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The Edible Adlay Seed Oil Containing Cinnamaldehyde and Gingerol Prevents DMBA/TPA-Induced Skin Carcinogenesis by Cooperative Ablation of Lipid Rafts

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Abstract

It is known that food intake was associated with decreased risk of cancer, we prepared a compounded adlay seed oil (C-ASO) containing 0.5% cinnamaldehyde and 1% 6-gingerol and evaluated the preventive effects of the oral C-ASO on DMBA/TPA-induced skin carcinogenesis. In contrast to untreated carcinogenic mice, skin tumor incidence and multiplicity were decreased to some extent in single ASO-, cinnamaldehyde- and gingerol-treated mice, whereas the oral C-ASO almost completely reduced susceptibility to skin carcinogenesis by cooperative ablation of enhanced lipid rafts, drastically suppressed phosphatidylinositol 3-Kinases (PI3K)/protein kinases A (Akt) signaling and decreased levels of nuclear transcription factor-kappa B (NF-κB) and cyclin D1 which reduced skin epithelial proliferation and epithelial-mesenchymal transition (EMT) during tumor genesis. This work supports C-ASO as a candidate functional food to prevent carcinogenesis.

Keywords: Adlay seed oil, Cinnamaldehyde, Gingerol, Skin carcinogenesis, Lipid rafts

Introduction

Despite improvements in screening and care, cancer has already become a leading cause of death worldwide [1]. In cancer cells, lipid rafts are viewed as important linchpins, from which signals essential for invasive growth, resistance to death-inducing stimuli and other malignant characteristics are launched [2,3]. Lipid rafts are specialized membrane microdomains of the plasma membrane that is enriched in sphingolipid and cholesterol [4]. Previous study demonstrated that lipid raft disruption results in their internalization dependent on cholesterol [5]. There are quantitative and qualitative raft changes in many cancers due to abnormal accumulation of cholesterol in malignant cells [6], indicating involvement of cholesterol-enriched lipid rafts in initiation and progression of human cancers. Chemoprevention represents the most effective cancer treatment [7], and an alternative strategy to prevent tumor cell is to combine edible composition with health care therapy [8]. Adlay seed has long been used in Asian countries for the treatment of rheumatism, neuralgia, and lung diseases and as a diuretic [9]. The adlay seed oil (ASO) has been shown to reduce blood lipids and have antineoplastic effect [10-12]. Cinnamaldehyde, a major active components of cinnamon which is a spice widely used in cooking since biblical times, has been reported to have anti-obesity and antineoplastic effect [13-15]. 6-gingerol, a major component of fresh ginger which is a commonly used herb and food-flavoring agent, has been reported to inhibit adipogenesis and lipid content accumulation and have also antineoplastic effect [16-18]. Therefore, it is possible that the composition containing three materials might cooperatively ablate lipid rafts to prevent carcinogenesis. To investigate if this hypothesis is correct, we prepared a compounded adlay seed oil (C-ASO) containing 0.5% cinnamaldehyde and 1% 6-gingerol and evaluated the preventive effects of the oral C-ASO on 9, 10-dimethylbenzanthracene (DMBA)/12-O-tetradecanolyphorbal-13-acetate (TPA)-induced skin carcinogenesis.

Materials and Methods

Materials

Adlay seed oil (ASO) was obtained from Guangzhou Masson Pharmaceutical Ltd. (Guangzhou, China). 12-O-tetradecanolyphorbal-13-acetate (TPA) and 9, 10-dimethylbenzanthracene (DMBA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Cinnamaldehyde and 6-Gingerol (purity >98%) were purchased from Xi-an Helin Biological Engineering Co. (Xi-an, Shanxi, China). Evans blue was purchased from Sigma Chemical Co. (Shanghai, China). The antibodies used include: proliferating cell nuclear antigen (PCNA), Phosphatidylinositol 3-Kinases (PI3K), protein kinases A (Akt), nuclear transcription factor-kappa B (NF-κB), cyclin D1, E-cadherin, N-cadherin and β-Actin were obtained from BD Pharmingen. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody, Peroxidase substrate 3′,3′-diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC) were from Nichirei Bioscience (Tokyo, Japan). The mouse quantitative ELISA kits (Tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6), High-sensitivity CRP (hs-CRP), reactive oxygen species (ROS), 8-hydroxy-2-deoxyguanosine (8-OHdG)) were obtained from R&D Systems. Cholesterol kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Filipin and Alexa 594-cholera toxin B (CTXB) were obtained from Molecular Probes. Standard rodent chow was purchased from Henan Provincial Medical Laboratory Animal Center (Zhengzhou, China). All the other reagents used were the highest purity and commercially available.

