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Original Article

Coptis chinensis inhibits growth and metastasis and induces cell apoptosis in non-small cell lung cancer cells

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Abstract: Objective: Coptis chinensis (COP), also known as Huang Lian, is a common herb used in Traditional Chinese Medicine. Little is known about its biological functions and mechanisms in cancer cells. This study aimed to evaluate the anti-tumor effects and biological mechanisms of COP in non-small cell lung cancer (NSCLC) cells. Methods: The quality of COP was determined by high performance liquid chromatography (HPLC). A549 and H1299 human NSCLC cells were used to explore the effect of COP *in vitro*. Effect of COP on cell proliferation, metastasis and apoptosis was measured by the MTT, cell invasion and Annexin V-conjugated FITC assay, respectively. Effect of COP on genes expression in NSCLC cells was determined by PrimeView Human Gene Expression Array. The *in vivo* anti-tumor effect of COP was evaluated using the tumor xenograft model. Results: The results showed that the components of the three batches of COP granules are identical. COP exhibited remarkable cytotoxic activities against the A549 and H1299 cells by inhibiting cell proliferation and clone formation. We also found that COP inhibited NSCLC cell migration and invasion ability. In addition, COP induced cell apoptosis and regulated the gene expression of cell growth, death, replication and repair of NSCLC cells. Moreover, experimental results on C57 mice showed that orally administration of COP could inhibit tumor growth without obvious toxicity. Conclusions: These results indicate that COP inhibits the growth and metastasis and induces cell apoptosis in NSCLC cells, suggesting that COP is a potential anti-tumor candidate in NSCLC.

Keywords: Non-small cell lung cancer, coptis chinensis, metastasis, apoptosis

Introduction

Lung cancer has become the most common cause of cancer-related mortality in the world population [1]. Non-small cell lung cancer (NSCLC) accounting for 80%, is the most common pathological pattern of lung cancer [2]. In recent years, although the diagnosis and treatment of lung cancer have been greatly improved, the survival rate of lung cancer still remains low [3]. Chemotherapy and radiotherapy remain as the main therapeutic methods for NSCLC [4, 5], but the efficacy are less than satisfaction. Natural products have been proved as an effective source of anticancer agents. It is up to 30-40% of the antitumor agents are derived from natural plant source [6]. The investigation of medicinal plants continues to hold promise for the prevention and treatment of cancer.

The dried rhizome of coptis chinensis (COP), also known as Huang Lian, is a popular herb used in Traditional Chinese Medicine (TCM). Due to its relaxant, pyretic, anti-diabetic, antiviral and antibacterial activity, COP has been used in the treatment of various diseases, such as carbuncles, cardiovascular diseases, ulcers, gastro enteric disorders, dysentery and diabetes [7]. In recent years, a number of studies

have reported the pharmacological properties of COP, including anti-oxidant, anti-inflammatory activity and neuroprotective effect [8, 9]. In addition, Ye et al. reported that the aqueous extract of COP can protect against carbon tetrachloride induced liver injuries [10]. Moreover, it was indicated that COP can activate MOLT-4 cell to Th1 cell and activate the Mitogen-activated protein kinase (MAPKs) signaling pathways [11]. Recent study revealed that component of COP extract possesses anticancer activities, as indicated by its ability to suppress cell growth and induce cell apoptosis in cancer cells [12]. All above studies indicate that COP displays positive effect on various diseases and shows potent anti-tumor activities. However, COP's role as an anti-cancer agent has not been fully established, and the anti-tumor effect of COP on non-small cell lung cancer cell still remains unclear.

Therefore, to investigate the potential anti-tumor effect of the COP on NSCLC, we first examined the quality of different batches of COP granules by HPLC. Then we examined the role of COP on the cell proliferation, metastasis and apoptosis in human A549 and H1299 cells. We also explored the effect of COP on gene expression of A549 cells. We further investigated the inhibitory action on tumor growth using tumor xenograft mice.

Materials and methods

Experiment reagents

COP granules were purchased from Tianjiang Pharmaceutical Ltd. (Jiangyin, China). The granules were dissolved in phosphate buffer saline (1 × PBS) at 80°C for 30 minutes, centrifuged at 1500 rpm for 5 minutes and then passed through 0.22 μm filter. Epiberberine (catalog number: 140621) and coptisine (catalog number: 140423) were purchased from Shengli Biological Technology Company (Sichuan Province, Chengdu, China) and acted as controls for COP granules. Palmatine chloride (catalog number: 110723-201510) and berberine hydrochloride (catalog number: 110713-201212) were purchased from National Institutes for Food and Drug Control and also served as controls for COP granules. RPMI1640 and Dulbecco's modified Eagle medium (DMEM) for cell culture were purchased from Gibco (Grand Irsland, NY, USA). Fetal bovine serum (FBS) was obtained from HyClone (South Logan, UT, USA) and trypsin was bought from Gibco (Grand Irsland, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Matrigel was purchased from BD Biosciences (San Jose, CA, USA). Propidium iodide (PI) and FITC-Annexin V were both purchased from BD Biosciences (San Jose, CA, USA). Other chemical agents were purchased from Guoyao Chemical Reagent Co., Ltd.

HPLC analysis of COP granules

The quality of COP granule was determined by high performance liquid chromatography (HPLC). Four components of COP including epiberberine, coptisine, palmatine chloride and berberine hydrochloride were prepared in methanol, respectively and served as the internal standard control. Different batches of COP were prepared in methanol-hydrochloric acid (100:1). Following filtration, 10 μl of standard control and COP solution were injected onto C18 column (kromasil, 250 mm × 4.6 mm, 5 μm). The mobile phase was composed of acetonitrile and 0.05 mol/L potassium dihydrogen phosphate solution (50:50), pH 4.0. The detection wavelength was set at 345 nm. The content of the main components of COP were calculated by comparing their peak areas to those of standard controls.

