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N-Acetylchitooligosaccharide is a potent angiogenic inhibitor both *in vivo* and *in vitro*

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Abstract

N-Acetylchitooligosaccharide (*N*-acetyl-COs) was prepared by *N*-acetylation of chitooligosaccharide (COs). *In vitro* study using human umbilical vein endothelial cells (HUVECs) revealed that both *N*-acetyl-COs and COs inhibited the proliferation of HUVECs by inducing apoptosis. Treatment of HUVECs by *N*-acetyl-COs resulted in a significant reduction of density of the migration cells and repressed tubulogenesis process. The antiangiogenic effects of the oligosaccharides were further evaluated using *in vivo* zebrafish angiogenesis model, and the results showed that both oligosaccharides inhibited the growth of subintestinal vessels (SIV) of zebrafish embryos in a dose-dependent manner, as observed by endogenous alkaline phosphatase (EAP) staining assay. In contrast, no cytotoxicity was found when treating the NIH3T3 and several other cancer cells with the oligosaccharides. Our results also confirmed the antiangiogenic activity of *N*-acetyl-COs was significantly stronger than the parent oligosaccharide, COs.

Keywords: *N*-Acetylchitooligosaccharide; Chitooligosaccharide; Human umbilical vein endothelial cells; Zebrafish; Antiangiogenesis

Angiogenesis, the formation of new blood vessels, is recognized as a fundamental process and an essential component of tumor growth and metastasis. Antiangiogenesis has become a strategy for the treatment of cancer [1]. The process of neovascularization involves coordinated endothelial cell proliferation, migration, and tube formation [2]. Meanwhile, the concept of an antiangiogenesis approach as a promising anticancer strategy has led to the discovery of many natural and synthetic anticancer agents with antiangiogenic properties [3]. Large numbers of antiangiogenic agents with various mechanisms of action are currently under clinical development [4].

Chitin is the second most abundant natural polysaccharide and chitosan can be readily obtained from chitin by deacetylation. Due to their biocompatibility and nontoxic nature, there is a growing interest in the therapeutic potential of them. Chitooligosaccharide (COs) and *N*-acetylchitooligosaccharide (*N*-acetyl-COs) have received increased attention for their water-soluble character and versatile functional properties, especially in cancer research. Antitumor activity of COs was first reported in 1970s [5]. *N*-Acetyl-COs display notable antitumor activity against Sarcoma 180 solid tumors in BALB/C mice as well as in MM-46 solid tumor implanted in C3H/HC mice [6]. The molecular and cellular mechanism of their anticancer properties remained to be determined. Many factors influenced the anticancer activities of these compounds including amino groups [5], molecular weight [7], immunostimulation property [6], induction of the synthesis of nitric oxide and tumor necrosis factor-alpha in macrophages [8], increased activity of natural killer lymphocytes [9], net electric charge [10] and hydrophobicity of the molecules [11], and

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induction cancer cell apoptosis [12]. Recently it was revealed that COs exerted anticancer activity by inhibiting tumor angiogenesis [13].

In the present report, antiangiogenic effects of *N*-acetyl-COs and COs were studied both *in vivo* and *in vitro*. Our studies provided direct evidence that the two oligosaccharides inhibited angiogenesis and influence of apoptosis of HUVECs may be one of the mechanisms of the oligosaccharides. In contrast, no cytotoxicity was found when treating the NIH3T3 cells with the two oligosaccharides. Further study revealed that the *N*-acetylation of COs significantly increased the antiagiogenic activity of the oligosaccharride.

Materials and methods

Preparation of COs and N-acetyl-COs. COs was prepared by hydrolysis of chitosan (Sigma, MO) with chitinase [14]. *N*-Acetyl-COs was subsequently prepared by *N*-acetylation [15] and purified by passing through a Sephadex G-25 column (Amersham Biosciences, Uppsala, Sweden). Method of end groups was used to determine the MW of the two oligosaccharides [16]. A dichroism method [17] was applied in determining the acetyl content of the samples. Also, the purity of samples was determined by HPLC (Waters, MA).

