogenic activity of obtained κ-carrageenan oligosaccharides (KOS), were explored. The results showed that KOS could inhibit the proliferation, migration and tube formation of ECV304 cells, and could inhibit the growth of new vessels in CAM model. KOS displayed strong anti-tumor activity in both S180 and MCF-7 xenograft models. Only human CD105 was detected in MCF-7 xenograft tumor, moreover KOS could decrease the growing of new blood vessels derived from tumor cell. Real-time PCR results showed that KOS could suppress the mRNA expression of human VEGF, bFGF, bFGFR and CD105 in MCF-7 xenograft tumor. All these results indicated that KOS has anti-tumor and anti-angiogenic activity in vivo and in vitro. Especially K has the potency to inhibit the differentiation of tumor cell to blood vessel endothelial cell.

1. Introduction

Angiogenesis – the formation of new blood vascular network from pre-existing vessels – is critical to the growth and progression of solid tumors (Folkman, 1990). In cancer, the vasculature is highly disorganized and resulted in poor blood flow and high vascular permeability, which may lead to increased potential for metastasis (Baluk, Hashizume, & McDonald, 2005; Gerber & Ferrara, 2005; Jain, 2005). Nearly 40 years ago, Folkman (1972) proposed that suppression of tumor blood vessel growth would offer a new option for cancer therapy, and lots of researches proved that interrupting the process of angiogenesis was an effective way for the treatment of cancer (Fischer, Mazzone, Jonckx, & Carmeliet, 2008; Grothey & Allegra, 2012; Jekunen & Kairemo, 1997). In the past decades, a number of potent endogenous angiogenesis inhibitors have been evaluated and brought to clinical trials of cancer (Bridges & Harris, 2011; Ribatti, 2011). But most of them are proteins, and the disadvantages are unavoidable, such as difficulties of manufacture, high costs and risk of transmission of microorganism toxins in recombinant proteins. Small molecule with anti-angiogenic activity from natural products can avoid these disadvantanges (Kim, Jung, & Kwon, 2012).

Carrageenans are a family of anion polymers extracted from certain marine red algae and share a common backbone of alternating (1 → 3)-linked β-D-galactopyranose and (1 → 4)-linked α-D-galactopyranose. Carrageenans are regarded as safe (GRAS) by the Food and Drug Administration in the US, so they are widely used as texturizing, viscosity-building and gel-forming ingredients in the food and pharmaceutical industry (Navarro & Stortz, 2005). Carrageenan can hardly be degraded and absorbed in human intestinal tract, but can be degraded into oligomer by carrageenases (Mu, Jiang, & Guan, 2003), acid (Yuan & Song, 2005), microwave (Zhou et al., 2004), active oxygen (Yamada, Ogamo, Saito, Uchiyama, & Nakagawa, 2000) and free radical depolymerization by H_2O_2 (Zuniga, Matsuhiro, & Mejias, 2006) or methanolysis (Knutsen & Grasdalen, 1992) in vitro. Compared to carrageenan, the oligomers are more absorbable, less toxic and have more biological activities, such as immunological (Stephaniea, Eric, Sophiea, Christiand, & Yue, 2010), anticoagulant (Silva et al., 2010), antiviral (Pujol et al., 2006), antibacterial (Wang et al., 2012), anti-angiogenic (Chen, Yan, Lin, Wang, & Xu, 2007) or antitumor activities (Alves de Sousa et al., 2007). The presence of sulfates in carrageenan makes them known to have valuable biological functions. In recent years, marine oligosaccharides are attracting increasing interests in developing potential antitumor drugs. In 2007, Chen et al. reported that the depolymerized products of lambda-carrageenan had anti-angiogenic activity. In the present study, κ-carrageenan oligosaccharides were prepared by enzymolysis, the antitumor and anti-angiogenic activity of oligomers were studied.