Cell culture

Lewis lung carcinoma (LLC) cells and human NSCLC cell lines A549 and H1299, were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured with RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL) at 37°C in a humidified incubator with 5% CO_2.

MTT assay

The effect of COP on cell proliferation was determined by MTT assay as previously described [13]. Briefly, A549 or H1299 cells (3×10^3 cells/well) were seeded in 96-well plates and allowed to attach overnight. COP was added to wells at various concentrations (0.16, 0.31, 0.62, 1.2, 2.5, and 5 mg/ml). After incu-

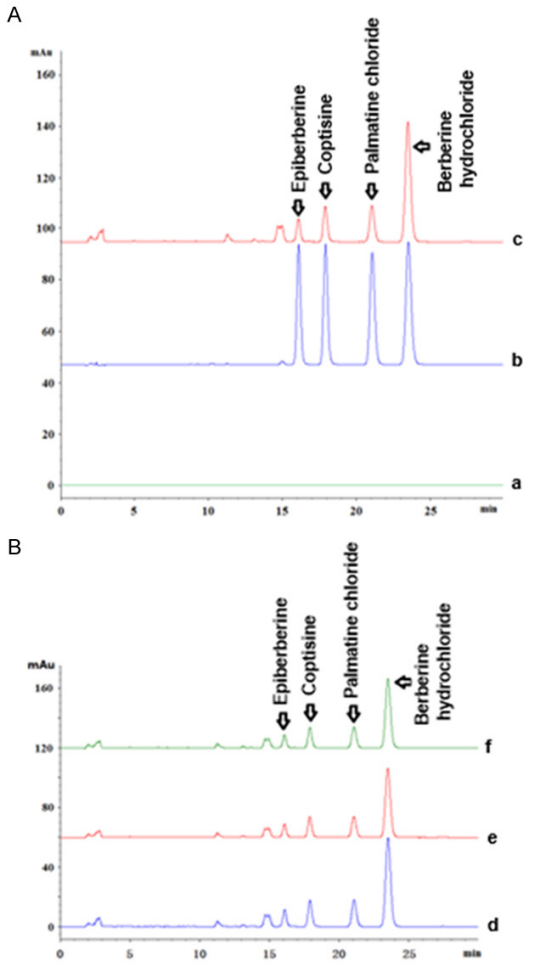

Figure 1. HPLC profile of three batches of COP granules. A. The chemical fingerprint of COP granules batch #2 was measured by HPLC analysis. a: blank; b: standard control; c: batch #2 COP granules. B. The chemical fingerprint of COP granules batch #1, #2 and #3. d: batch #1; e: batch #2; f: batch #3.

bation for 24, 48 and 72 h, respectively, 10 μL of the MTT solution was added to each well at a concentration of 5 mg/mL and incubated for 4 h. The culture medium was aspirated and 150 μL of Dimethyl sulfoxide (DMSO) was added to each well. Optical density (OD) value was determined at 570 nm. Cell viability was calculated using the following formula: cell viability (%) = $OD_{570\ nm}$ in cells treated with COP/ $OD_{570\ nm}$ in control cells × 100%. The IC_{50} value is defined as the concentration of drug required to inhibit cell growth by 50% when compared to control cells. All assays were performed in triplicate with at least 3 independent experiments.

Clone formation assay

A549 and H1299 cells were plated in 6-well plates at a density of 500 cells/well. On the following day, cells were treated with COP (0.08, 0.16, 0.31 and 0.62 mg/ml) for 24 h. After an additional 10 days, colonies were fixed with crystal violet before counting colonies > 50 cells.

Wound-healing assay

A549 or H1299 cells (6 × 10^5 cells per well) were seeded on 6-well plates. Sixteen hours later, the cells were wounded with a sterile 200 μl pipette tip to remove cells and were treated with COP (A549 cells: 0.16 and 0.31 mg/ml; H1299 cells: 0.08 and 0.16 mg/ml) for 24 h. The progress of migration was photographed (after identification of each wounded zone) in six regions, immediately and 24 hours after wounding.

Cell invasion assay

To determine the effect of COP on NSCLC cell invasion, transwell chamber with 24 wells was used and 40 μl matrigel (BD Biosciences) was inserted in each chamber. NSCLC cells were treated with various concentrations of COP (A549 cells: 0.16 and 0.31 mg/ml; H1299 cells: 0.08 and 0.16 mg/ml) for 24 h. Then 2 × 10^4 A549 or H1299 cells in 200 ml medium with 5% FBS were seeded into each chamber. Culture medium (750 μl) with 20% FBS was injected into lower chamber and maintained in 37°C, 5% CO_2 incubator. After 24 h, cells without invasion were erased by cotton balls. The invading cells were fixed with 95% ethanol and stained with 1% crystal violet. Photographs of the stained cells were taken with microscope (× 10) and 10 visions were selected randomly.

Detection of apoptosis

Annexin V-conjugated FITC was used to determine the effect of COP on apoptosis of NSCLC cells. A549 and H1299 cells were incubated with various concentrations of COP (A549 cells: 0.16, 0.31 and 0.62 mg/ml; H1299 cells: 0.08, 0.16 and 0.31 mg/ml) for 24 h. Cells were harvested, stained with Annexin V and PI, and analyzed on facscalibur cytometer (BD Biosciences).

Table 1. Main components of three different batches of COP granules

No.	Epiberberine (%)	Coptisine (%)	Palmatine chloride (%)	Berberine hydrochloride (%)
#1	1.58	2.23	2.15	8.1
#2	1.56	2.18	2.14	8.06
#3	1.58	2.2	2.15	8.1

PrimeView human gene expression array

PrimeView Human Gene Expression Array was used to determine the effect of COP on genes expression of NSCLC cells. A549 cells were treated with COP (0.31 mg/ml) for 24 h. Total RNA was extracted with Trizol reagent (Invitrogen, Life Technologies). Gene chip was hybridized and then scanned with Affymetrix Scanner 3000.

