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Berberine Reverses the Tumorigenic Function of Colon Cancer Cell-Derived Exosomes

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Exosomes derived from colon cancer cells has been found to elevate viability and metastasis of recipient cells. Berberine is a plant-derived natural compound that has shown anti-colon cancer potential. However, berberine's impacts on the tumorigenic functions of tumor exosomes have yet to be evaluated. To elucidate whether berberine modulates exosomal pro-tumor activity, we evaluated the effects of exosomes released by berberine-treated colon cancer cells against viability, migration, and invasion of recipient cancer cells. The human colon cancer HCT116 cells were treated or not treated with berberine, and culture media were collected following 48 h of treatment. Exosomes released by treated or untreated cells were isolated from collected media via ultracentrifugation. To study effects of berberine on tumor exosomes, HCT116 cells were co-cultured with exosomes derived from berberine-treated and non-treated cells, followed by monitoring changes in cell viability, migration, and invasion. The treatment with 100, 150, and 200 μg/ml of berberine-primed exosomes could dose-dependently decrease the viability [by −35.4% ($p < 0.0001$), −47% ($p < 0.0001$), and −65.5% ($p < 0.0001$), respectively], migration [by −24% ($p = 0.0001$), −43.5% ($p = 0.0001$), and −65.2% ($p = 0.0001$), respectively], and invasion [by −29% ($p < 0.0001$), −58.8% ($p < 0.0001$), and −69.7% ($p < 0.0001$), respectively] of HCT116 cells compared to the control. However, non-primed exosomes exerted significant inducing effects on the viability and metastatic ability of HCT116 cells. In conclusion, berberine can reverse the tumorigenic function of colon cancer exosomes and, thus, exert a remarkable suppressive impact against the survival and metastatic ability of colon cancer cells.

Keywords: berberine; colon cancer; exosome; invasion; migration
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Introduction

Colon cancer, ranking third among malignant types of cancer worldwide, has an increasing morbidity rate (Ulaganathan et al. 2018; Ocalewski et al. 2021; Kumar and Lewis 2022). At present, the common choices for the treatment of colon cancer are molecular targeted therapy and chemotherapy as well as radiotherapy and surgery. However, there is a high rate of post-treatment tumor metastasis, due to the invasion and migration of the colon cancer cells to the adjacent and distal tissues (Yamaguchi et al. 2013; Muslim and Al-Obaidi 2021). Hence, the investigation of novel therapeutic approaches and drugs blocking colon cancer cells from metastasis has great importance.

The aggressiveness of tumor cells is associated with the influence of intercellular signaling networks in the tumor microenvironment. Studies have revealed that cancer cell-secreted extracellular vesicles (EVs) such as exosomes play important roles in the cell-to-cell communication and tumor microenvironment, inducing tumor development and metastasis, because of their capacity to deliver cancer-inducing molecules to the recipient cells (Umwali et al. 2021). Exosomes include a population of membrane-enclosed vesicles that are homogenous in shape and size (ranging from 40 to 150 nm) and are widely distributed in various body fluids (van der Meel et al. 2014).

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Exosome vesicles secreted by colon cancer cells have been shown to modulate local and systemic tumor microenvironments by transferring oncogenic nucleic acids and proteins (Umwali et al. 2021). They are implicated in tumorigenesis, the tumor cell survival and metastasis, as well as chemotherapy resistance. Colon cancer cell-derived exosomes can promote tumor growth via inducing the proliferation and inhibiting the apoptosis mechanistically by influencing the expression or function of the important oncogenic genes/proteins or signaling pathways (Umwali et al. 2021). Notably, there is evidence indicating that exosomes induce metastasis in colon cancer. For example, exosomes derived from colon cancer cells could induce *in vitro* cell invasion and migration (Liu et al. 2020a). In addition, the plasma levels of exosomal miR-106b-3p in patients with metastatic colon cancer were detected to be remarkably more than that of non-metastatic patients, and elevated levels of plasma exosomal miR-106b-3p were associated with poor prognosis of colon cancer (Umwali et al. 2021). Of note, the plasma exosomes containing miR-106b-3p could enhance lung metastasis of colon cancer cells in an animal model (Liu et al. 2020a). Tumor-derived exosomes have been also found to induce a highly aggressive cancer phenotype in many other types of tumors, including prostate (Ramteke et al. 2015) and ovarian ones (Dorayappan et al. 2018). These findings indicate the critical role of exosomes in exacerbating malignant progression and metastasis of cancers. Thus, to successfully treat metastatic colon cancer, it is essential to explore efficient therapeutic agents that target tumor-cell exosomes.