Animals

Cohorts of 5- to 6-wk old female ICR mice were obtained from Henan Provincial Medical Laboratory Animal Center. All mice were

housed in individual ventilated cages under a 12 h light-dark cycles (lights on 7:00 AM to 7:00 PM). The animals were fed standard rodent chow and water. All animal procedures in this study were approved by the Animal Experimentation Ethics Committee of Henan University (the permission number HD20141016), all procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals and the regulation of animal protection committee to minimize the suffering and injury. The animals were monitored daily and euthanized humanely by overdose carbon dioxide at the end of the experiment or the first sign of shortness of breath, reduced locomotion and reduced body weight (greater than 20% total body weight). All surgery was performed under general anesthesia by intraperitoneal injection of 45 mg/kg pentobarbital sodium, and all efforts were made to minimize suffering.

DMBA/TPA-induced skin cancer model

The dorsal skin on the back area of the ICR female mice was shaved 1 day before the commencement of the experiment. The mice were randomly divided into six groups (Normal; DMBA/TPA (control); DMBA/TPA+ASO (5 ml/kg body weight); DMBA/TPA+6-Gingerol (50 mg/kg body weight); DMBA/TPA+Cinnamaldehyde (25 mg/kg body weight); DMBA/TPA+C-ASO (5 ml/kg body weight, containing 0.5% cinnamaldehyde and 1% 6-gingerol), each group included twenty mice. The mice except normal were treated on their shaven backs with topical application of DMBA (150 μg) dissolved in 0.2 mL of acetone once weekly for three times, and the animals in the normal group were treated only with vehicle (0.2 mL acetone). One week after initiation with DMBA, the mice except normal were treated topically with TPA (5 μg) twice weekly for 10 weeks. Following the first DMBA application, mice were placed on the standard rodent diet and received once daily oral tested compounds for 20 weeks. During the studies, food and water were provided ad libitum, the health of the mice was monitored daily, and body weights were measured weekly. Papillomas appearing on the skin were recorded every week during the experimental period, with only those having diameter >1 mm considered as positive. Mice were sacrificed 21 weeks after the first DMBA applications under anesthesia with pentobarbital sodium (45 mg/kg), skin carcinoma multiplicity (average carcinomas per mouse) were determined, and skins were biochemically processed.

Clinical chemistry

Blood samples were collected from the orbital venous plexus at sacrifice after 6 h of fasting for chemical analyses. The serum concentrations of cholesterol, IL-6, hs-CRP and TNF-α were determined according to the manufacturer's protocol.

Oxidative stress analysis

Serum 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were measured using an ELISA assay kit. Serum levels of ROS, a marker for oxidative stress, were also determined using an ELISA assay kit.

Immunohistochemistry

For immunohistochemical (IHC) analyses, serial sections (5 μm) of skin were cut and attached to superfrost plus microscope slides. The slides were warmed at 60°C for 1 h and deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. After blocking with 3% hydrogen peroxide for 10 min, the sections were incubated with 0.1% TritonX-100 in phosphate-buffered saline (PBS) for 20 min at room temperature and next immersed in a blocking solution

of 5% bovine serum albumin (BSA) for 20 min at room temperature. The sections were incubated with primary antibodies (1:100) against PCNA, E-cadherin and N-cadherin overnight at 4°C. Antigen–antibody binding was detected by using Dako Cytomation labeled streptavidin biotin (LSAB) System-HRP kit. The signal was developed with the peroxidase substrate DAB which appears as a brown reaction product or with AEC which appears as a red reaction product. All sections were counterstained with hematoxylin and were imaged under a microscope. A semi-quantitative histological score (HSCORE) was used as described previously.