Antitumor activity in vivo

Animal experiments involving in this study were conducted in accordance with the internationally accepted principles for laboratory animal use and care. The study was approved by the Committee on the Ethics of Animal Experiments of Shanghai University of Traditional Chinese Medicine. Female C57 mice (5 weeks) were purchased from SLRC Shanghai and fed in a standard feeding atmosphere in Shanghai University of Traditional Chinese Medicine. LLC cells (3×10^6 cells per mouse) were inoculated subcutaneously in C57 mice at the right axillary region, after three days, mice were randomly assigned to three different treatment groups (5 mice per group): (A) vehicle control (water); (B) COP 100 mg/kg; (C) COP 200 mg/kg. The mice were administered drug orally once daily for 3 weeks. Tumor size was measured on two axes with the aid of Vernier calipers and tumor volume (mm^3) was calculated using the formula: $1/2 (a \times b^2)$, where a is the longest and b is the shortest axis. Mice were euthanized at the end of the study and/or when tumor size exceeded 2,000 mm^3.

Statistical analysis

Statistical software SPSS version 13.0 was used for analysis. All data are presented as mean ± SD unless otherwise indicated. The data was analyzed by Student t test or a one-way analysis of variance (ANOVA), followed by pairwise multiple comparisons to determine any difference between groups Values of $P < 0.05$ were considered statistically significant.

Results

Batch to batch comparison chemical fingerprints of COP granules

Three different batches of COP granules were determined by HPLC. Four main components of COP including epiberberine, coptisine, palmatine chloride and berberine hydrochloride serve as the standard control. As shown in **Figure 1A**, epiberberine, coptisine, palmatine chloride and berberine hydrochloride are detectable in COP granules. We also found that the magnitude, number, and retention time of the peaks were highly similar among the three batches of COP (**Figure 1B**). In addition, the standard linear curve for epiberberine, coptisine, palmatine chloride and berberine hydrochloride was Y = 3074.2X-2.7816 (X: 0.017-2.18 μg, R = 1), Y = 3476.6X + 10.254 (X: 0.0163-2.088 μg, R = 0.9999), Y = 4025.4X-1.8929 (X: 0.0157-2.004 μg, R = 1), Y = 3895.4X-2.9426 (X: 0.0197-2.52 μg, R = 1), respectively. The content (μg) of control solution was recorded as X and the peak area (mAu) of chromatographic peak was Y. The content of four standard controls in each batch of COP sample was calculated as follows: epiberberine, #1: 1.58%, #2: 1.56%, #3: 1.58%; coptisine, #1: 2.23%, #2: 2.18%, #3: 2.20%; palmatine chloride, #1: 2.15%, #2: 2.14%, #3: 2.15%; berberine hydrochloride, #1: 8.1%, #2: 8.06%, #3: 8.1% (**Table 1**). These findings suggest that the three batches of COP granules are identical.

COP inhibits cell proliferation of NSCLC cells

To study the effect of COP on cell proliferation of NSCLC cells, A549 and H1299 cells were exposed to various concentrations of COP for 24, 48 and 72 h, respectively, and cell viability was analyzed by the MTT assay. As shown in **Figure 2A** and **2B**, COP (0.16 mg/ml) significantly inhibited cell growth of A549 and H1299 cells. We also observed that COP treatment resulted in cell growth inhibition in a dose- and time- dependent manner (**Figure 2C** and **2D**). A549 and H1299 cells were incubated in the presence of COP for 48 h, the IC_{50} values were 0.29 mg/ml and 0.23 mg/ml, respective-