Natural compounds and their derivatives include about 37% of approved anticancer agents because of diversity in their chemical structure as well as high biocompatibility and drug-like features (Newman and Cragg 2016). Berberine is a plant natural product, a Chinese herb extract presented abundantly in various parts of medicinal plants such as goldenthread, barberry, and goldenseal (Arayne et al. 2007; Kumar et al. 2015). Clinically, berberine has been applied for treating gastroenteritis and diarrhea (Kumar et al. 2015). In recent years, berberine has growingly received substantial attention due to its considerable pharmacological properties including anti-cancer (Ayati et al. 2017; Mortazavi et al. 2020; Rauf et al. 2021), hypolipidemic and anti-diabetic (Lan et al. 2015; Ma et al. 2018; Fatahian et al. 2020), suppressing liver fibrosis (Feng et al. 2009), neuroprotective (Pirmoradi et al. 2019), anti-oxidant (Kazaz et al. 2020), and anti-inflammatory effects (Ehteshamfar et al. 2020; Shen et al. 2020; Mohammadian Haftcheshmeh and Momtazi-Borojeni 2021; Haftcheshmeh et al. 2022).

Preclinical investigations has shown that oral administration of berberine has the safety window at 25 mg/kg (Ye et al. 2009). No significant toxic effects of berberine, such as cytotoxic, genotoxic, and mutagenic activities, have been reported (Yin et al. 2008). However, the middle-term consumption of berberine has potential to promote gastrointestinal complications (Yin et al. 2008). Due to binding to macrolides, berberine has been proposed to induce life-treating arrhythmias (Jiang et al. 2011; Caliceti et al. 2015). Notably, administration of berberine in pregnant women and jaundiced neonates is strongly not recommended, due to displacement of bilirubin from albumin by berberine (Chan 1993; Affuso et al. 2010). However, several meta-analyses of randomized controlled trials recruiting patients with hypercholesterolemia and type 2 diabetes showed safety of berberine (Lan et al. 2015; Millán et al. 2016; Wei et al. 2016; Ju et al. 2018).

Recent studies have demonstrated that berberine is able to exert anti-tumor impacts against colon cancer through interacting with various molecular targets, including arylamine N-acetyltransferase (Lin et al. 1999), Wnt/β-catenin (Wu et al. 2012), epidermal growth factor receptor (EGFR) (Wang et al. 2013), transforming growth factor-β (TGF-β) (Huang et al. 2019, 2020), AMP-activated protein kinase (AMPK) (Park et al. 2012; Li et al. 2015), reactive oxygen species (ROS) and c-Jun N-terminal kinase (JNK)/p38 pathway (Hsu et al. 2007; Wang et al. 2012), nuclear receptor retinoid X receptor alpha (Ruan et al. 2017), microRNAs (miRs) (Lü et al. 2018; Liu et al. 2019), cyclooxygenase-2 (Fukuda et al. 1999), members of "mitochondrial ribosomal protein L" (Li et al. 2021b), pyruvate kinase isozyme type M2 (Yan et al. 2022), inflammation and apoptosis mediators (Chidambara Murthy et al. 2012; Xu et al. 2012; Yan et al. 2022; Chen et al. 2022), basolateral potassium voltage-gated channel subfamily Q member 1 (KCNQ1) channels (Alzamora et al. 2011), glucose metabolism (Mao et al. 2018; Yan et al. 2022), polyamines (Wu et al. 2018), as well as signaling pathways like COX-2/PGE2-JAK2/STAT3 (Liu et al. 2015), Notch1/PTEN/PI3K/AKT/mTOR (Li et al. 2021a), and miR-21/ITGβ4/PDCD4 (Liu et al. 2022).

The above-mentioned studies indicate the remarkable application potential of berberine in colon cancer treatment. However, does berberine act on colon cancer cell-derived exosomes? There has been no study that reported the impact of berberine on tumor exosomes. Herein, for the first time, we investigated the impact of exosomes isolated from berberine-treated colon cancer cells on proliferation, migration, and invasion of recipient colon cancer cells, and found that berberine significantly reversed the tumorigenic properties of tumor-derived exosomes.

Materials and Methods

Cell culture

The human colon cancer HCT116 cell line was purchased from Cybertron Biotechnology Co., Ltd, Shanghai, China. The cells were incubated in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (Gibco, Waltham, MA, USA) at 37°C and 5% CO_2 air humidified.

Berberine treatment and exosome extraction

Berberine was dissolved in 0.001% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to prepare berberine stock solution (100 mM). HCT116 cells were cultured in a T75 flask (2-2.5 × 10^6 cells) and cell culture medium was collected after 48 h of treatment with or not with berberine (10 μM). The berberine concentrations and treatment duration were chosen based on previous publications (Lü et al. 2018; Li et al. 2021a). As an untreated control, cells were exposed to DMSO. Exosomes released by treated or untreated cells were harvested from collected culture media via differential centrifugation and ultracentrifugation as previously published (Osterman et al. 2015; Wu et al. 2016; Qiu et al. 2020; Li et al. 2021c; Habibian et al. 2022). In brief, the cell debris and other impurities in the collected medium were removed by three sequential steps of centrifugation (Hettich, Kirchlengern, Germany), first for 8 min at 500 × *g* followed by 15 min at 2,500 × *g*, and finally for 25 min at 12,000 × *g*, all at 4°C. After differential centrifugation, exosomes were pelleted via ultra-centrifugation (Hettich) of the supernatant at 110,000 × *g* for 80 min at 4°C. Pellets were dispersed in phosphate-buffered saline (PBS), filtered through a sterile 22-μm filter, and centrifuged again at 110,000 × *g* for another 80 min at 4°C. Finally, the pellet was re-suspended in PBS buffer containing 25 mM Trehalose and stored at −80°C until utilization in subsequent assays. The concentrations of isolated exosomes were measured using a BCA protein assay kit in accordance with the manufacturer's instructions (Abcam, Cambridge, UK).