Lipid raft detection

Skin single cell suspensions of were prepared by collagenase IV digestion. The cells were attached to superfrost plus microscope slides and were fixed with 3.7% paraformaldehyde in PBS at 4°C for 20 min, rinsed with PBS, and treated with 1.5 mg/ml glycine in PBS to quench free aldehyde group. Cells were then stained with filipin (0.05 mg/ml) and Alexa 594-CTXB (0.04 μg/ml) for plasma membrane cholesterol and monosialotetrahexosylganglioside (GM1) staining, respectively. Cells were visualized using confocal imaging with a ZEISS LSM 510 Meta/UV.

Western blot analysis

Skin extracts were prepared in immunoprecipitation cell lysis buffer and an equal amount of protein was separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electroblotted on nitrocellulose membranes and probed with specific antibodies against PI3K, Akt, NF-κB, cyclin D1 and GAPDH. Antibody binding was detected by enhanced pico chemiluminescence (Pierce, Rockford, IL).

Statistical analyses

The measures are presented as mean ± SD and were statistically analyzed using the GraphPad Prism software program, Version 5.0 (San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to compare groups by the Mann-Whitney U test. The differences were considered significant when the two-sided P value was less than 0.05.

Results

C-ASO almost completely reduced susceptibility to skin carcinogenesis

Natural phytochemicals derived from dietary sources have gained significant recognition in the potential management of several human clinical conditions [19], and the combination of dietary phytochemicals are considered more promising for higher efficacy [20]. In this study, a two-stage skin carcinogenesis experiment was conducted. One week after initiation with DMBA, the mice were treated topically with TPA for 10 weeks. Following the first DMBA application, mice received once daily oral tested compounds for 20 weeks. Tumor incidence and multiplicity were calculated after mice were sacrificed at 21 weeks. Under these experimental conditions, oral treatment of ASO, cinnamaldehyde and gingerol alone or combined had no effect on body weight, whereas C-ASO led to lean body phenotypes (<fifteen percent of normal body weight) (Figure 1A). In contrast to untreated carcinogenic mice, skin tumor incidence and multiplicity were decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol-treated mice. However, C-ASO almost completely reduced susceptibility to skin carcinogenesis (Figure 1B and C).
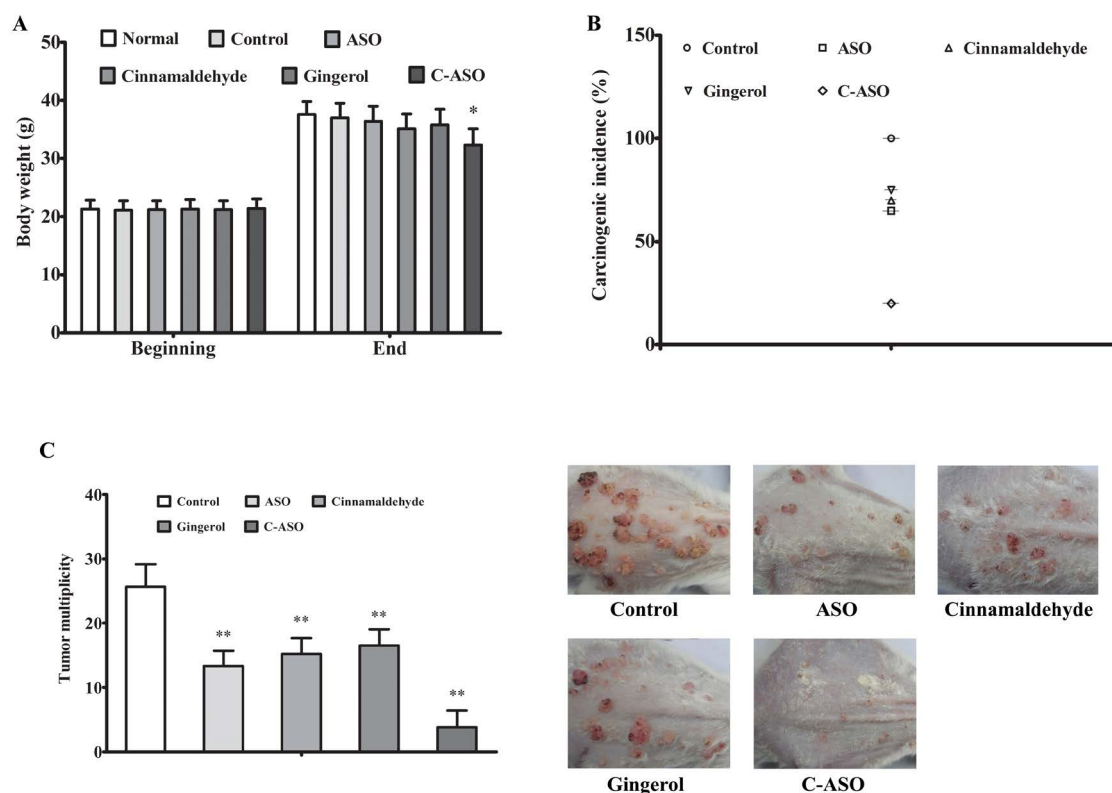
Figure 1: C-ASO synergistically prevented DMBA/TPA-induced skin carcinogenesis. The mice were treated on their shaven backs with topical application of DMBA once weekly for three times, one week after initiation with DMBA, the mice were treated topically with TPA twice weekly for 10 weeks. Following the first DMBA application, mice received once daily oral tested compounds for 20 weeks. ASO, cinnamaldehyde and gingerol alone or combined had no effect on body weight, whereas C-ASO led to lean body phenotypes (A) (n=20). Skin carcinoma incidence (B) and multiplicity (C) were decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol-treated mice, and C-ASO almost completely reduced susceptibility to skin carcinogenesis (n=20). The data present Mean ± SD and statistical significance was determined by the Mann-Whitney U test. *P<0.05, **P<0.01 *vs.* control.