Cell line and cell culture. NIH3T3 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured with DMEM (Invitrogen, NY) supplemented with 10% fetal bovine serum (Invitrogen, NY), and 100 IU/ml penicillin (Invitrogen, NY), 100 IU/ml streptomycin (Invitrogen, NY) in a humidified incubator at 37 °C with 5% CO_2.

HUVECs were isolated from human freshly delivered umbilical cords by collagenase I (Roche, IN) digestion [18] and maintained in medium DMEM/F12 (Invitrogen, NY) containing 20% fetal bovine serum supplemented with 2 mM L-glutamine (Invitrogen, NY), 1 mM sodium pyruvate (Invitrogen, NY), 100 IU/ml penicillin, 100 IU/ml streptomycin, 10 U/ml heparin (Invitrogen, NY), and 30 μg/ml ECGF (Roche, IN). HUVECs at 80–90% of confluency and passage between 3 and 5 were used for all the experiments.

Cell proliferation assay. NIH3T3 cells, HUVECs were plated in 96-well flat-bottomed tissue culture plates (1×10^4 cells/100 μl/well). After 24 h incubation, cells were treated with two oligosaccharides at various concentrations (125, 250, 500, and 1000 μg/ml) and were incubated for another 48 h. After incubation, 10 μl of MTT (Sigma, MO) solution was added to each well for additional 4 h incubation. Two hundred microliters of DMSO was added to each well and optical density (OD) was determined at 570 nm using a spectrophotometer (Bio-Tek Instruments, VT) with subtracted background absorbance [19].

Apoptosis assay. DNA fragmentation [20] was used to study the apoptosis of HUVECs treated with oligosaccharides. HUVECs were seeded to a T25 flask (1×10^5 cells/ml) and cultured for 24 h. Cells were incubated for another 48 h in the absence or presence of both oligosaccharides at a concentration of 500 μg/ml and then detached by Trypsin-EDTA (Invitrogen, NY) solution. Genomic DNA was isolated by Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China). DNA concentrations were determined using UV–vis spectrophotometer (Beckman, CA). Same amount samples were electrophoresed on a 1.5% agarose gel (Bio-Rad, CA) at 10 V/cm. Gel was visualized and photographed on a UV transilluminator (BioImaging Systems, UVP, CA).

HUVECs migration assay. To assess the migration ability of HUVECs after treatment with *N*-acetyl-COs, polystyrene transwell plates (24-well) (Corning Costar, MA) were used [21]. Cells were detached by trypsinization, and suspension at a density of 5×10^4 cells/insert was placed into the upper chamber in 100 μl of serum-free medium without or with oligosaccharides (125, 250, 500, and 1000 μg/ml concentration). SU5416 was used as positive control. Medium containing VEGF (20 ng/ml) was used as a chemoattractant. After 6 h of incubation, cells migrated to the lower surface of the filters were fixed in 90% ethanol and then stained with 0.1% crystal violet. Cells attached to the bottom side of the membrane were counted under the microscope. Cellular images were taken on the entire tissue culture dish using a stereo-microscope (Zeiss, Jena, Germany).

Morphogenesis assay: tube formation. To assess the effect of *N*-acetyl-COs on angiogenesis potential, tube formation assay was performed using Matrigel (Becton–Dickinson, MA). Fifty microliters per well of Matrigel (10 mg/ml) was added to a 96-well plate. The gel was overlaid with 1.5×10^4 HUVECs in the absence or presence of oligosaccharides (125, 250, 500, and 1000 μg/ml), SU5416 was used as positive control. Cells were incubated for 8 h [22]. Tube formation was observed and photographed using a stereo-microscope (Zeiss, Jena, Germany). Vascular index was quantified as the ratio of existing cell-free areas delimited by the network and the total surface of cell culture on fibrin gel at the onset of cell reorganization.

Zebrafish maintenance, embryo generation, and staging. Zebrafish strains were maintained as described in the zebrafish handbook [23]. Embryos were obtained from natural spawnings and staged according to morphology [24]. Zebrafish embryos were generated by natural pairwise mating, collected and maintained in distilled water at 27 °C for approximately 20 h until the 21 somite stage before sorting for viability, using both morphology and developmental stage as criteria.