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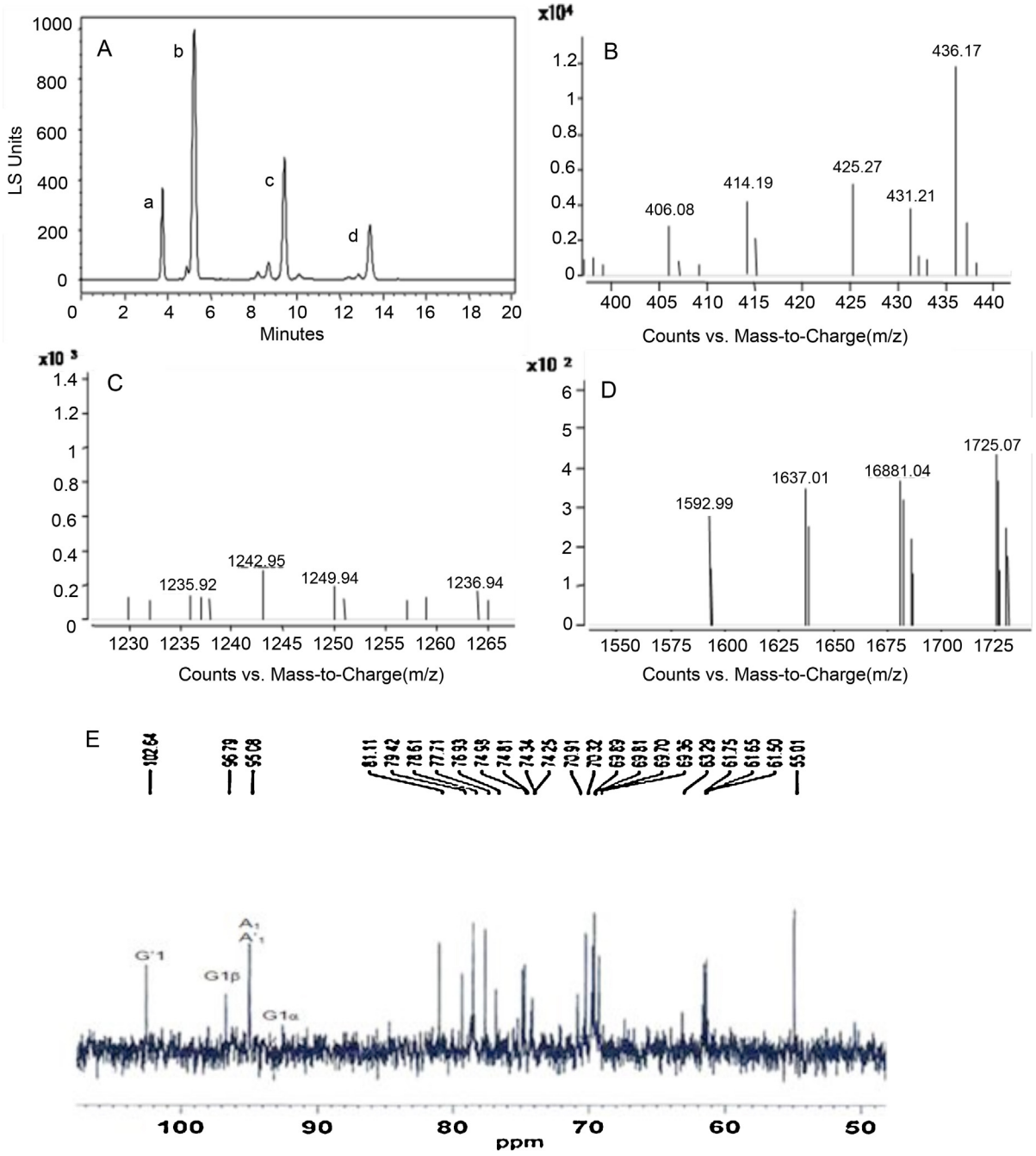

Fig. 1. HPLC (A), MSI-TOF-MS (B–D) and ^{13}C NMR spectrum (E) analysis of κ-carrageenan oligosaccharides. (A) HPLC spectrum of κ-carrageenan oligosaccharides. κ-Carrageenan was hydrolyzed with the enzyme under standard condition for 48 h, and the product was separated with HPLC. Three components were detected and separated (b, c and d). (a) Solvent peak. (B–D) MSI-TOF–MS analysis of κ-carrageenan oligosaccharides. The components obtained from HPLC were analyzed with ESI-TOF–MS to determine the molecular mass distribution. (E) κ-Carrageenan oligosaccharides were dissolved in $^{2}H_2O$ and processed at 30 °C. Spectrum was recorded on an AVANCE 500 MHz apparatus (Bruker, Germany). G:β-D-galactopyranose, A:3,6-anhydro-α-D-galactopyranose.

2. Materials and methods

2.1. Cells and animals

Human umbilical vein endothelial cells (ECV304) and Sarcoma 180 cells were purchased from the China Center for Type Culture Collection, Human breast cancer cells (MCF-7) were a generous gift from Professor Changqian Zeng (Dalian University, Dalian, China). All of these were maintained in RPMI 1640 medium 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.

5-Week-old female Kunming mice were purchased from the Experimental Animal Center of Dalian Medical University and maintained in a standard animal room for 1 week before the experiment began.

6-Week-old female nude mice were purchased from the Experimental Animal Center of Dalian Medical University and the relevant experiment was carried out in the SPF Experimental Animal Center of Dalian Medical University.

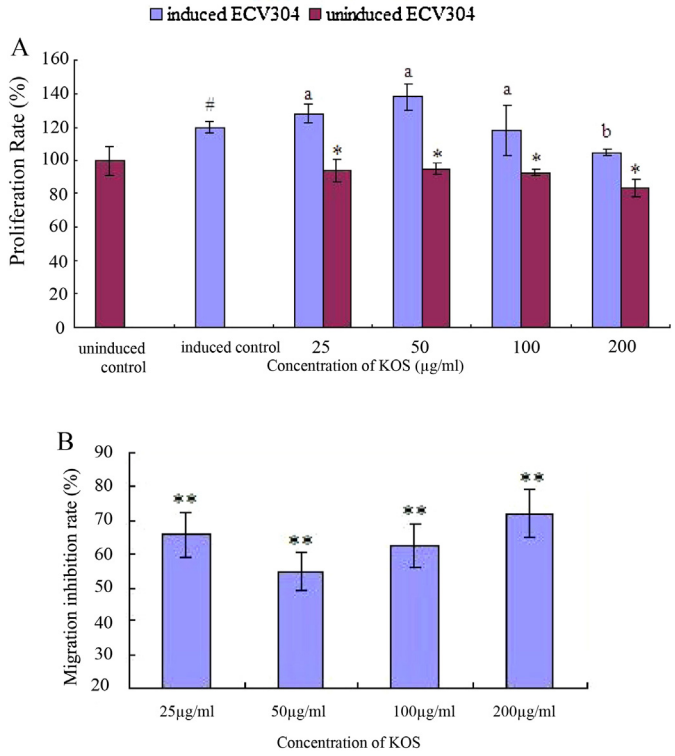

Fig. 2. Effect of KOS on the proliferation (A) and migration (B) of ECV304 cells (*t*-test). (A) Different concentration of KOS was applied to normal and MCF-7 culture medium-induced ECV304 cells for 24 h and cell viability was detected by using MTT method. Statistical analysis was performed by Student's *t*-test. $^{*}p > 0.05$ and $^{\#}p < 0.05$ vs. uninduced control; (a) $p < 0.05$ and (b) $p < 0.01$ vs. induced control. (B) ECV304 cells monolayer interrupted by cell scraper were further incubated with or without 5% MCF-7 culture fluid (induced and non-induced control respectively), and with different concentration of KOS. Migration of the cells was photographed under an inverted microscope (Leica, Germany). The number of cells migrating to the clear area in the induced control sample was regarded as 100%. Migration inhibiting of different concentration KOS was calculated. Statistical analysis was performed by Student's *t*-test. $^{**}p < 0.01$ vs. induced control.