C-ASO synergistically decreased inflammation and oxidative stress during carcinogenesis

To further verify above results, we carried out clinical chemistry test and oxidative stress analysis at 21 weeks. Consistent with above results, serum levels of IL-6, hs-CRP and TNF-α were decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol-treated mice but were very significantly suppressed by C-ASO compared to untreated carcinogenic mice (Figure 2A-C). Similarly, serum levels of 8-OHdG and ROS were only decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol-treated mice but were very significantly suppressed by C-ASO (Figure 2D and E).

C-ASO synergistically decreased skin cell lipid rafts and suppressed cellular signaling during carcinogenesis

To explore why C-ASO synergistically reduced susceptibility to skin carcinogenesis, we examined DMBA/TPA-associated changes in serm cholesterol, cell membrane lipid rafts by plasma membrane cholesterol and GM1 staining, and cellular signaling such as PI3K, Akt, NF-κB and cyclin D1 by western blot analyses at 21 weeks. As shown in Figure 3A, serum levels of IL-6 were decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol-treated mice but were very significantly suppressed by C-ASO compared to untreated carcinogenic mice. Consistently, the skin cell lipid rafts were decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol-treated mice but were very significantly suppressed by C-ASO

compared to untreated carcinogenic mice (Figure 3B and C). Similarly, the skin protein expression of PI3K, Akt, NF-κB and cyclin D1 was only decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol -treated mice but were very significantly suppressed by C-ASO (Figure 3D).

C-ASO synergistically decreased skin epithelial proliferation and epithelial-mesenchymal transition (EMT)

Carcinogenesis is the final results of epithelial proliferation and EMT [21]. To verify the synergistical effect of C-ASO on cancer prevention, we also examined DMBA/TPA-associated changes in skin epithelial proliferation and EMT by immunohistochemistry at 21 weeks. Consistent with above results, PCNA, a skin epidermal proliferation marker, were decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol-treated mice but were very significantly suppressed by C-ASO compared to untreated carcinogenic mice (Figure 4A and B). Similarly, lung EMT indicated by E-cadherin and N-cadherin were only decreased to some extent in single ASO-treated, cinnamaldehyde-treated and gingerol-treated mice but were very significantly suppressed by C-ASO (Figure 4A and B).