Antiangiogensis activity using zebrafish embryos. Healthy embryos were dechorionated with 1 mg/ml protease (Sigma, MO). Oligosaccharides were added directly to the culture media at 24 hpf [25]. Treated embryos were maintained in 24-well plates (20 embryos/well) for additional 48 h. Vessel staining was performed as described previously [26] with slight modifications. Briefly, the 72 hpf embryos were fixed in 4% paraformaldehyde for 2 h, dehydrated by immersing in 25%, 50%, 75%, and 100% methanol in PBT, and rehydrated stepwise to 100% PBT. The embryos were then equilibrated in NTMT buffer (0.1 M Tris–HCl, pH 9.5; 50 mM $MgCl_2$; 0.1 M NaCl; 0.1% Tween 20). Three hundred seventy-five micrograms of NBT and 200 μg of X-phosphate were added per ml of NTMT. After staining, reaction was stopped by adding PBST. Embryos were then immersed in a solution of 5% formamide and 10% hydrogen peroxide in PBS. Embryos were then examined on a stereo-microscope (Zeiss, Jena, Germany).

Whole-embryo endogenous alkaline phosphatase (EAP) assay. EAP staining was performed as described previously with slight modifications [27]. Briefly, embryos were treated with ice-cold 70% ethanol for 10 min, dehydrated, and permeabilized in 100% ethanol for 30 min. The embryos were washed three times with diethanolamine buffer (Pierce, IL), and incubated in substrate containing 0.5 mg/ml *p*-nitrophenyl phosphate disodium salt (Pierce, IL) for 30 min at RT. Two molar NaOH was added to stop the reaction. The OD of soluble end product was measured at 405 nm using a microplate reader (Bio-Tek Instruments, VT). Vessel growth was presented as percentage of change in OD compared with control [% vessel growth = $(OD_{\text{treated day 3}} - OD_{\text{control day 1}})/(OD_{\text{control day 3}} - OD_{\text{control day 1}}) \times 100\%$].

Results

Properties of COs and N-acetyl-COs

COs (MW = 1454 Da, 5% *N*-acetylation) was prepared by chitosan using the enzymatic method. COs was *N*-acetylated with acetic anhydride in aqueous acetic acid to obtain *N*-acetyl-COs. As shown in Table 1, the *N*-acetyl content of COs was only 5%. After *N*-acetylation, the *N*-acetyl content increased to 81%. Of note, the MW of the *N*-acetyl-COs was increased to 1565 Da due to increased *N*-acetyl group. After the purification by Sephadex chromatography, the purity of the samples was above 95% as determined by HPLC.

Table 1
The property of COs and *N*-acetyl-COs

Oligosaccharides	Molecular weight (Da)	*N*-Acetyl content (%)	Purity
Chitooligosaccharide	1454	5	≧95
N-Acetylchitooligosaccharides	1565	81	≧95

N-Acetyl-COs inhibited proliferation of HUVECs

As angiogenesis involves local proliferation of endothelials, we initially investigated the effects of both oligosaccharides on the proliferation of HUVECs. MTT assay was performed to determine if *N*-acetyl-COs could cause inhibition of HUVECs. Our results showed that treatment of HUVECs with COs or *N*-acetyl-COs resulted in significant repression of HUVEC growth in a dose-dependent manner (Fig. 1A), with IC_{50} values of about 1000 μg/ml (COs) and 500 μg/ml (*N*-acetyl-COs), respectively. This result suggested that *N*-acetylation of COs could increase the activity of COs. In contrast, both of the two oligosaccharides do not affect the growth of fibroblasts (NIH3T3) *in vitro* at the same concentrations (Fig. 1A).

N-Acetyl-COs induced apoptosis of endothelial cells

Anti-proliferation and subsequent antiangiogenesis have been correlated with several underlying mechanisms, including induction of apoptosis. Accordingly, we used DNA fragmentation to investigate whether treatment with *N*-acetyl-COs induced endothelial cell apoptosis. Our results revealed that, compared with control (Fig. 1B, lane 1), HUVECs exposed to 500 μg/ml *N*-acetyl-COs for 48 h resulted in a clear induction of apoptosis (Fig. 1B, lane 3), while a slight induction of apoptosis was also observed when cells were treated with COs in the same concentration (Fig. 1B, lane 2).