2.2. Chemicals

κ-Carrageenan was purchased from the Changhang Colloid Technological Co., Ltd. (Jiangsu, China). Cell culture reagents were obtained from Invitrogen. Fertilized chicken eggs were obtained from the breeding bird farm of Dalian Fengqiyuan Industry Association (Liaoning, China). All through the experiment 5% MCF-7 human breast cancer cells culture fluid was used as an inducing factor, and equal volume of ECV304 culture fluid was used as a control. All other reagents were of analytical grade.

2.3. Preparation of κ-carrageenan oligosaccharides

K-carrageenan oligosaccharides (KOS) were prepared by enzymic hydrolysis κ-carrageenan according to our previous method. In brief, the mixture of crude enzyme (2%) and κ-carrageenan (0.5%) was incubated for 48 h at 30 °C. The reaction was stopped by boiling for 10 min. The enzyme protein in the reaction mixture was removed by ethanol precipitation, and salts and other small molecule impurities were removed by dialysis method. The obtained KOS were concentrated to dryness with a rotary evaporator under diminished pressure. An aliquot of KOS was analyzed with a high performance liquid chromatography (HPLC) and the molecular weight of KOS was tested with ESI-TOF–MS (G1969A, Agilent, UN) operated in the positive-ion mode. The structure of KOS was identified with ^{13}C NMR spectroscopy (AVANCE500MHz, Bruker, Germany).

2.4. The subcutaneous S180 xenograft model in Kunming mice

The experimental protocol was approved by the China Institutional Ethics Review Committee for Animal Experimentation. The S180 mouse sarcoma cells with ascites were harvested sterilely (the concentration was adjusted to 2×10^7 ml^{-1} with sterilized physiological saline) and subcutaneously injected into the right flank region of female 6-week-old Kuming mice (200 μl per mouse) as described by Lu et al. (2003). 5 days after tumor cell implantation, a palpable tumor mass was confirmed and the mice were randomly divided into five groups (at least 10 mice per group) to start the antitumor experiment. KOS in dose of 50, 100 and 200 mg/kg body weight/day were intragastric administration to the experimental group mice for 15 days. Physiological saline and cyclophosphamide (20 mg/kg body weight/day, Hengrui Medicine Co. Ltd., China) were used as negative and positive controls respectively. Then the mice were sacrificed and the tumor mass were excised and weighed.

The tumor growth inhibiting rate was calculated as: tumor growth inhibiting rate (%) = (1 − tumor weight of test group/tumor weight of negative control group) × 100%.

2.5. Determination of the effect of KOS on ECV304 cells viability by MTT method

In order to determine the effect of KOS on the viability of ECV304 cells, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used. In short, ECV304 cells were seed (5×10^3 per well) in 96-well plate and cultured for 24 h, then the medium was renewed with different concentrations KOS and cultured for another 24 h. 4 h before the end of culture 20 μl MTT (5 mg/ml) solution was added into each well, and plates were incubated for 4 h. After the medium was removed, 150 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the blue crystals and the absorbance value (490 nm) was measured by using an ELISA microplate reader (Rayto instrument). Six replications were used for each concentration of KOS. The viability of control group ECV304 cells was regarded as 100%.

Then the effect of KOS on the viability of MCF-7 culture fluid-induced ECV304 cells was determined. The method was as described above, but in addition 5% (v/v) MCF-7 culture fluid was added to each well as an inducing factor. Six replications were used for each concentration of KOS. The viability of control group ECV304 cells was regarded as 100%.

2.6. Determination of the effect of KOS on induced ECV304 cells migration by wound healing assay

To evaluate the effect of KOS on MCF-7 medium-induced ECV304 cells migration, a "wound healing" method was used (Sato & Rifkin, 1988). ECV304 cells were seeded (2×10^5 cells per well) in a 24-well plate and incubated till the cells had grown to confluence. Then a 0.5 mm cell scraper was used to interrupt the monolayer. After the 24-well plate was washed twice with PBS, ECV304 cells were further incubated with or without 5% MCF-7 culture fluid (induced and non-induced control respectively), and with different concentration of KOS. The migration of cell was photographed under an inverted microscope (Leica, Germany). The number of cells migrating to the clear area in the induced control sample was regarded as 100%, i.e. no inhibition