Discussion

The typical foods and spices containing various phytochemicals have been used for prevention and treatment of a variety of human ailments since time immemorial [22], there has been growing interest
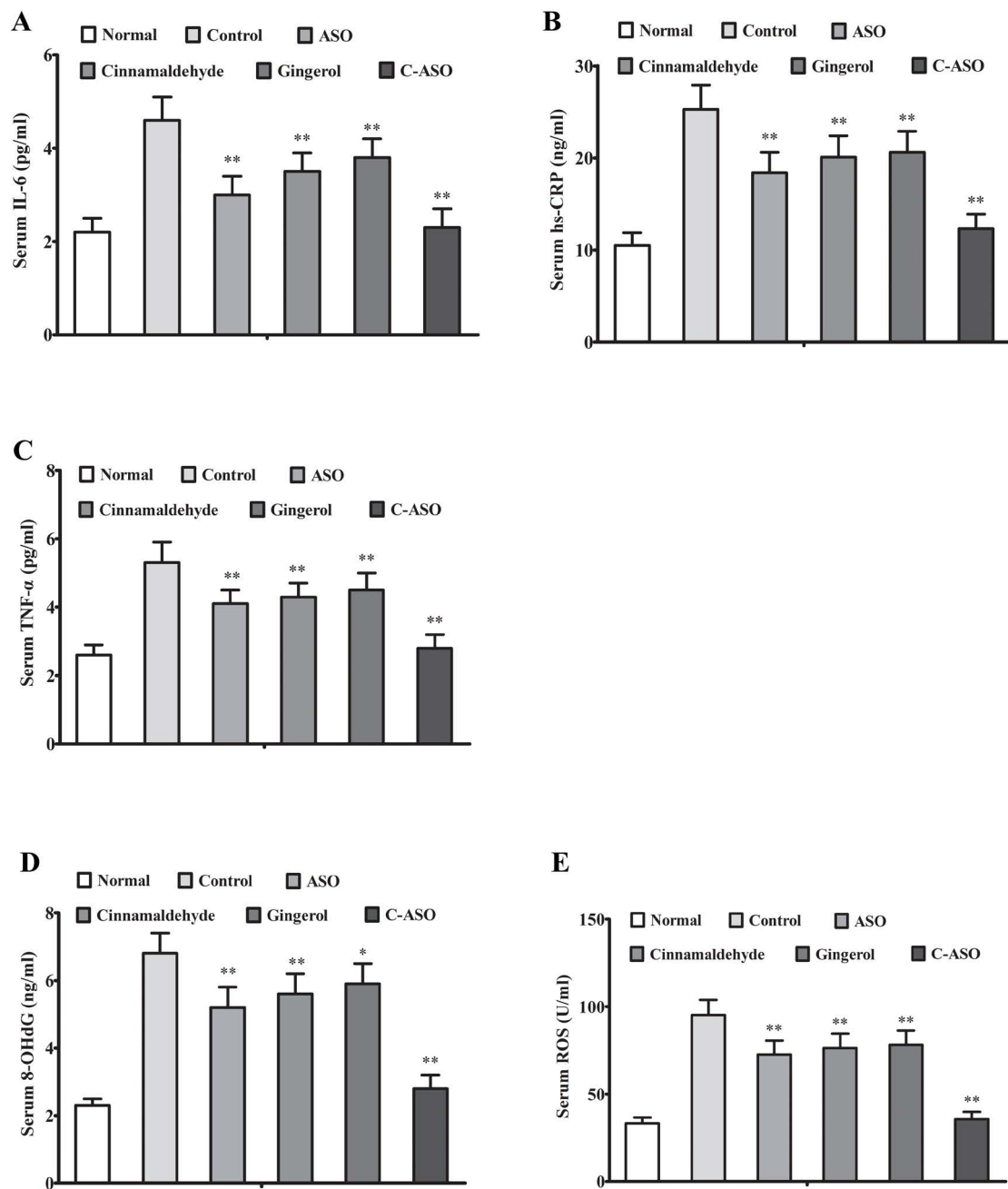
Figure 2: C-ASO synergistically decreased inflammation and oxidative stress during carcinogenesis. C-ASO synergistically decreased inflammation indicated by increased serum levels of IL-6 (A), hs-CRP (B), TNF-α (C) (n=5). C-ASO synergistically decreased oxidative stress indicated by increased serum levels of 8-OHdG (D) and ROS (E) (n=5). The data present Mean ± SD and statistical significance was determined by the Mann-Whitney U test. *P<0.05, **P<0.01 *vs.* control.