Hereinafter, exosome isolates of berberine-treated cells and untreated cells will be called berberine-primed exosomes and non-primed exosomes, respectively.

Acetylcholinesterase activity assay in isolated exosomes

Since the acetylcholinesterase enzyme is presented in exosomes (Rieu et al. 2000), an acetylcholinesterase activity assay was employed in accordance with the previously published protocols (Savina et al. 2003; Lancaster and Febbraio 2005) to confirm the presence of exosomes in isolated samples. This assay is based on the quantification of the thiocholine produced from the hydrolysis of acetylthiocholine by acetylcholinesterase. DTNB (5,5′-dithio-bis[2-nitrobenzoic acid]) is used to measure the amount of thiocholine production by enzymatic activity of acetylcholinesterase. In summary, 37.5 μL of the isolated sample, 112.5 μL of 1.25 mM acetylthiocholine (Sigma-Aldrich), and 150 μL of 0.1 mM DTNB (Sigma-Aldrich) were loaded into each well of a 96-well plate. Since the absorption intensity of DTNB (412 nm) is proportional to the acetylcholinesterase activity, a spectrophotometer (Bio-Tek, Winooski, VT, USA) was used to detect alterations in absorbance at 412 nm in 5 min intervals for 30 min.

Nanoparticle tracking analysis in isolated exosomes

To measure the size and concentration of isolated exosomes, the nanoparticle tracking analysis (NTA) using the Nanosight NS300 system (Malvern Instruments, Worcestershire, UK) was employed. Exosome isolates were diluted in PBS buffer (1:200) and measurements were recorded at 25°C for 30 s. Three replicates of each isolate were measured and averaged to derive the representative size and concentration profiles.

Transmission electron microscopy in isolated exosomes

The size and morphology of isolated exosomes were monitored by transmission electron microscopy (TEM). Exosome particles were covered using negative staining via adding the isolated solution onto parafilm and a formvar (polyvinyl formal)-carbon-coated 400 copper mesh grid, followed by 1 min of incubation with 2% phosphotungstic acid and drying at room temperature for 15 min. Exosomes were visualized using a Philips CM10 TEM (Philips, Corvallis, OR, USA) with ×200,000 magnification.

Evaluating antitumor activity of berberine-primed exosomes

To study the anti-tumor activity of berberine-primed exosomes against colon cancer cells, we isolated exosomes from HCT116 cells that were treated with or not with 10 μM berberine for 48 h. In the independent experiments, HCT116 cells were incubated with isolated exosomes (berberine-primed exosomes or non-primed exosomes) at increasing concentrations 100, 150, and 200 μg/ml based on the previous reports (Zhang et al. 2007; Kalani and Chaturvedi 2017; Ren et al. 2019), followed by monitoring changes in cell viability, migration, and invasion.

Cell viability

To evaluate the viability of treated HCT116 cancer cells, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay (Sigma-Aldrich) was employed. HCT116 cells were seeded and incubated at a density of 2 ×10^4 cells/well in the 96-well plates at 37°C and 5% CO_2 air humidified for 24 h. Afterward, the cultured cells were treated with the isolated non-primed exosomes (200 μg/ml) and berberine-primed exosomes at different concentrations (100, 150, and 200 μg/ml), followed by a 48 h of incubation at 37°C and 5% CO_2. As a control, cells were exposed to PBS buffer containing 25 mM Trehalose. Each concentration was repeated three times. After the treatment period, MTT solution was loaded at a final concentration of 0.5 mg/ml, followed by a 4 h of incubation. The medium containing MTT was then replaced by DMSO and gently mixed to dissolve produced formazan crystals. The optical density (OD) was measured at 560 nm using an ELISA plate reader (Bio-Tek). Three independent experiments were conducted to acquire data.

Wound healing assay

HCT116 cells (6 × 10^5 cells/ml) were incubated overnight in six-well plates to reach 80% confluence. To create

a wound, the cell monolayers were scratched with a scalpel, and detached cells were then eliminated by washing via a serum-free medium. Afterward, cells were treated with the isolated non-primed exosomes (200 μg/ml) and berberine-primed exosomes at different concentrations (100, 150, and 200 μg/ml), and then migrating of cells from the leading edge was photographed using an inverted light microscope (BX40, Olympus, Tokyo, Japan) at 0 and 24 h post-treatment times. The area covered by the migrated cells was measured with the Image J software. The area covered by cells in the treatment group with respect to the control group was employed to evaluate the percentage of migrated cells. As a control, cells were treated with PBS buffer containing 25 mM Trehalose. The results were presented by mean values of three independent experiments.