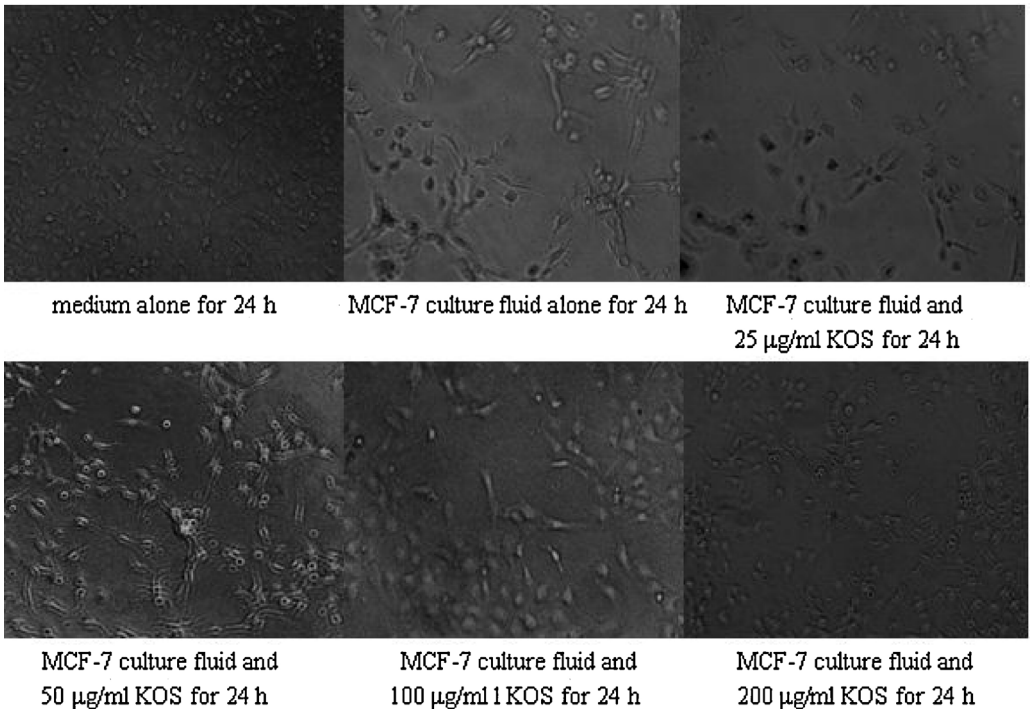

Fig. 3. Effect of KOS on tube formation of induced ECV304 cells. ECV304 cells were seeded in collagen-coated 96-well plates and subsequently incubated for 24 h with RPMI-1640 medium (non-induced control), with RPMI-1640 medium containing 5% MCF-7 culture fluid (induced control) or with RPMI-1640 medium containing 5% MCF-7 culture fluid in presence of different concentration of KOS. The enclosed networks of tubes were photographed under an inverted microscope.

of migration. Six replications were used for each concentration of KOS.

2.7. Determination of the effect of KOS on the tube formation of ECV304 cells

ECV304 cells tube-structure formation on type I collagen was modified from a previous method (Jones et al., 1999). In brief, 50 μl type I collagen (5 mg/ml) at 4 °C was added to a 96-well plate and allowed to polymerize at 37 °C for about 20 min. ECV304 cells were seeded (2×10^4 per well) on the surface of the gel and incubated for 24 h in RPMI1640 medium in the presence of 5% MCF-7 culture fluid and different concentration of KOS. The formation of tube-like structures by ECV304 cells on type I collagen was photographed under an inverted microscope (Leica, Germany).

2.8. Effect of KOS on the angiogenesis of CAM (chicken chorioallantoic membrane) model

The CAM assay was modified from a method described previously (Zhao, Miao, Zhao, Zhang, & Yin, 2005). Briefly, after incubated at 37 °C for 6 days in a humidified atmosphere, the fertilized eggs were opened a window (~1.5 cm in diameter) at the air space end on the seventh day, so that a part of the CAM was exposed. Then sterilized gelatin sponges with 50 μl different concentration of KOS were placed on the CAM. PBS and hydrocortisone (5 mg/mL, Jinyao Co. Ltd., China) were used as negative and positive controls respectively. The windows were covered, after the eggs were incubated for additional 24 h at 37 °C, 50 μl of the same samples were added again onto the gelatin sponge, and the eggs were incubated for another 24 h at 37 °C. So the final doses of KOS were 2.5, 5 and 20 μg/egg. At the end of the experiment, the neovascular zones of the CAM under the sponges were photographed under a stereomicroscope (Olympus, Japan) and the pictures were analyzed by using a Microsoft Auto CAD (Autodesk, US). The area of blood vessels in the negative control was regarded as 100% (i.e. 0% inhibition of blood vessel growth/angiogenesis), and the vessel growth inhibiting of positive control and different concentration KOS were calculated.

2.9. The subcutaneous MCF-7 xenograft model in nude mice

The experimental protocol was approved by the China Institutional Ethics Review Committee for Animal Experimentation. The sterilely harvested MCF-7 cells were adjusted to 1×10^7 ml^{-1} with sterilized physiological saline and implanted subcutaneously in the front flank region of nude mice (200 μl per mouse). Tumors were allowed to grow for 2 weeks to reach average diameter of 0.5 cm, then treatment commenced.

The mice were randomly divided into 5 groups (at least 10 mice per group) and treated with physiological saline or with different concentrations of KOS (50, 100 and 200 mg/kg body weight/day) by intragastric administration for 28 days. Physiological saline and thalidomide (76 mg/kg body weight/day, Changzhou Pharmaceutical Factory Co. Ltd., China) were used as negative and positive controls respectively. Mice were weighed and tumors were measured with vernier caliper every 3 days. The estimated tumor volume (V) was calculated using the longitudinal (L) and transverse (W) cross-sections according to the formula: $V = (L \times W^2)/2$.

24 h after the last administration, the mice were sacrificed and the tumor mass were excised and weighed. Then a portion of every group tumor mass was fixed in phosphate-buffered 10% formalin for the following immunohistochemical experiment, and the rest tumor mass was stored at −80 °C refrigerator for QRT-PCR experiments.