in cancer prevention by food plants and their products in recent years [23]. Adlay seed is a kind of nourishing food which has also been used in traditional Chinese medicine [24], and ASO has been proved to reduce the abdominal fat tissue and increase the total antioxidant capacity in hyper-lipidemic rats [25]. In addition, a pharmaceutical grade extract of adlay seed is currently the most commonly used treatment for cancer in China [26]. Although clinical data also support the use of coix seed and its extracts for cancer treatment [27], a single agent based treatments coax seed and its extracts may not always be sufficient to provide chemo preventive efficacy, therefore, the new concept of combination

chemoprevention by multiple agents has become an increasingly attractive area of study. In this study, we showed that C-ASO could reduce susceptibility to skin carcinogenesis with higher efficacy.

Targeting malfunctioning molecules along the disrupted signal transduction pathway in cancer represent a rational strategy in chemoprevention [28]. A wide number of signal transduction processes related to cell adhesion, migration, as well as to cell survival and proliferation, which play major roles in cancer development and progression, are dependent on lipid rafts [29,30]. In this study, we
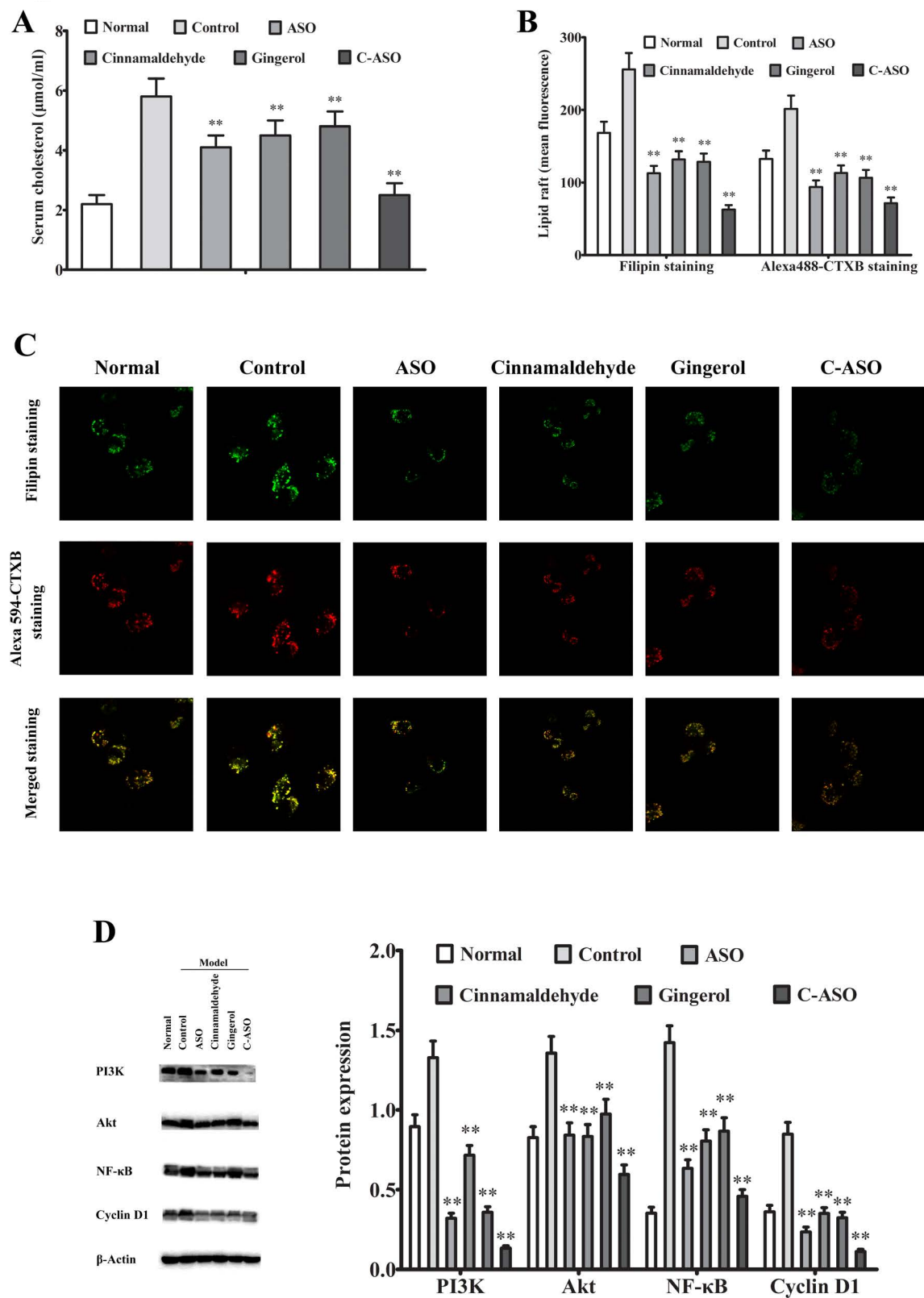
Figure 3: C-ASO synergistically decreased skin cell lipid rafts and suppressed cellular signaling during carcinogenesis. C-ASO synergistically decreased serum cholesterol (A) and skin cell lipid rafts (B and C), and suppressed protein expression of PI3K, Akt, NF-κB and cyclin D1 examined by western blot (D). The data present Mean ± SD of triplicate assays and statistical significance was determined by the Mann-Whitney U test. *P<0.05, **P<0.01 *vs.* control.
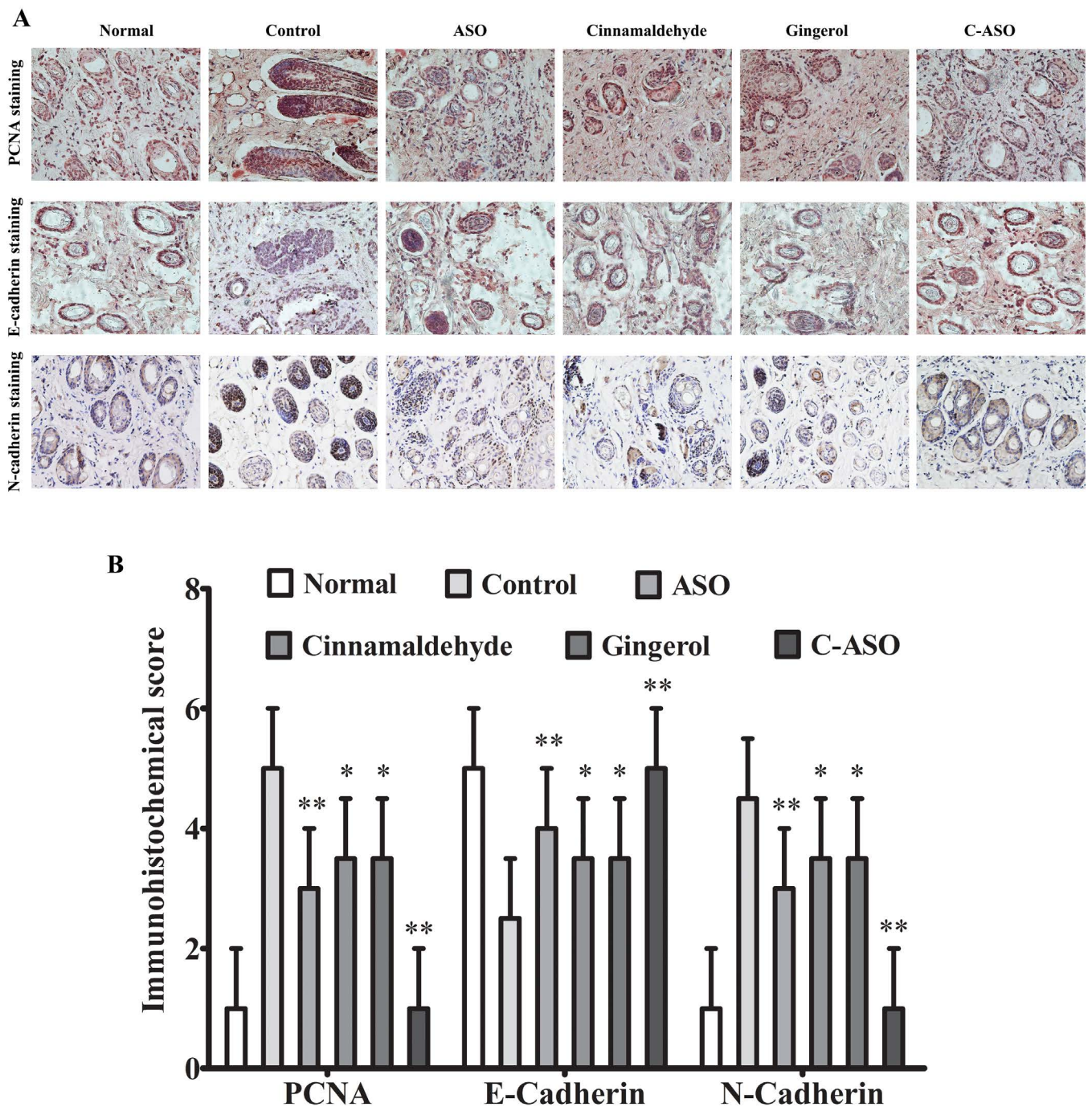
Figure 4: C-ASO synergistically prevented skin epithelial proliferation and EMT during carcinogenesis examined by immunohistochemistry. (A) C-ASO synergistically prevented skin epithelial proliferation indicated by increased PCNA and EMT indicated by decreased E-cadherin and increased N-cadherin. (B) The semi-quantitative histological score. Images were taken at ×40 magnification. The data present Mean ± SD of triplicate assays and statistical significance was determined by the Mann-Whitney U test. *P<0.05, **P<0.01 *vs.* control.