Transwell invasion assay

The cell invasion was assayed using a 24-well Transwell chamber coated with 1 mg/ml Matrigel matrix. 1×10^5 HCT116 cells treated with non-primed exosomes or berberine-primed exosomes were seeded into the upper chamber, while 500 μl medium containing 10% FBS was loaded to the lower compartment of the chamber. After 24 h of incubation at 37˚C, the cells that invaded the lower side of the filter were fixed by 5% glutaraldehyde for 10 min and then stained with 1% crystal violet in 2% ethanol for 20 min. The filter membranes were photographed using the inverted light microscope and the number of stained cells was counted in six randomly selected fields. The mean of the counted cells from the treatment group compared to the control group was employed as the percentage invasive cell number. As a control, cells were treated with PBS buffer containing 25 mM Trehalose.

Statistical analysis

All statistical analyses were carried out using either an unpaired one-tailed Students' t-test or one-way ANOVA with a post-hoc uncorrected Fisher's LSD test using the Prism (Graphpad) software. A probability of less than a 95% confidence limit ($p < 0.05$) was considered to be significant. Data are presented as mean ± standard deviation (SD).

Results

Detection, validation, and quantification of isolated exosomes

The resultant data indicated the significantly elevated enzymatic activity of acetylcholinesterase in exosome isolates than in control (berberine-primed exosomes vs. control; $p = 0.0007$, non-primed exosomes vs. control; $p = 0.0017$). No statistical difference was found in acetylcholinesterase activity between non-primed exosomes isolated from untreated HCT116 and berberine-primed exosomes isolated from HCT116 cells treated with 10 μM of berberine (Fig. 1A). The exosome isolation was further confirmed by evaluating particle size and morphology in exosome isolates using the NTA and TEM techniques. The results showed that both exosome isolates (non-primed exosomes and berberine-primed exosomes) encompassed vesicles within the exosome size range, with diameters between 40-150 nm (Fig. 1B, C). As demonstrated in Fig. 1C, TEM visualized the "cup-shaped" vesicles in exosome isolates, morphologically verifying the presence of exosomes. Quantifying exosome isolates using NanoSight NTA indicated that the number (particles/ml) of exosomes released by berberine-treated HCT116 cells was not significantly different from those by untreated HCT116 cells (Fig. 1D).

Berberine-primed exosomes reduce the viability of recipient colon cancer cells

Resultant data indicated a significant dose-dependent decrease in viability of colon cancer HCT116 cells treated with berberine-primed exosomes (100, 150, 200 μg/ml) by −35.4%, −47%, and −65.5%, respectively, when compared to untreated control cells ($p < 0.001$), and by −62.3%, −74%, and −93%, respectively, when compared to cells treated with non-primed exosomes ($p < 0.0001$). Inversely, non-primed exosomes (200 μg/ml) significantly increased colon cancer cell viability by 29% compared to the control ($p < 0.001$) (Fig. 2). The inducing effect of non-primed exosomes can be supported by the findings that tumor-derived exosomes can transport cancer-driving mediators to recipient cells, exacerbating aggressive properties (Ren et al. 2019). Therefore, our results demonstrate that berberine can reverse the tumorigenic function of tumor-derived exosomes, thereby reducing the viability of recipient colon cancer cells.

Berberine-primed exosomes inhibit migration and invasion of recipient colon cancer cells

Cell invasion and migration are key steps of cancer cell metastasis and donate druggable targets for cancer therapy. To evaluate the impact of non-primed and berberine-primed exosomes on colon tumor metastasis, wound healing and transwell invasion assays were applied.

While non-primed exosomes significantly increased the migration ability of colon cancer HCT116 cells (by 31%, $p = 0.0001$), berberine-primed exosomes significantly and dose-dependently impeded the capability of colon cancer cells to move throughout the wounded area. The treatment with 100, 150, and 200 μg/ml of berberine-primed exosomes reduced the migrative ability of HCT116 cells by −24% ($p = 0.0001$), −43.5% ($p = 0.0001$), and −65.2% ($p = 0.0001$) compared to untreated control (Fig. 3).

In addition, HCT116 cells were cultured non-primed or berberine-primed exosomes and permitted to motivate through Matrigel-coated transwell chambers, indicating a significant inducing effect of non-primed exosomes (by 36.6%, $p < 0.0001$) and a significant dose-dependent inhibitory effect of berberine-primed exosomes on the invasive ability of HCT116 cells. Of note, the treatment with 100, 150, and 200 μg/ml of berberine-primed exosomes signifi-