2.10. Immunohistochemical analysis

MCF-7 tumor tissues fixed in 10% formalin were embedded in paraffin. Then all paraffin-embedded tissue was sectioned with a

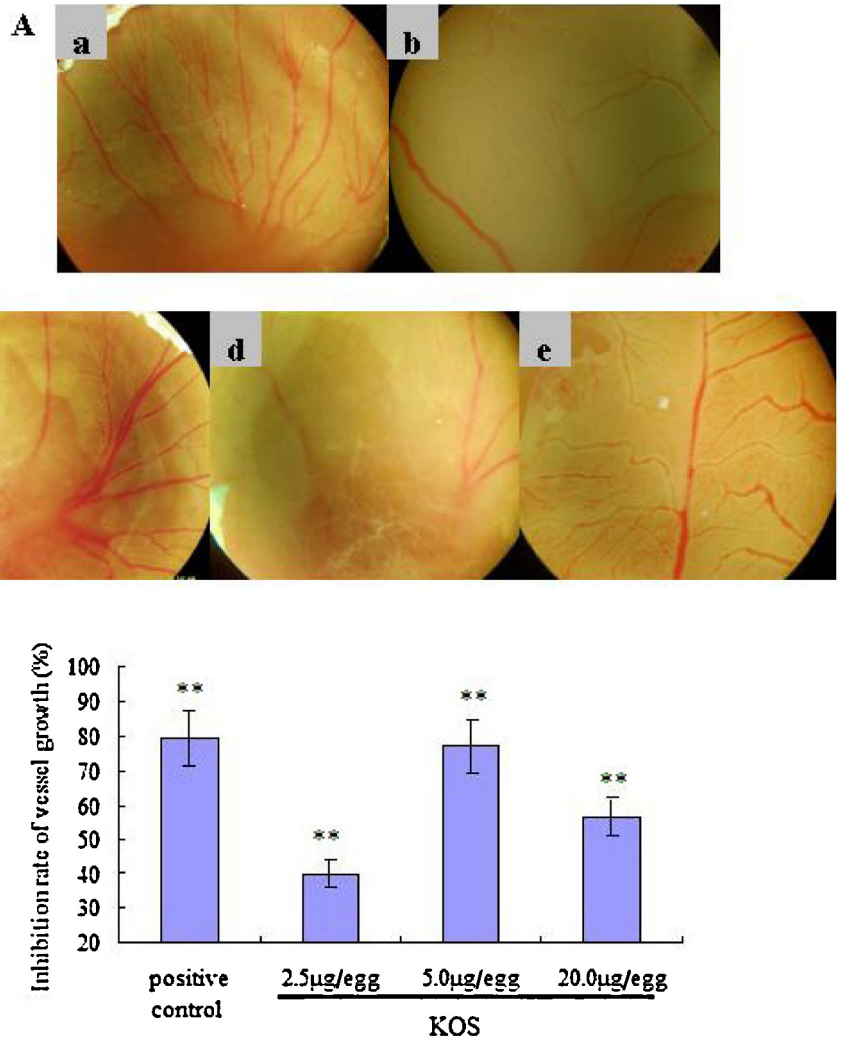

Fig. 4. Effect of KOS on CAM angiogenesis. CAM were treated with different amount of KOS for 48 h and photographed. (A) Morphological micrographs. (a) Control, CAM treated with PBS; (b) positive control, CAM treated with 500 μg hydrocortisone; (c–e) CAM treated with 2.5, 5 and 20 μg KOS respectively. (B) The inhibition of vessel growth. The neovascular area of the CAM under the sponges was analyzed by the Microsoft of Auto CAD. The neovascular area under the sponges of negative control CAM was regarded as 100%. Statistical analysis was performed by Student's *t*-test. $^{**}p < 0.01$ vs. negative control.

microtome, deparaffined with xylene and rehydrated by ethyl alcohol in decreasing concentration (100%, 95%, 85% and 75%). Antigen retrieval was performed by using hot water bath (95–99 °C). After complete washing, the sections were incubated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. Then the sections were incubated with corresponding primary antibody (monoclonal mouse anti-CD105 or human anti-CD105) at 4 °C overnight, and incubated with secondary antibody at room temperature for 10 min. After washed with PBS, immunohistoreactivity was visualized by using diaminobenidine (DAB) and photographed under a micro-imaging system (Olympus BX41, Japan). The stained sections were examined at 40× and 100× magnification to identify the area of highest neovascularization (so-called hot spots) of the tumor. In each section, five hot spots were chosen to count the microvessels number, and the averages were calculated.

2.11. Real-time PCR assay

MCF-7 tumor tissues stored at −80 °C refrigerator was used for the real-time PCR assay. Total RNA of the tumor tissues was isolated by using guanidine isothiocyanate method and was transcribed to cDNA with Prime Script™ RTase (Takara, Dalian, China). The real-time PCR was performed with Mighty Amp for Real Time (Takara, Dalian, China) and the PCR conditions were 95 °C for 1 min to pre-denaturation, 95 °C for 15 s and final extension was performed at 60 °C for 1 min for 40 cycles. Primers used for human cells were: GAPDH, sense 5′-AGAAGGCTGGGGCTCATTTG-3′, anti-sense 5′-AGGGGCCATCCACAGTCTTC-3′; VEGFA, sense 5′-GAGTACATCTTCAAGCCATCCTA-3′, anti-sense 5′-TGCTCTATCTTTCTTTGGTCTC-3′; KDR, sense 5′-CAACACAGCAGGAATCAGTCA-3′, anti-sense 5′-AACCATACCACTGTCCGTCTG-3′; bFGF, sense 5′-GAAGAGCGACCCTCACATCAA-3′, anti-sense 5′-CGTTTCAGTGCCACATACCAA-3′; FGFR1, sense 5′-ATGATGCCACAGAGAAAGACCT-3′, anti-sense 5′-GCAGGCTCCAAGAAGATTTATG-3′; CD105, sense 5′AGAGGACAGGGTGACAAGTT-3′, anti-sense 5′-AAGTGTGGGCTGAGGTAGAGG-3′. The relative gene expression data was analyzed by using $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.12. 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.