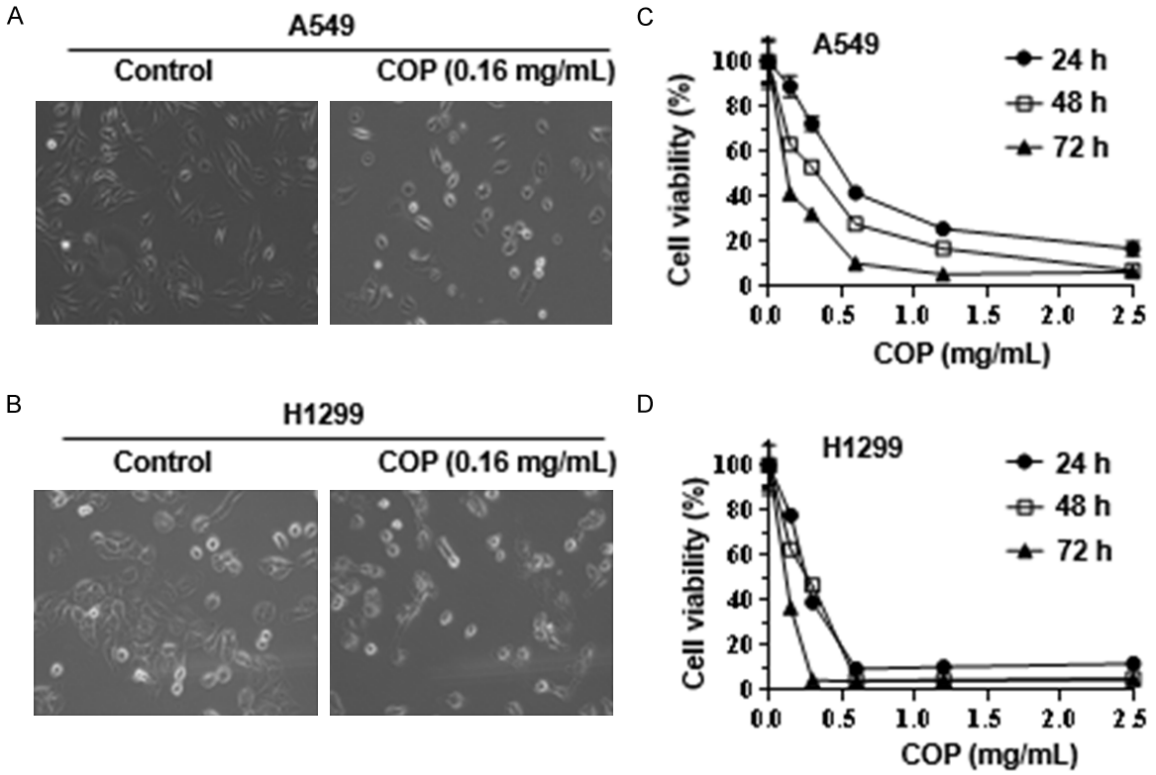

Figure 2. Effect of COP on proliferation of NSCLC cells. A549 (A) and H1299 (B) cells were treated with COP (0.15 mg/ml) for 24 h, respectively. Photographs of cell morphology from a representative experiment are shown. A549 (C) and H1299 (D) cells were treated with COP (0.16, 0.3, 0.6, 1.2, 2.5, and 5 mg/ml) for 24, 48 and 72 h, respectively, and cell viability was analyzed by the MTT assay. All values represent the mean ± SD from 3 independent experiments.

ly. In addition to the MTT assay, colony formation assays were performed on A549 and H1299 cells. We found that low dose of COP (0.16 mg/ml for A549 cells and 0.08 mg/ml for H1299 cells) significantly decreased growth of NSCLC cells in colony formation assays (**Figure 3A** and **3B**). These observations indicated that COP inhibits cell proliferation of NSCLC cells *in vitro*.

COP suppresses migratory and invasive ability in NSCLC cells

Given that COP inhibits growth of NSCLC cells, we postulated that COP could affect the migratory and invasive ability of NSCLC cells. To further explore the effect of COP on NSCLC metastasis, using wound healing assay, we determined changes in cell migration after 24 h of incubation with COP. Compared with the vehicle control treated cells, COP treated A549 and H1299 cells both showed significantly decreased migratory ability (**Figure 4A** and **4C**). We also tested the effect of COP in NSCLC invasion using a transwell chamber. The results further confirmed that COP inhibits NSCLC cells invasion ability (**Figure 4B** and **4D**). Taken together, these *in vitro* results suggest that COP inhibits NSCLC cell migration and invasion ability.

COP induces apoptosis of NSCLC cells

We further investigated the effect of COP on NSCLC cell apoptosis. A549 and H1299 cells were treated with COP at different concentrations for 24 h, then stained with Annexin V-fluorescein isothiocyanate (FITC)/PI. As shown in **Figure 5A** and **5B**, treatment of A549 and H1299 cells with COP induced a dose-dependent increase in the number of late apoptotic cells. The relative percentage of A549 cells in late apoptosis in response to 0.31 and 0.62 mg/ml of COP was 6.2 ± 2.2% and 15.6 ± 2.9%, respectively, while the relative percentage of H1299 cells in late apoptosis in respon-