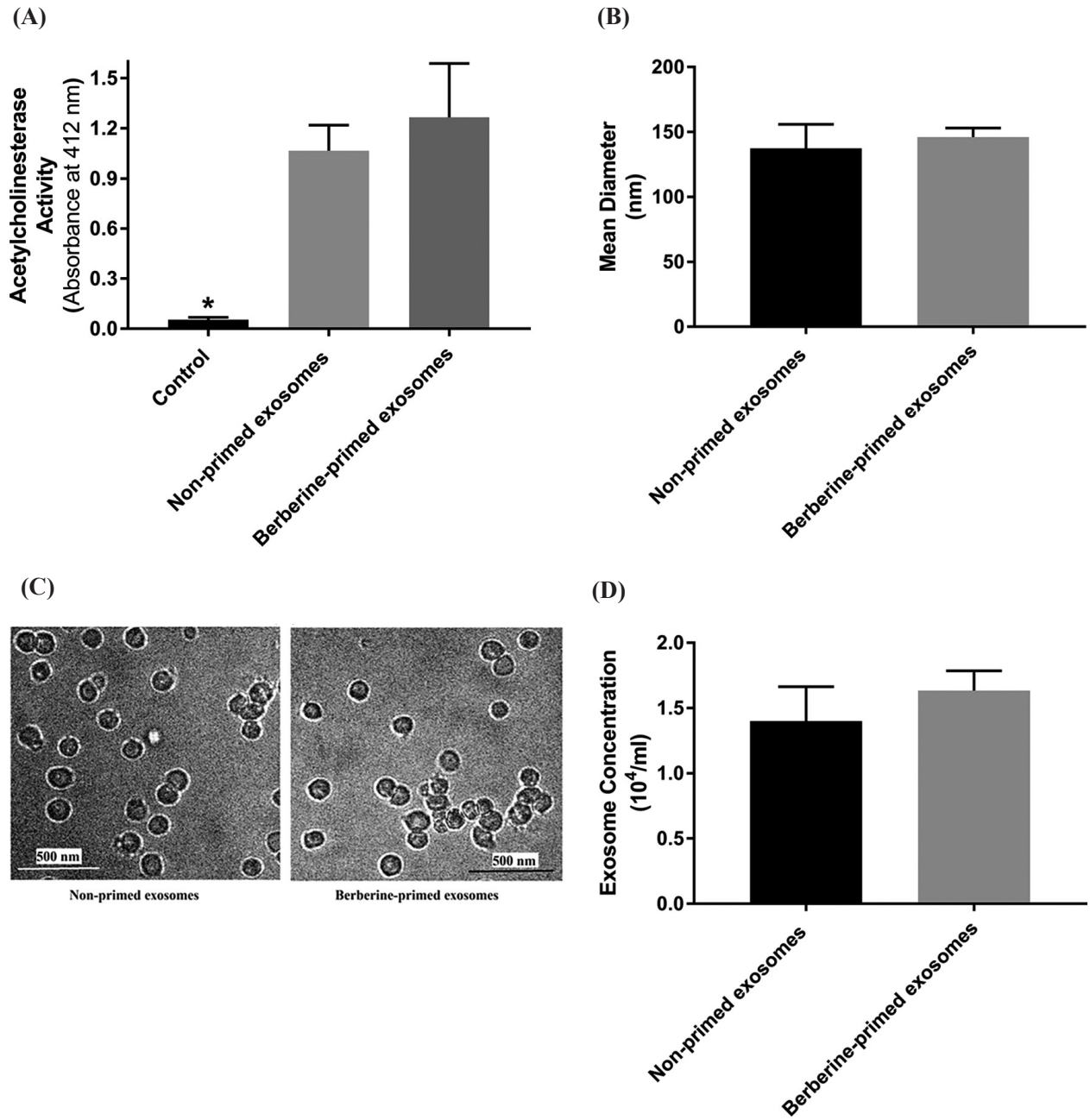

Fig. 1. Detection and characterization of isolated exosomes.
(A) Acetylcholinesterase activity assay was employed to detect exosomes in isolates from untreated HCT116 cells (non-primed exosomes) or HCT116 cells treated with 10 μM of berberine for 48 h (berberine-primed exosomes) compared to the control (PBS buffer). (B) The size distribution of exosomes was evaluated using nanoparticle tracking analysis (NTA). (C) Representative photomicrographs show the isolated exosomes visualized by electron microscopy (× 200,000 magnification; scale bar, 500 nm). (D) Exosome concentration (particles/ml) was evaluated by NTA. No significant differences were detected in acetylcholinesterase activity, size distribution, or concentration of vesicle between non-primed exosomes and berberine-primed exosomes. Results are expressed as mean ± SD of three independent experiments. *$p < 0.001$ regarding the significant difference of control vs. exosome isolates.

cantly decreased the invasive HCT116 cells by −29% ($p < 0.0001$), −58.8% ($p < 0.0001$), and −69.7% ($p < 0.0001$) compared to the control (Fig. 4).

Discussion

In the present study, non-primed exosomes and berberine-primed exosomes secreted by colon cancer HCT116 cells could alter viability and metastasis of recipient HCT116 cells.