N-Acetyl-COs repressed migration of HUVECs

As endothelial cell migration is a prerequisite for angiogenesis, we explored the effect of *N*-acetyl-COs on directional cell motility using a transwell system. As shown in Fig. 2AI, incubation of HUVECs for 8 h with non-treated cells resulted in large-scale migration of endothelial cells to the lower side of the filter. In contrast, treatment of HUVECs with 500 μg/ml of both COs and *N*-acetyl-COs resulted in a significant reduction of density of the migration cells (Fig. 2AII and III). With the increase of the concentration of the two oligosaccharides to 1000 μg/ml, the inhibition rate of *N*-acetyl-COs reached 89.74%, while the inhibition rate of COs was only 67.95% (Fig. 2B).

N-Acetyl-COs disrupted the capillary tube formation of HUVECs

As organization of endothelial cells into a network of tubes is a late event during angiogenesis, we used a Matrigel-induced tube formation assay to determine whether the *N*-acetyl-COs inhibited tubulegenesis. HUVECs were plated onto matrigel in 96-well plates. After incubation for 8 h, the morphology and network structure of the cells could be clearly found in the absence of oligosaccharides (Fig. 2C). However, when cells were treated with 500 μg/ml *N*-acetyl-COs, the capillary structure of HUVECs was significantly inhibited (Fig. 2CIII). Similar effect was found when HUVECs were exposed with COs (Fig. 2CII).

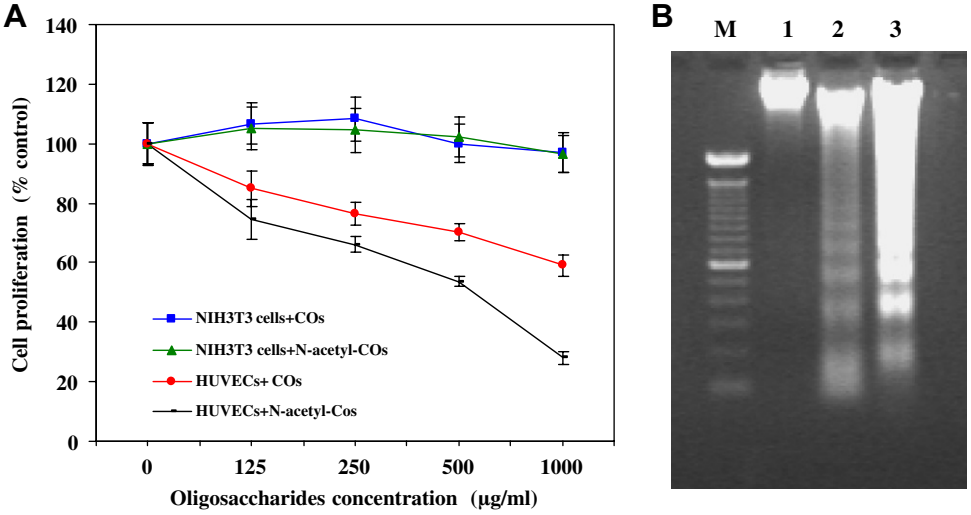

Fig. 1. (A) Influence of COs and *N*-acetyl-COs (0–1000 μg/ml) on NIH3T3 cells and HUVECs proliferation. The cells were incubated with oligosaccharide in basal medium for 48 h. Cell numbers were determined by MTT assay. Results are normalized to untreated cells. All experiments were repeated six or more times. (B) Treatment of HUVECs with COs or *N*-acetyl-COs induced DNA fragmentation. HUVECs were exposed to COs or *N*-acetyl-COs for 48 h, and the total DNA was isolated and analyzed in 1.5% agarose gel. (M) Hundred base pair marker, (1) native HUVECs intact DNA, (2) COs (500 μg/ml) treated, and (3) *N*-acetyl-COs (500 μg/ml) treated. Each treatment was repeated at least three times.