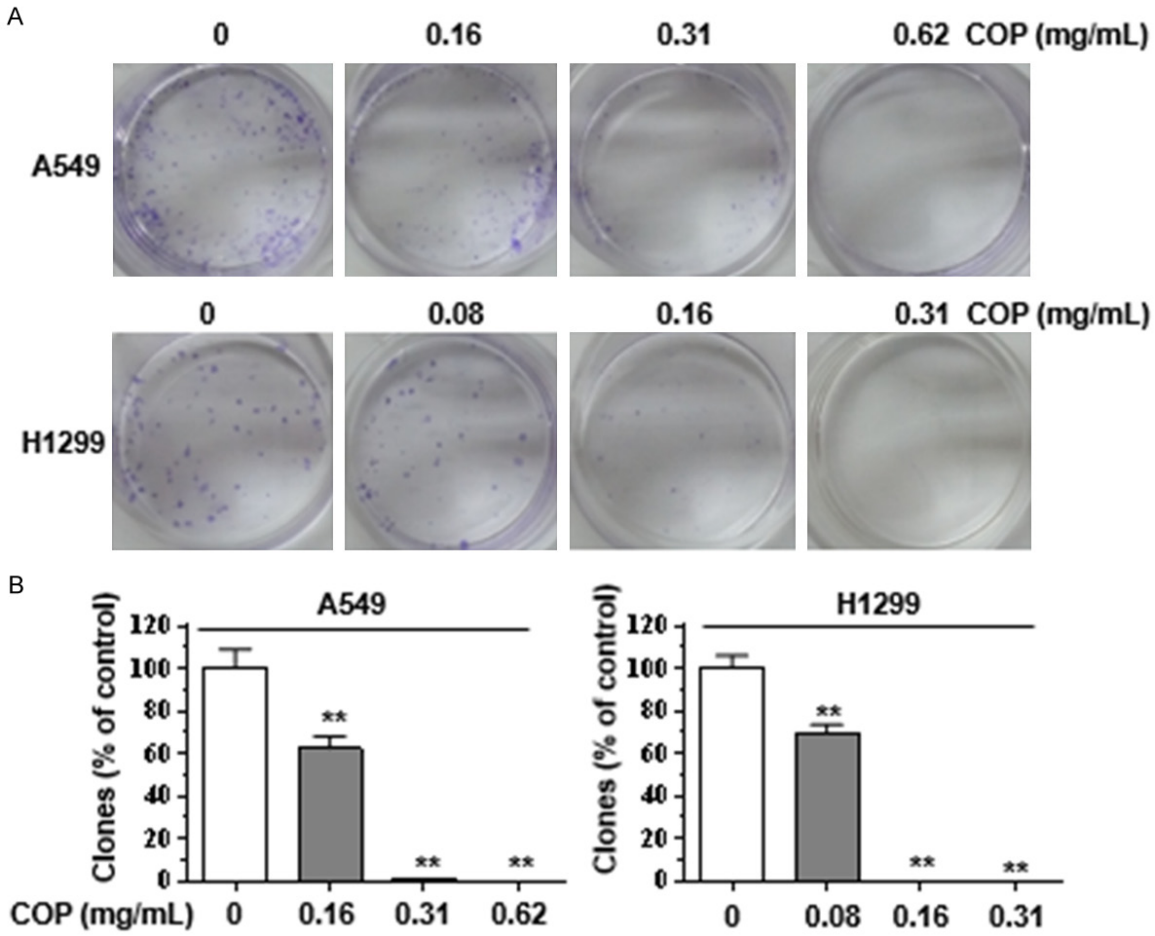

Figure 3. Effect of COP on colony formation of NSCLC cells. A. Cells were treated with COP (A549 cells: 0.16, 0.31 and 0.62 mg/ml; H1299 cells: 0.08, 0.16 and 0.31 mg/ml) for 24 h and allowed to grow for an additional 10 days. Photographs of cell culture dishes from a representative experiment are shown. B. Colony percentages represent the mean ± SD from 3 individual experiments. The number of colonies after treatment with vehicle control was normalized to 100%. **, $P < 0.01$ compared with the control group.

se to 0.16 and 0.31 mg/ml of COP was 9.2 ± 3.4% and 27.9 ± 3.9%, respectively. These data indicate that COP induces apoptosis of NSCLC cells.

Effect of COP on gene expression of NSCLC cells

Flow cytometry results indicated that COP can induce the apoptosis of NSCLC cells. To address the molecular mechanism of COP's anti-tumor action, PrimeView Human Gene Expression Array was used to determine the effect of COP on the expression of different genes. A549 cells were treated with COP (0.31 mg/ml) for 24 h. Total RNA was extracted and performed ChIP analysis. Heat map is shown in **Figure 6A**. According to the parameters of 2-fold change and $P < 0.05$, 16843 genes showed differential expression. Among them, 8187 genes were up-regulated and 8656 genes were down-regulated. Further analysis showed that 8 of 11 biological processes including cell cycle, DNA replication, p53 signaling pathway, oocyte meiosis, mismatch repair, base excision repair, homologous recombination and nucleotide excision repair were involved in cell growth, death, replication and repair (**Figure 6B**). These results suggest that COP regulates the gene expression of cell growth, death, replication and repair of NSCLC cells.

COP suppresses the growth of xenografts tumor

Since COP inhibits the cell proliferation of NSCLC cells, we further investigated whether COP can suppress the growth of xenografts tumor. To validate this, LLC cells were inoculated subcutaneously in C57 mice at the right