Of note, when HCT116 cells were incubated with non-primed exosomes, the proliferation, migration, and invasion were found to be significantly increased. Tumor aggres-

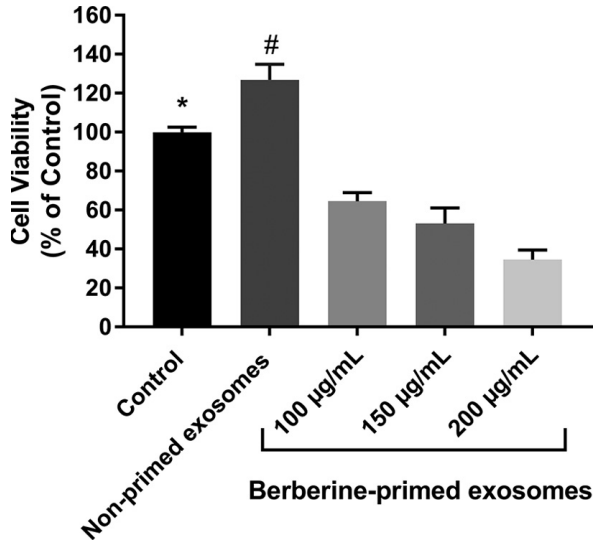

Fig. 2. Effects of non-primed and berberine-primed exosomes on the viability of human colon cancer HCT116 cell line after 48 h of treatment.
Non-primed exosomes (200 μg/ml) derived from untreated colon cancer cells significantly increased the viability of recipient colon cancer cells compared to the control. In a separate experiment, berberine-primed exosomes secreted by cancer cells treated with 10 μM of berberine significantly and dose-dependently decrease the viability of recipient cancer cells compared to the control. Results are expressed as mean ± SD of three independent experiments. $^{*}p < 0.0001$ regarding the significant difference of control versus exosome isolates. $^{\#}p < 0.0001$ regarding the significant difference between non-primed exosomes vs. other groups.

siveness is mainly ascribed to the effects exerted by signaling networks directed in part by tumor-secreted exosomes. Exosomes have the ability to affect recipient cells by transferring oncogenic proteins or genetic materials that exacerbate cancer progression. Besides, by delivering cancer-inducing material, tumor-secreted exosomes were also found to participate in the formation of a suitable metastatic niche that induces the transformation of non-metastatic cancer cells into metastatic ones (Jung et al. 2009; Rana et al. 2013). There is evidence showing the promotion roles of colon cancer cell-derived exosomes on the proliferation and metastasis of recipient colon cancer cells. It was reported that human colon cancer cell lines HCT116 and SW480 could increase their own growth or proliferation through exosome secretion which reduces the duration of mitosis and activates STAT3 (Ren et al. 2019). The other investigation indicated that exosomes from patients with colon cancer could enhance the proliferation potential of the recipient HCT116 cells *in vitro* and in animal models (Soldevilla et al. 2014). Moreover, it was also reported that exosomes from colon cancer patients with metastasis induced colon cancer cell migration and invasion *in vitro*, and enhanced lung metastasis of colon cancer cells *in vivo* (Liu et al. 2020a).

Berberine has been found to inhibit progression of colon cancer by inducing the cell cycle arrest. It was reported that berberine could inhibit the cell proliferation by promoting G0/G1 cell cycle arrest via modulating the expression of cyclin D1 (CCND1) and cyclin-dependent kinase 4 (CDK4) and inhibiting telomerase activity in HCT116 cells (Liu et al. 2020b; Zhang et al. 2020; Samad et al. 2021). Moreover, berberine was also found to inhibit growth of colon cancer cells by modulating G1-phase cell cycle delay (Soffar 2019). In our study, it was found that exosomes isolated from berberine-pretreated HCT116 cells exert anti-tumor activity. We isolated berberine-primed exosomes after treating colon cancer cells with 10 μM berberine for 48 h. Our results indicated that berberine-primed exosomes significantly reduced the viability, migration, and invasion of colon cancer cells. The study outcome suggests that berberine can reverse the tumor-promoter effects of exosomes released from colon cancer cells. To the best of our knowledge, these findings represent the first evidence of berberine treatment that leads to the modulation of the tumorigenic exosomes. Our findings are consistent with the results of already reported studies on other natural compounds such as curcumin that showed anti-cancer effects by affecting exosomes-mediated cross-talk between pancreatic cancer cells (Osterman et al. 2015). Curcumin was found to change the pro-survival impact of exosomes secreted by pancreatic cancer cells toward a pro-death impact, leading to a significant decrease in viability of recipient pancreatic cancer cells (Osterman et al. 2015). The other investigation indicated that exosomes secreted by curcumin-pretreated lung cancer cells could decrease the survival, colony formation, and migration of recipient lung cancer cells (Wu et al. 2016).

Another finding of the present study was no significant difference between the number of exosomes released by colon cancer cells treated with or without berberine, indicating that berberine does not affect exosome secretion and, thus, may alter cargo content of tumor-derived exosomes to exert anti-tumor effect against colon cancer cells. This can be supported by the notion that the modulation in the function of tumor exosomes is related to alteration in their cargo content such as miRs and proteins/enzymes. For instance, curcumin was found to promote selective packaging of miR-21 in exosomes and, thereby, change cellular signature of the post-transcriptional network in recipient cells (Taverna et al. 2015).