firstly supposed that the combination of dietary lipid-reducing agents could target lipid rafts to prevent cell signaling cascades, we found that C-ASO synergistically decreased inflammation and oxidative stress during carcinogenesis, indicating a possible endeavor in cancer chemoprevention because chronic inflammation is associated with increased risk of cancer. Next, we showed that C-ASO synergistically decreased skin cell rafts and suppressed PI3K/Akt, NF-κB and cyclin D1 signaling during carcinogenesis, indicating a lipid raft disruption and an abortion of cell signaling cascades. Finally, we showed that C-ASO synergistically decreased skin epithelial proliferation and EMT during carcinogenesis, indicating the final result induced by C-ASO.

Early interruption of the carcinogenic process is a priority strategy to reduce the burden of cancer; however, the intervention may be

ineffective if pre-neoplastic lesions with cellular changes cannot be reversed [31]. Recent research efforts have been greatly emphasized on the recognition of naturally occurring plant derived substances that are capable of inhibiting, retarding or reversing the development of cancer [32], and comparative effectiveness research designs and the value of information obtained from large-scale prevention studies are necessary in order for preventive interventions to become a routine part of cancer management [33]. Our results opened up a new avenue for cancer chemoprevention and suggested that C-ASO is promising and inexpensive in this endeavor.

Acknowledgments

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