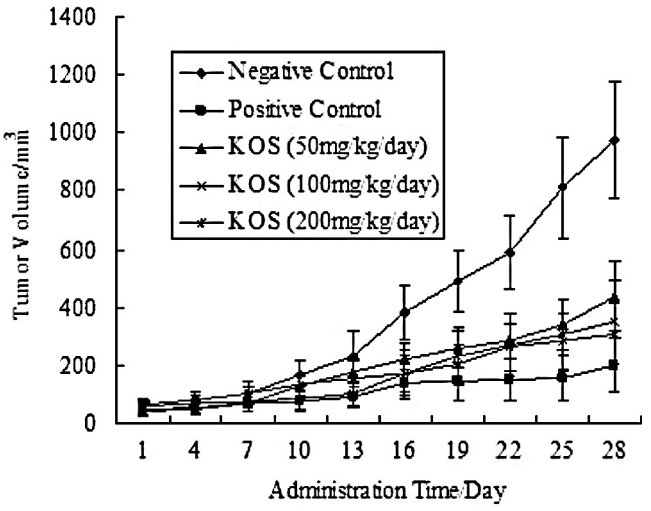

Fig. 5. Effect of KOS on the growth of MCF-7 xenograft in nude mice. The MCF-7 cells were implanted subcutaneously in the front flank region of nude mice, and allowed to grow for 2 weeks. Then the mice were treated with physiological saline or with different concentrations of KOS (50, 100 and 200 mg/kg body weight/day) by intragastric administration for 28 days. Physiological saline and thalidomide (76 mg/kg body weight/day) were used as negative and positive controls respectively. Tumors were measured with vernier caliper every 3 days. Statistical analysis was performed by Student's *t*-test. Positive control vs. negative control $p < 0.05$ from the seventh day and $p < 0.01$ from the thirteenth day. KOS groups vs. negative control $p < 0.05$ from the tenth day and $p < 0.01$ from the sixteenth day.

3. Results

3.1. Preparation of KOS

As shown in Fig. 1, the KOS obtained by enzymic hydrolysis was mixture of three components and the molecular mass of the three components were 425.27 Da, 1242.95 Da and 1681.04 Da respectively. ^{13}C NMR was used to obtain the structure information of the products. As shown in Fig. 1E, only one signal was observed at around 102.64 ppm, which is the characteristic signal of the G-unit at non-reducing end of the oligosaccharides. The resonance at 95.08 ppm was assigned to be the carbon of the A-unit. The signal at 92.5 ppm and 96.79 ppm were assigned to be the carbon of the G-unit at the reducing end for α and β configuration respectively. According to above results, the reducing end of the oligosaccharides was G-unit. So the hydrolyzed products of the κ-carrageenase were κ-neocarrabiose-sulfate (dp2), κ-neocarrahexaose-sulfate (dp6) and κ-neocarraoctaose-sulfate (dp8) respectively. The molar ratio of each oligosaccharides in the mixture was about 4:2:1 (dp2:dp6:dp8).

3.2. Anticancer activity of KOS on S180 xenograft tumor in Kunming mice

The effects of three doses of KOS (50, 100 and 200 mg/kg body weight/day) on the growth of S180 sarcoma were shown in Table 1. Positive control (cyclophosphamide) displayed a strong tumor inhibiting function, and its tumor inhibiting rate was 69.5%. KOS also could inhibit tumor growth in a dose-dependent manner. When the administration dose of KOS was 200 mg/kg body weight/day, its tumor inhibiting rate was 56.8%.

3.3. Effect of KOS on the proliferation and migration of ECV304 cells

To evaluate the influence of KOS on the proliferation of normal and MCF-7 culture fluid-induced ECV304 cells, MTT method was used. As shown in Fig. 2A, all concentrations of KOS showed no influence on the proliferation of normal ECV304 cells. On the other hand, MCF-7 could promote the proliferation of ECV304 cells evidently, KOS (100 and 200 μg/ml) could inhibit the proliferation of induced ECV304 cells in a concentration dependent manner. When the concentration of KOS was up to 200 μg/ml, the inhibiting activity was significantly evident. That is to say, KOS showed no toxicity toward ECV304 cells, but could counteract the promoting of MCF-7 culture fluid toward ECV304 cells.

"Scratch wound" assay was used to investigate the effect of KOS on MCF-7 culture fluid-induced ECV304 cells migration. The results were shown in Fig. 2B. After being induced by MCF-7 culture fluid, massive cells migrated to the scratch area. All concentrations of KOS could suppress the migration of induced cells. The migration of induced control was set to 100% migration (or 0% migration inhibiting) and the inhibiting effect of different concentration KOS was calculated. When the KOS concentration was 200 μg/ml, the migration of cells was inhibited 71.5 (±8)%.

Table 1
Effect of KOS on the weight of S180 sarcoma growing in Kunming mice.

Groups	Dose (mg/kg d)	Tumor weight (mg)	Tumor inhibition rate (%)
Negative control	–	290.67 ± 6.88	–
Positive control	20	88.67 ± 6.46**	69.50
KOS	50	247.25 ± 9.10*	14.94
KOS	100	176 ± 4.77**	39.45
KOS	200	125.67 ± 2.98**	56.77

* $p < 0.05$ vs. negative control.
** $P < 0.01$ vs. negative control.