In conclusion, berberine-primed exosomes may provide a novel and promising therapeutic approach as the monotherapy or the combination therapy for treating colon cancer. Notably, although our study results clearly indicate a potential future therapy by berberine-primed exosomes, the study was restricted by not evaluating the impact of berberine on the cargo content of tumor-derived exosomes as well as the molecular mechanisms through which berberine-primed exosomes offered protection against proliferation and metastasis of colon cancer cells. Therefore, investigating the pathways and molecular signaling mechanisms

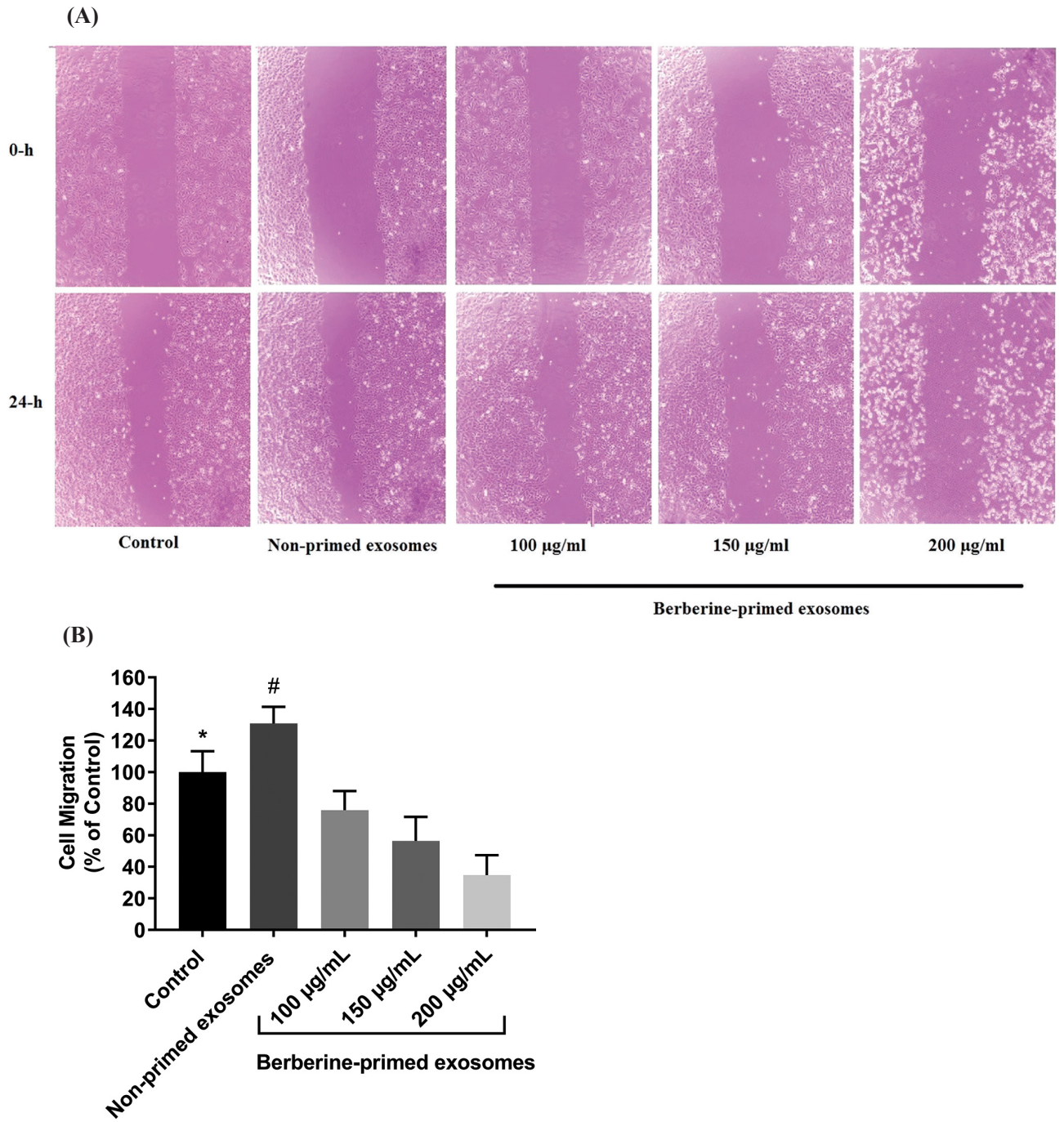

Fig. 3. Evaluating the effects of non-primed and berberine-primed exosomes on migrative ability of human colon cancer HCT116 cell line using wound healing assay.
Non-primed exosomes (200 μg/ml) derived from untreated colon cancer cells significantly increased the migrative ability of recipient colon cancer cells compared to the control. In a separate experiment, berberine-primed exosomes secreted by cancer cells treated with 10 μM of berberine significantly and dose-dependently reduce the migrative ability of recipient cancer cells compared to the control. (A) Representative images of the wound area were obtained by phase-contrast microscopy (200 × magnification) at 0 and 24 h post-scratching. (B) Statistical analysis of migrated cells. Results are expressed as mean ± SD of three independent experiments. *$p < 0.0001$ regarding the significant difference of control versus exosome isolates. $^{\#}p < 0.0001$ regarding the significant difference between non-primed exosomes vs. other groups.

can further assist to clear the mechanistic basis of the therapy and assist in the development of promising therapy against colon cancer. Besides, it is possible that the preparations of primed-exosomes can also be practicable with other therapeutic compounds that will equally be effective in donating benefits as berberine.