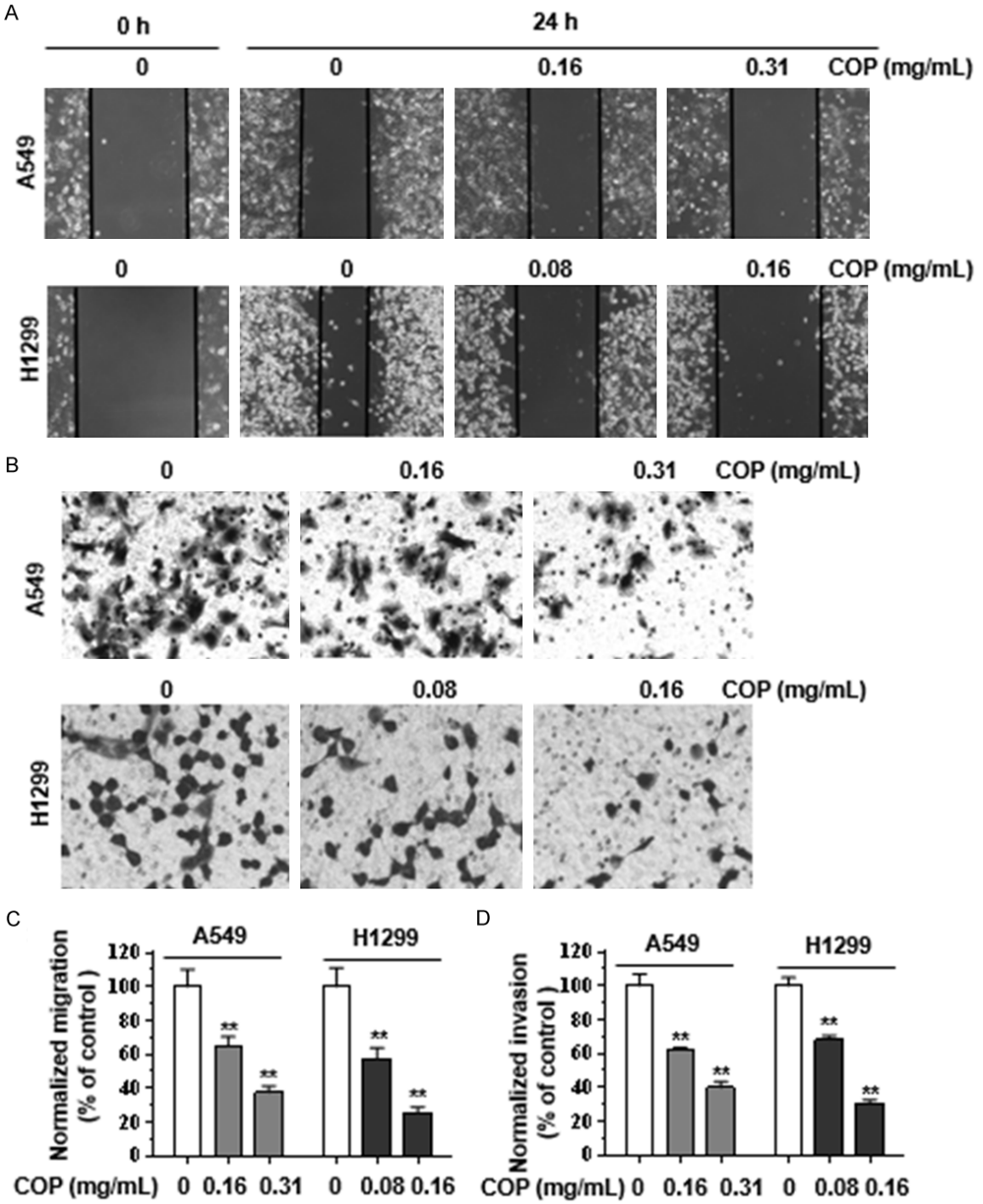

Figure 4. Effect of COP on NSCLC cells migratory and invasive ability. Scratch wound healing (A) and cell invasion (B) assays were performed, and cells were treated with COP (A549 cells: 0.16 and 0.31 mg/ml; H1299 cells: 0.08 and 0.16 mg/ml) for 24 h. Photographs of cell culture dishes from a representative experiment are shown. Migration (C) and invasion (D) rate histograms of each COP treated group. Data are presented as mean ± SD from three independent experiments. **, $p < 0.01$ compared with the control group.

axillary region. As shown in **Figure 7A**, compared with the control group, orally administration of COP at dose of 100 mg/kg and 200 mg/kg could significantly suppress the growth of LLC xenografts tumor. In addition, animal weight loss is commonly used as a surrogate marker of toxicity, and as seen in **Figure 7B**, treatment with COP was well-tolerated without weight loss when compared to control mice. These data suggest that COP suppresses the

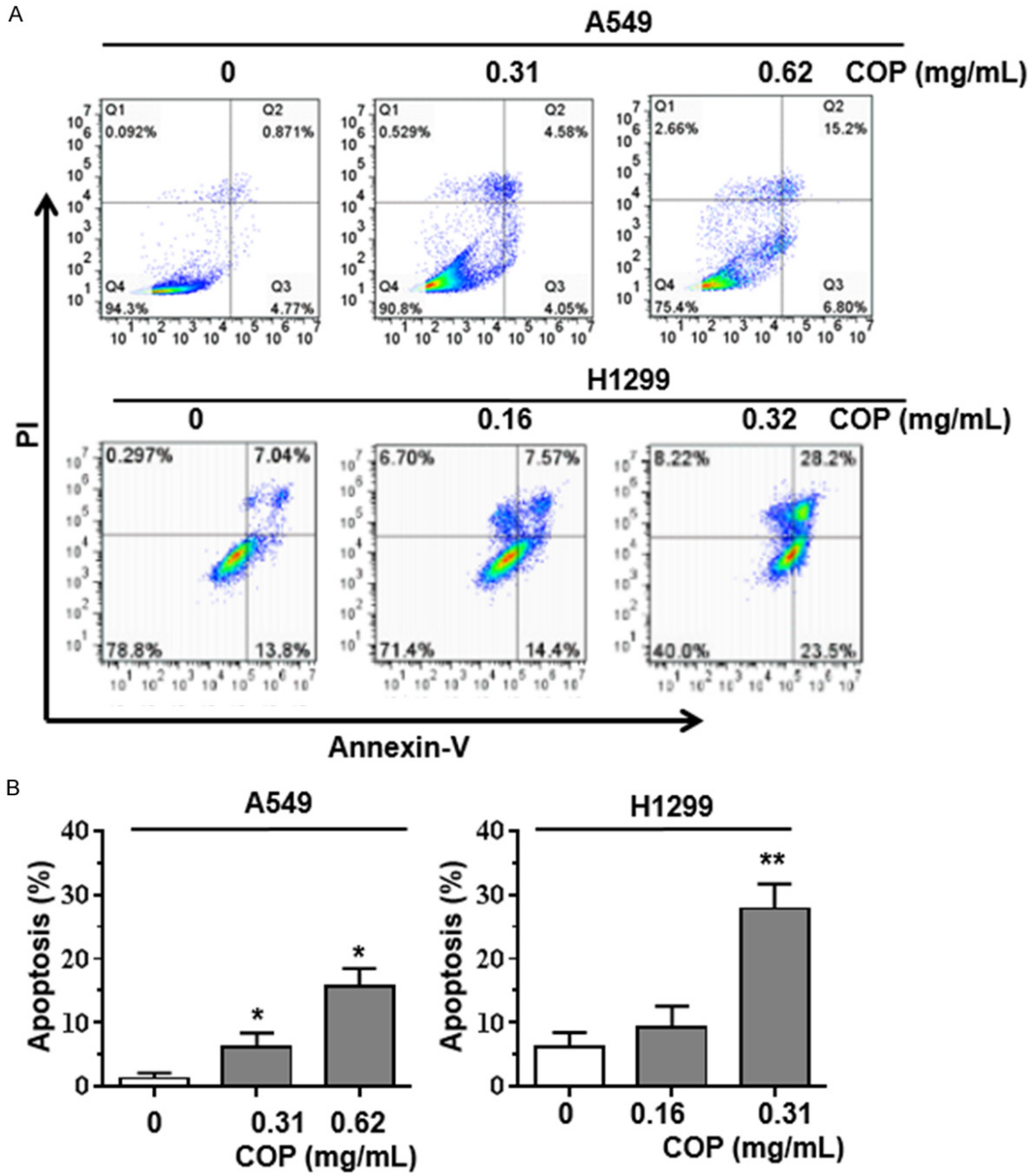

Figure 5. Effect of COP on cell apoptosis. A. A549 and H1299 cells were treated with COP at different concentrations (A549: 0.16, 0.31 and 0.62 mg/ml; H1299: 0.08, 0.16 and 0.31 mg/ml) for 24 h, then stained with Annexin V-fluorescein isothiocyanate (FITC)/PI. Top right quadrant, cells in late stage of apoptosis (Annexin V-FITC$^+$/PI$^+$). B. Quantitative analysis of dose-dependent alteration of COP on late apoptosis. Data are presented as mean ± SD from three independent experiments. **, $p < 0.01$ compared with the control group.

growth of xenografts tumor without obvious toxicity.