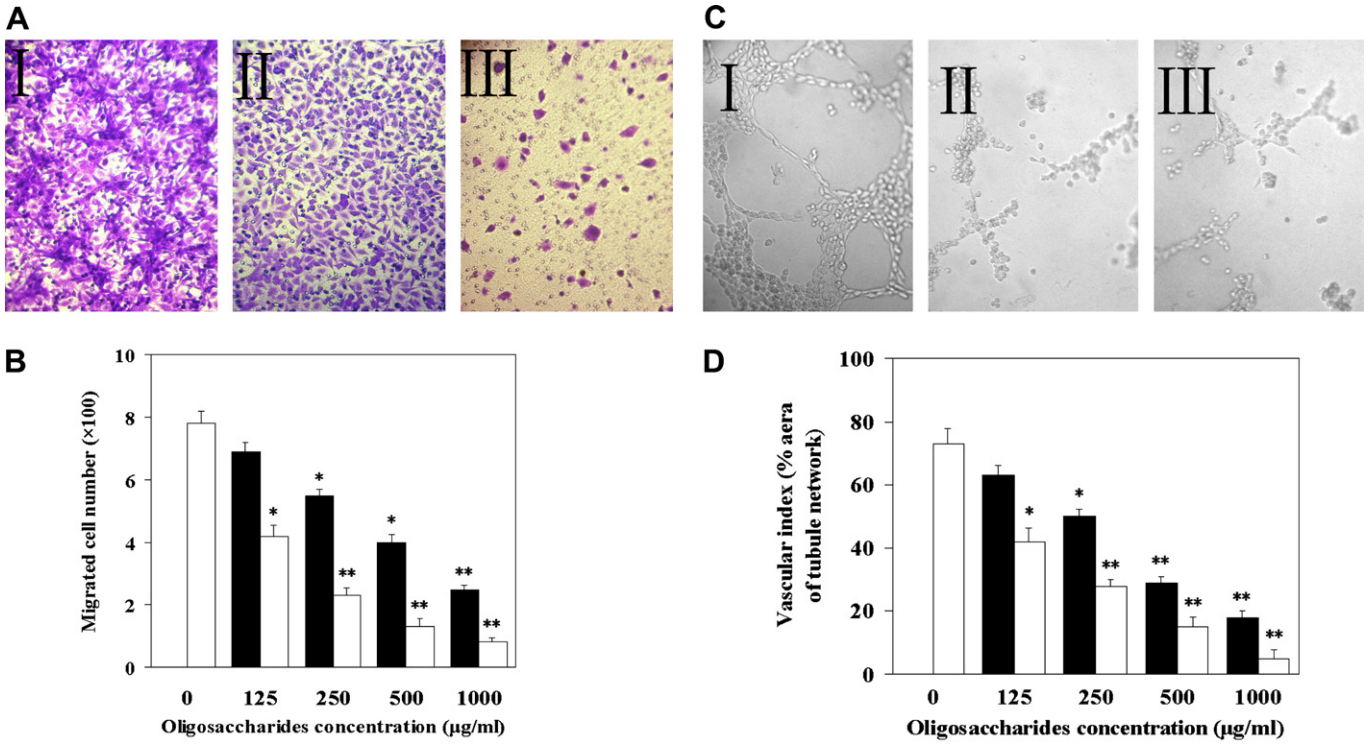

Fig. 2. (A) *N*-Acetyl-COs inhibited the migration of HUVECs. Migrated cells were observed using stereo-microscope with 100× magnification. (I) In the absence of oligosaccharides. (II) In the presence of 500 μg/ml COs. (III) Treated with *N*-acetyl-COs 500 μg/ml. (B) The dose-dependent effect of COs (■) and *N*-acetyl-COs (□) on the cell migration. The migrated cells were counted, and data are expressed as means of cell counts from replicate experiments. Each treatment was repeated at least three times. $^{*}p < 0.05$, $^{**}p < 0.01$. (C) Effect of the two oligosaccharides on endothelial cell tubulogenesis *in vitro*. HUVECs were seeded on the surface of the Matrigel of 96-well plate. After incubation for 8 h, the network structures of HUVECs were viewed by stereo-microscope. (I) In the absence of oligosaccharides. (II) Five hundred micrograms per liter COs was included in the incubation buffer. (III) Treated with *N*-acetyl-COs 500 μg/ml. (D) Quantification of the dose-dependent relation about different concentration of COs (■) and *N*-acetyl-COs (□) inhibited tube formation of HUVEC was shown. Each treatment was repeated at least three times. $^{*}p < 0.05$, $^{**}p < 0.01$.