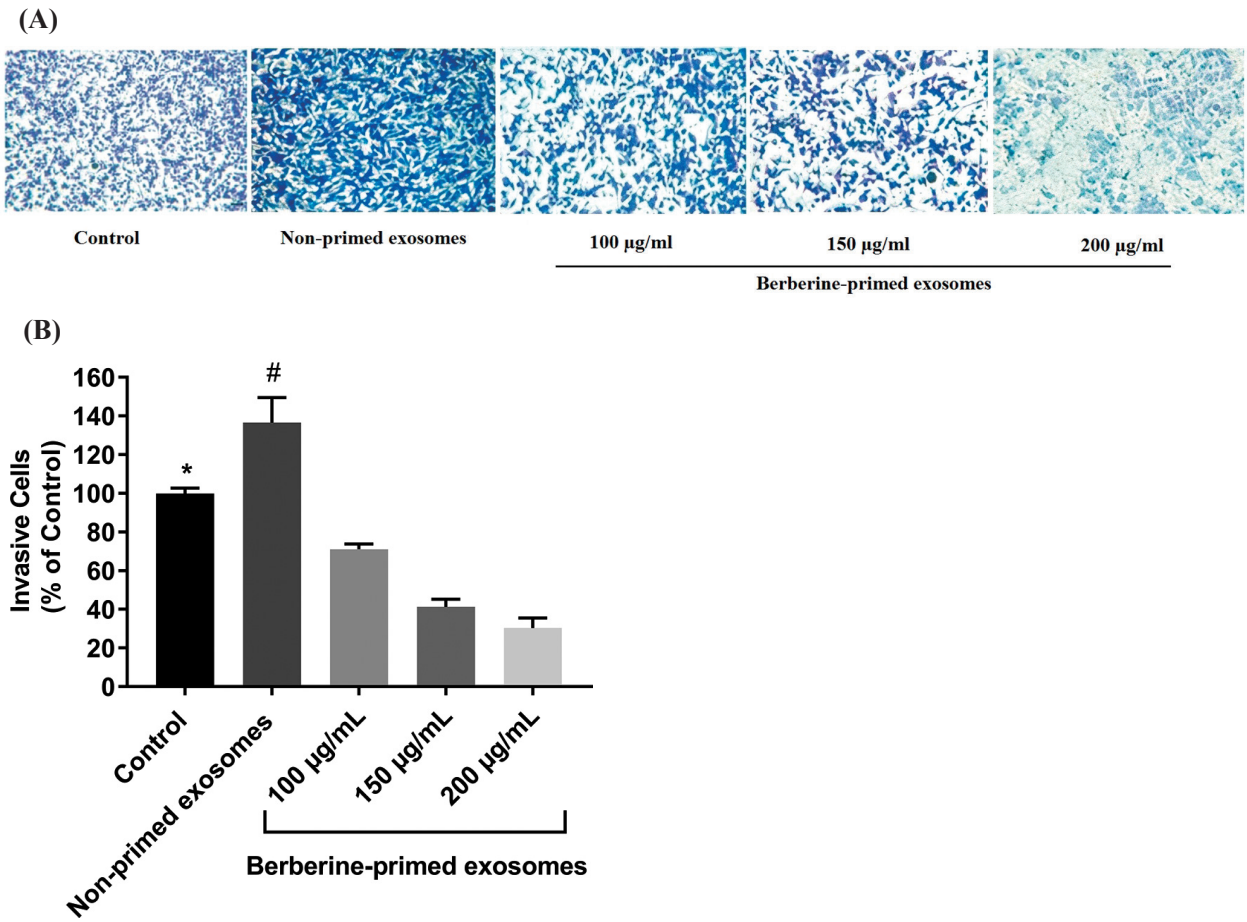

Fig. 4. Evaluating the effects of non-primed and berberine-primed exosomes on invasive ability of human colon cancer HCT116 cell line using transwell invasion assay.
Non-primed exosomes (200 μg/ml) derived from untreated colon cancer cells significantly increased the invasive ability of recipient colon cancer cells compared to the control. In a separate experiment, berberine-primed exosomes secreted by cancer cells treated with 10 μM of berberine significantly and dose-dependently reduce the invasive ability of recipient cancer cells compared to the control. (A) Representative images and (B) statistical analysis of transwell invasion assay. Results are expressed as mean ± SD of three independent experiments. $^{*}p < 0.0001$ regarding the significant difference of control versus exosome isolates. $^{\#}p < 0.0001$ regarding the significant difference between non-primed exosomes vs. other groups.

Conflict of Interest

The authors declare no conflict of interest.

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