Discussion

NSCLC is very difficult to treat because of its low therapeutic and survival rates [14]. Patients with NSCLC are primarily treated by surgical resection, and subsequently by chemotherapeutic drugs with curative intent. Previous reports indicated that natural herbs can reduce the side effects of chemotherapy for patients with NSCLC [15-17]. Our recent study showed that Trichosanthes kirilowii fruits display inhibitory effect on NSCLC cells through cell-cycle and mitosis arrest [18]. In the present study, we

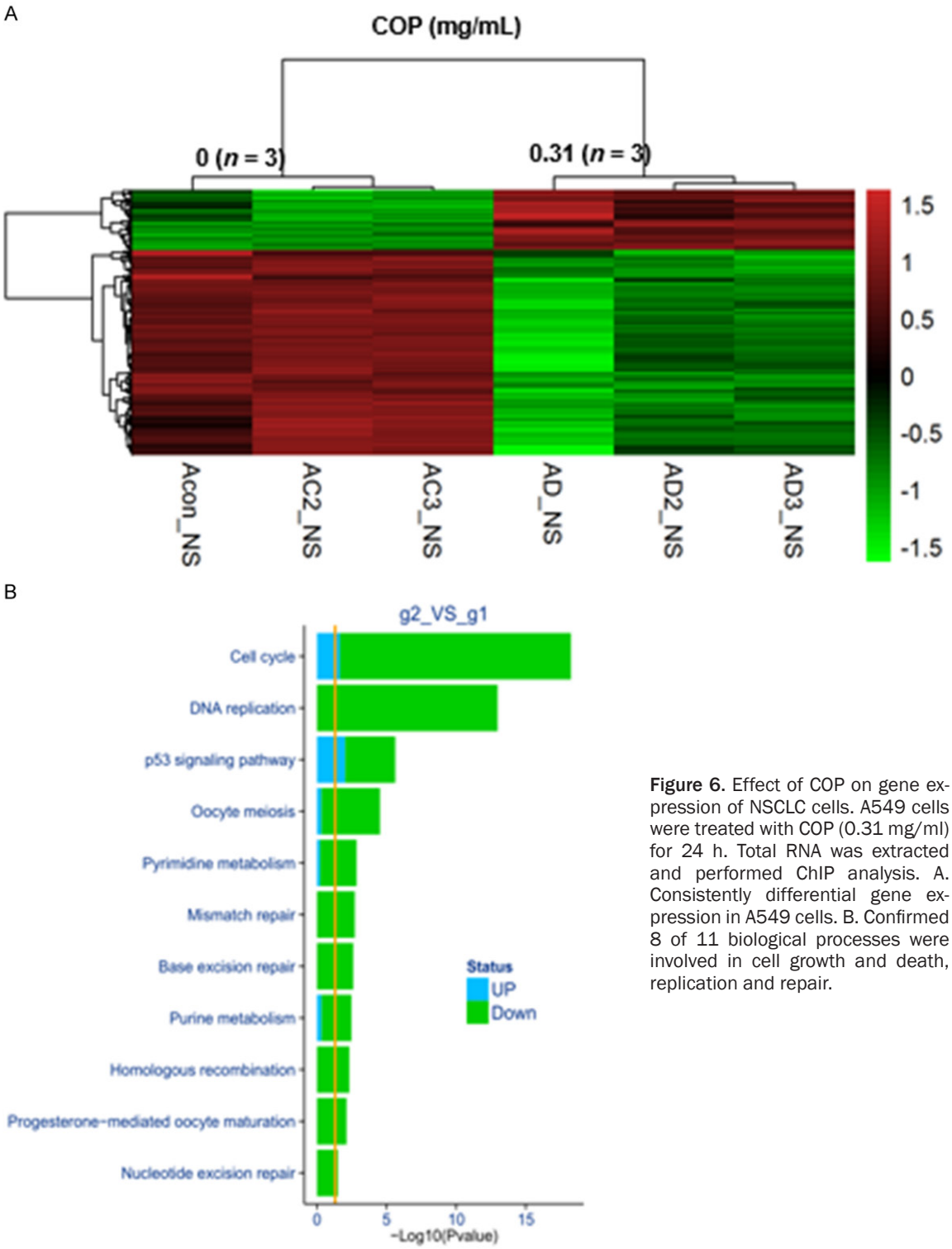

Figure 6. Effect of COP on gene expression of NSCLC cells. A549 cells were treated with COP (0.31 mg/ml) for 24 h. Total RNA was extracted and performed ChIP analysis. A. Consistently differential gene expression in A549 cells. B. Confirmed 8 of 11 biological processes were involved in cell growth and death, replication and repair.

showed that the three batches of COP granules are identical. COP displayed cytotoxic activities against the A549 and H1299 cells via suppressing cell proliferation and clone formation. In addition, COP was found to inhibit NSCLC cell migration and invasion ability. Moreover, COP induced cell apoptosis and regulated the gene expression of cell growth, death, replication and repair of NSCLC cells. Further, orally administration of COP could suppress xeno-