Further study revealed that the repression of tube formation of HUVECs by COs or *N*-acetyl-COs displayed in a dose-dependent manner (Fig. 2D).

N-Acetyl-COs inhibited the angiogenesis of the zebrafish embryo in vivo

Embryos were exposed to different concentrations of the COs and *N*-acetyl-COs at 20 hpf and collected for staining at 72 hpf. Phenotypic changes were assessed under convert microscope. When the embryos were treated with 1000 μg/ml COs, the circulation of blood cells was slower compared with the normal embryos, while a complete lack of circulation with a beating heart was observed when the cells were treated with *N*-acetyl-COs at the same concentration.

As seen in Fig. 3A, both oligosaccharides blocked angiogenic and vasculogenic vessel formation in zebrafish embryos. Treatment with 500 μg/ml of *N*-acetyl-COs was sufficient to reduce SIV growth by nearly 62% (Fig. 3AIII and B). A dose-dependent effect was observed when the embryos were treated with *N*-acetyl-COs or COs (Fig. 3B).

To confirm the effects of the two oligosaccharides on the growth of the blood vessel in various organs, including the heart, brain, intestine, pancreas, cartilage, liver, and kidney, endogenous alkaline phosphatase (EAP) staining was performed. The results showed that both *N*-acetyl-COs or COs significantly repressed the growth of blood vessel in EAP staining assay (Fig. 3C).

Discussion

In the present report, *N*-acetyl-COs was prepared by *N*-acetylation of COs. Antiangiogenic study revealed that *N*-acetyl-COs inhibited the proliferation of HUVECs by inducing apoptosis. Exposure of HUVECs in *N*-acetyl-COs resulted in decreased migration and tubulogenesis. Further studies indicated that treatment of the zebrafish embryos with *N*-acetyl-COs resulted in a significant reduction in vessel formation when introduced to zebrafish embryos prior to the onset of angiogenesis. Compared with COs, the *N*-acetyl-COs showed more potent antiangiogenic activity both *in vitro* and *in vivo*.

Previous studies had shown that natural polysaccharide chitin and its derivatives had antitumor activities. Deploymerized products of chitosan, particularly the hexamer and heptamer, display notable antitumor activity against S180 solid tumors [6]. Further studies showed that COs possesses antiangiogenesis activity, causing inhibition of angi-