3.4. Effect of KOS on the tube formation of MCF-7 culture fluid-induced ECV304 cells

As shown in Fig. 3, ECV304 cells in non-induced control group could not form enclosed networks of tube-like structure on collagen gel. In induced control group, after treated with MCF-7 culture fluid, ECV304 cells shaped enclosed networks of tube-like structure. All concentrations of KOS could inhibit the tube formation of cells, and no tube-like structure was observed at the concentration of 200 μg/ml.

3.5. Effect of KOS on CAM angiogenesis

To evaluate whether KOS have anti-angiogenic activity in vivo, a chicken CAM model was used. The result of KOS suppressing CAM angiogenesis was shown in Fig. 4. In negative control group, the blood vessels under the gelatin sponge grew productively. In positive control group, almost no blood vessels were observed under the gelatin sponge. After treated with different amount of KOS, the blood vessels under the gelatin sponge of CAM grew rarely and were short of branches. The area of blood vessels in the negative control was regarded as 100%. The growth inhibition of positive control was 78.3 (±6.2) %, and KOS were of 38.5(±5.5) % (2.5 μg/egg), 74.4 (±7.5) % (5 μg/egg) and 50.5 (±4.3) % (20 μg/egg) respectively.

3.6. Effect of KOS on the growth of MCF-7 xenograft in nude mice

The suppression of KOS on the growth of MCF-7 xenograft in nude mice was shown in Fig. 5. As shown in Fig. 5, there was no evident difference among different groups tumor volume till KOS administration for 1 week. After that, the tumor of negative control group grew fastest, and the growth of KOS and positive control groups tumor slow down. Among the total, the suppression activity of thalidomide (positive control) was most intensive,

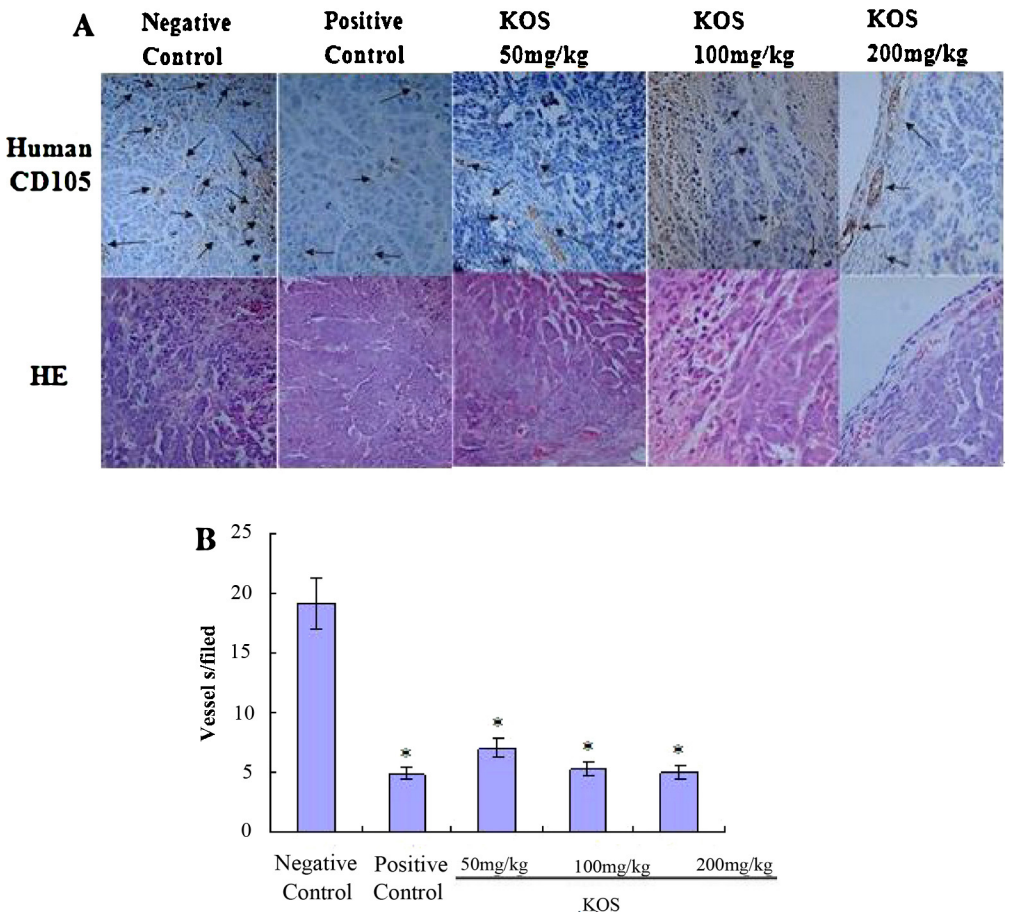

Fig. 6. KOS reduced the microvessel density (MVD) of MCF-7 xenograft in nude mice. (A) Immunohistochemical staining of human CD105. MCF-7 cells were inoculated into nude mice. Thalidomide (76 mg/kg body weight/day) or KOS (50, 100 and 200 mg/kg body weight/day) was given to the mice by intragastric administration for 28 days. After the final administration, paraffin slices of tumor were immunostained with monoclonal antibody to human and mouse CD105. The stained slices were photographed at 40× magnification. (B) Effect of KOS on the MVD (microvessel density) MCF-7 xenograft in nude mice. The stained sections were examined at 40× and 100× magnification to identify the area of highest neovascularization (so-called hot spots) of the tumor. In each section, five hot spots were chosen to count the microvessels number, and the averages were calculated. Statistical analysis was performed by Student's *t*-test. $^{*}p < 0.01$ vs. negative control.

and the following was 100 mg/kg, 200 mg/kg and 50 mg/kg KOS successively.