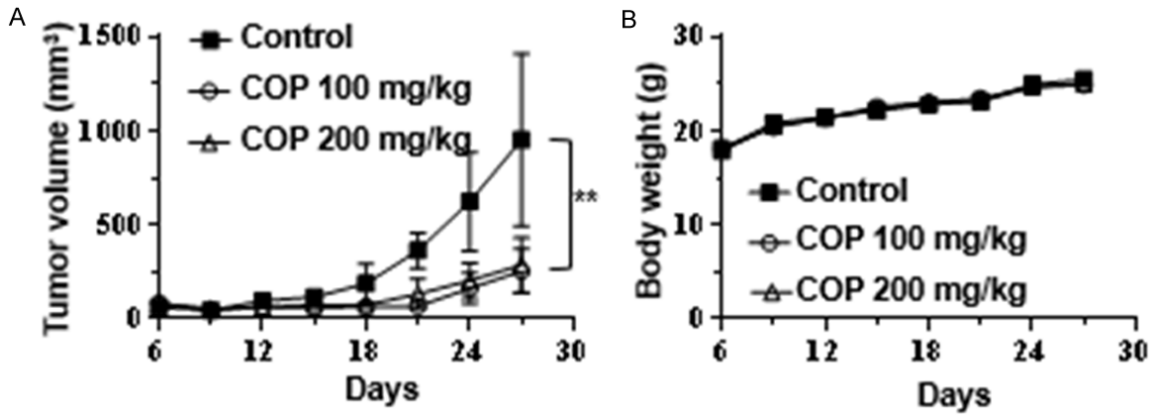

Figure 7. Antitumor activity of COP in C57 mice bearing LLC tumors. LLC cells (3×10^6 cells per mouse) were inoculated subcutaneously in C57 mice at the right axillary region, after three days, mice were randomly assigned to three different treatment groups (5 mice per group): vehicle control (water); COP 100 mg/kg and COP 200 mg/kg. The mice were administered drug orally once daily for 3 weeks. Tumor volume (A) and mice body weight (B) were recorded at different time. Data in the graphs represent the mean ± SD (n = 5 mice per group).

grafts tumor growth without obvious toxicity. These results suggested that COP may be a novel potential anti-tumor candidate in the NSCLC cells related lung cancer.

Given the batch to batch consistency of natural herb extracts is fundamental for basic research and clinical studies, at the beginning of our study, three different batches of COP granules were compared by HPLC. We found that four of main components including epiberberine, coptisine, palmatine chloride and berberine hydrochloride are detectable, and the content of them are highly similar in COP granules. These results indicate that the components of the three batches of COP granules are identical. It has been identified that the extract of COP contains several bioactivity components [19] and berberine has been recognized as the dominant one [20]. Interestingly, previous studies reported that purified berberine was less effective than the whole COP extract [21, 22]. These reports indicated that it is unusual for a single component to be isolated from an herbal medicine with optimal biological activity. Our previous study also provided evidence that an extract is more effective *in vivo* than a single isolated component [13]. In this regard, it seems better to develop the whole herbal medicine extract rather than its single dominant component for cancer treatment. COP has various pharmacological activities and caused high interests on its anti-cancer activity.

It has been shown that COP extract inhibits tumor progression via suppressing cell proliferation, inducing cell death and arresting cell cycle [23]. In this study, we found that COP displayed cytotoxic activities against the NSCLC cells (A549 and H1299) by inhibiting cell proliferation and clone formation. Previous reports demonstrated the effects of COP in suppressing cancer cell invasion, which in turn inhibits cancer metastasis [23]. In agreement with this published data, we also observed that COP inhibits the migration and invasion ability of A549 and H1299 cells. The anti-tumor actions of some natural products have been proved to be the result of their ability to induce cell apoptosis [24]. Recently, it has been reported that COP and its main active component, berberine, could initiate human cancer cell apoptosis, causing to death of tumor cells. Treatment of COP extract and berberine could induce the intrinsic pathway of apoptosis [25]. In the present study, flow cytometric analysis showed that exposure to COP resulted in a significant increase in the percentage of apoptosis cells, suggesting that COP initiates apoptosis in A549 and H1299 cells. It is now well recognized that understanding of molecular effect (i.e. gene expression) of an herb is very important for assessing its efficacy and safety [22]. A clearer understanding as to how COP regulates the cellular pathway would provide important insights into the potential mechanisms for the development of anti-cancer herbs with improved selectivity. Microarray technology is qui-

te useful in this regard. Our human gene expression array data clearly showed that COP regulated the gene expression of cell growth, death, replication and repair of NSCLC cells. These results may help identifying novel therapeutic effect of COP. Our strategy used in this study could serve as a framework to study medicinal herbs. Taken together, our results suggest that there are potentially multiple mechanisms by which COP is able to inhibit the growth of NSCLC cancer cells.

Our *in vivo* data further showed that COP (100 mg/kg and 200 mg/kg) treatment significantly reduced tumor volume. In addition, we observed that the COP treatment did not affect the body weight of mice. This indicates that COP is a low toxicity herb. COP has been widely used in China for several thousand years for the treatment of infectious conditions. COP has been shown to be safe for human consumption [26]. This advantage plus the accumulating evidence of its anti-tumor effects make COP a promising candidate for being an anti-cancer agent. This study may shed light on future direction of studies featuring COP as a novel anti-tumor agent, which should be further proven in future animal and clinical studies.

In conclusion, our studies demonstrated that COP displayed anti-tumor activity both *in vitro* and *in vivo*. COP may represent a novel therapeutic herb for NSCLC treatment. Further studies are needed to elucidate the whole signaling pathway of the anti-tumor action of COP and to determine the role of metabolism as it relates to the *in vivo* activity of COP.

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Disclosure of conflict of interest

None.

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