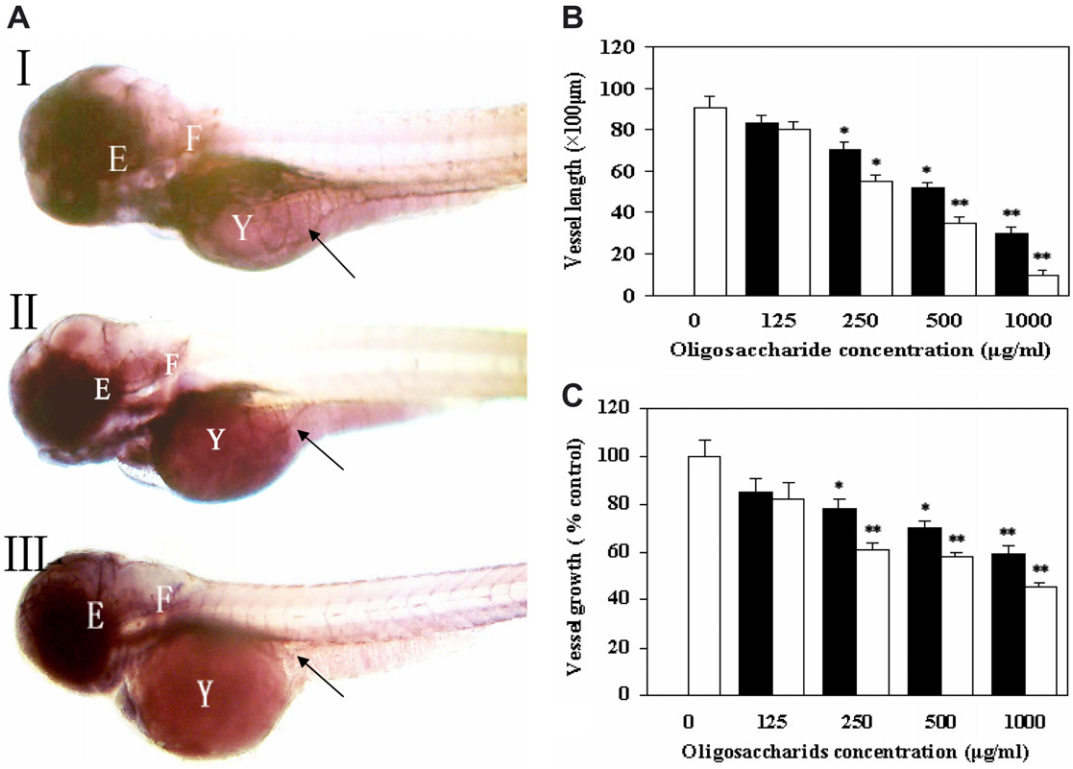

Fig. 3. Antiangiogenic activity of the two oligosaccharides using zebrafish embryo model. (A) Lateral view of alkaline phosphatase-stained embryos at 72 hpf. Zebrafish embryos were treated with COs 500 μg/ml (II), *N*-acetyl-COs 500 μg/ml (III), and without the sample (I). The SIVs of zebrafish embryos are indicated by arrow. (B) Quantification of the growth inhibition of COs (■) and *N*-acetyl-COs (□) on SIVs of zebrafish embryos. After alkaline phosphatase staining, the total SIV vessel length was determined by point-to-point measurement. Each histogram represents the mean of 3–4 separate experiments. The eye (E), yolk (Y), and fin (F) are labeled for orientation. $^{*}p < 0.05$, $^{**}p < 0.01$. (C) The dose-dependent effect of the two oligosaccharides on the growth of blood vessel of whole-embryos. Zebrafish embryos were incubated without or with COs (■) and *N*-acetyl-COs (□) (0–1000 μg/ml) separately. Soluble EAP substrate was analyzed using a quantitative microplate reader. Three to four experiments were performed for each concentration. $^{*}p < 0.05$, $^{**}p < 0.01$.

ogenesis in CAM model, as well as EAC bearing mice [13]. In the present report, *N*-acetyl content was increased to 81% by *N*-acetylation of COs. Results showed that the newly prepared oligosaccharide, *N*-acetyl-COs, displayed more powerful antiangiogenic activity than COs *in vitro* and *in vivo*. Our results suggested that the *N*-acetyl group may be in part responsible for the antiagiogenic activity of the COs or *N*-acetyl-COs.

To confirm the specificity of *N*-acetyl-COs on antiangiogenic activity, we determined the cytotoxicity of the oligosaccharide on other cell lines. Our results showed that no cytotoxicity was found when exposing NIH3T3 on *N*-acetyl-COs. In addition, the oligosaccharide did not affect the proliferation of human breast cancer MCF-7 cells, human hepatoma cancer BEL-7402 cells, as well as human colon cancer HCT-18 cells (data not shown). Recent study [13] suggested that COs did not display any cytotoxicity to several cancer cell lines, including HeLa, Hep3B, and SW480. However, highly charged COs derivatives, including positively charged quaternized amino COs and negatively charged carboxylated COs, displayed potent cytotoxicity by inducing necrosis. Further study is needed to address how the different modified COs affects the viability of cancer cells. Neovascularization is a complex process characterized by penetration of basement membrane by capillary EC, migration of cells through matrix towards a stimulus, and subsequent proliferation [28]. Various growth factors, including FGF [29], VEGF [30], and [31], were included in the process of tube formation. Study has shown that angiogenic inhibitors exert their activity by interfering in different process of neovascularization, including targeting the matrix mellaproteinase; interfering with the VEGF signaling pathway or repressing the process of proliferation of endothelial cells. Our present study revealed that both COs and *N*-acetyl-COs could repress the migration and tube formation of HUVECs and *N*-acetyl-COs showed stronger antiangiogenic activity. Study is ongoing in our laboratory to investigate the cellular mechanism(s) of the oligosaccharides that mediate these antiangiogenesis effects.

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