3.7. Immunohistochemical analysis

In order to identify the mechanism of anti-angiogenic activity of KOS, we stain the tumor sections with human anti-CD105 monoclonal antibody and mouse anti-CD105 monoclonal antibody respectively. As shown in Fig. 6A, only human CD105 was detected. That is the new vessels in tumor are mainly derived from MCF-7 cells in this experiment. According to immunohistochemistry of human CD105, the microvessels density (MVD) was calculated. As shown in Fig. 6B, KOS could decrease the MVD value of tumor with dose dependent manner, and the inhibiting activity of 100 mg/kg and 200 mg/kg KOS were near to that of thalidomide (positive control).

3.8. Real-time PCR assay

The mRNA expression of human VEGF1, KDR, bFGF, bFGFR and CD105 was determined by using real-time PCR method, and the results were shown in Fig. 7. All in all, KOS could inhibit the mRNA expression of all above mentioned cytokines and receptors. Among these, the inhibiting effect of KOS on VEGF mRNA expression was more evident than that of thalidomide (positive control) with dose dependent manner. The inhibiting effects of KOS on bFGF, bFGFR and CD105 mRNA expression were coincident, that is the inhibiting effects of 50 and 100 mg/kg KOS were more evident than that of thalidomide (positive control), and that of 200 mg/kg KOS was the weakest. The inhibiting effect of KOS on KDR mRNA expression was irregular.

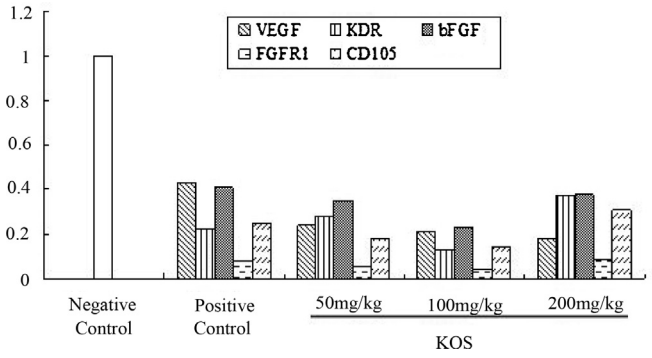

Fig. 7. Effect of KOS on the mRNA expression of human VEGF, KDR, bFGF, FGFR1 and CD105 in MCF-7 tumor mass. MCF-7 cells were inoculated into nude mice. Thalidomide (76 mg/kg body weight/day) or KOS (50, 100 and 200 mg/kg body weight/day) was given to the mice by intragastric administration for 28 days. After the final administration, mRNA expression of human VEGF, KDR, bFGF, FGFR1 and CD105 in MCF-7 tumor mass were detected by real-time PCR. The relative gene expression data was analyzed by using $2^{-\Delta\Delta CT}$ method.

4. Discussion

The skeleton structure of κ-carrageenan is alternately connected with 1, 3-β-D-galactose and 1, 4-α-D-galactose, so different degradation site would generate different production. In this research, the oligosaccharides used in anti-angiogenic activity detecting were the mixture of κ-neocarrabiose-sulfate, κ-neocarrahexaose-sulfate and κ-neocarraoctaose-sulfate.

In order to determine if KOS has anti-tumor activity, S180 xenograft tumor in Kunming mice model was used. The result showed that KOS had evident anti-tumor activity, and the inhibiting activity of 200 mg/kg KOS was near to that of positive control (cyclophosphamide).

As angiogenesis is indispensable for the growth and metastasis of tumor, new vessel in tumor has been a target of tumor therapy. In recent years, several marine saccharides have been reported to have anti-angiogenic activity (Ma et al., 2008; Matsubara et al., 2005; Wu, Yao, Bai, Du, & Lin, 2008). In order to determine if KOS has anti-angiogenic activity, the effect of KOS on the proliferation, migration and tube formation of ECV304 cell and on the new vessel formation of CAM were analyzed. And the results showed that KOS could inhibit the proliferation, migration and tube formation of ECV304 induced by MCF-7 culture fluid. It is well known that the proliferation, migration and tube formation of vascular endothelial cells are the main steps of angiogenesis, and have become the main target of anti-angiogenesis study. These results suggest that KOS have anti-angiogenic potency. The following CAM experiment further showed that KOS could inhibit the generation of new vessel.

Tumor vessels can grow by various mechanisms: (1) the vascular network expands; (2) the insertion of interstitial tissue columns into the lumen of pre-existing vessels; (3) endothelial cell precursors home to tumors; (4) derived from tumor stem cells (Carmeliet, 2005; Carmeliet & Jain, 2000). CD105 (Endoglin) is a receptor of TGF-β family, is mainly expressed in the vascular endothelial cell of new vessels. So it is usually considered as a mark of new vessels (Gilbert, Bauer, Gilbert, & Banek, 2012). As in this research, the human breast cancer cell MCF-7 was implanted into mice. In order to determine which kind of new vessels was suppressed by KOS, the tumor sections was stained with human anti-CD105 monoclonal antibody and mouse anti-CD105 monoclonal antibody respectively. It is very interesting that no mouse CD105 was detected in the tumor tissue. Maybe the period of the tumors allowed to grow was too short to permit the host vessels to expand into the tumor. The MVD result showed that KOS could inhibit the growth of new blood vessel derived from tumor cell. Maybe KOS could inhibit the differentiation of tumor cell into vascular endothelial cell. Real-time PCR result showed that KOS could inhibit the mRNA expression of human VEGF, bFGF, bFGFR and CD105. So KOS have anti-tumor and anti-angiogenic activity, its anti-angiogenic activity should be related with its inhibiting activity on the mRNA expression of human VEGF, bFGF, bFGFR, and should be related with its decreasing effect on the differentiation of tumor cell into vascular endothelial cell, and the further mechanism was undergoing in our lab.

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