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HERB-DRUG INTERACTION

Apoptotic Effect of Cisplatin and Cordycepin on OC3 Human Oral Cancer Cells*

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ABSTRACT **Objective**: To evaluate apoptotic effects of cisplatin and cordycepin as single agent or in combination with cytotoxicity in oral cancer cells. **Methods**: The influences of cisplatin (2.5 μg/mL) and/or cordycepin treatment (10 or 100 μmol/L) to human OC3 oral cancer cell line were investigated by morphological observation for cell death appearance, methylthiazoletetrazolium (MTT) assay for cell viability, flow cytometry assay for cell apoptosis, and Western blotting for apoptotic protein expressions. **Results**: Data demonstrated that co-administration of cisplatin (2.5 μg/mL) and cordycepin (10 or 100 μmol/L) resulted in the enhancement of OC3 cell apoptosis compared to cisplatin or cordycepin alone treatment (24 h), respectively (P<0.05). In flow cytometry assay, percentage of cells arrested at subG1 phase with co-treatment of cordycepin and cisplatin (30%) was significantly higher than cisplatin (5%) or cordycepin (12%) alone group (P<0.05), confirming a synergistically apoptotic effect of cordycepin and cisplatin. In cellular mechanism study, co-treatment of cordycepin and cisplatin induced more stress-activated protein kinase/Jun terminal kinase (JNK), the expressions of caspase-7, and the cleavage of poly ADP-ribose polymerase (PARP) as compared to cisplatin or cordycepin alone treatment (P<0.05). **Conclusion**: Cisplatin and cordycepin possess synergistically apoptotic effect through the activation of JNK/caspase-7/PARP pathway in human OC3 oral cancer cell line.

KEYWORDS cisplatin, cordycepin, apoptosis, OC3, synergistic

Betel quid-related oral cavity cancer is a unique type of the head and neck squamous cell carcinoma (HNSCC) with areca nut chewing habit, which is endemic in certain areas including Taiwan.[1] The cause of HNSCC is possibly induced by mechanical irritation and carcinogenic components from betel quid.[2,3] Remarkably, the incidence rate of intra-oral cancers related to betel quid chewing is still increasing in Taiwan.[4]

Clinically, surgery and radiotherapy are main treatments for early-staged human HNSCC. However, surgery will damage patient's face, and radiotherapy will affect their salivary secretion and taste. For late-staged patients, chemotherapy is often used in various combinations with surgery and radiotherapy in order to improve the poor survival rate, or to increase organ integrity for these patients.[5,6] Raltitrexed, cis-platinum, carboplatin, 5-fluorouracil and docetaxel etc., are often used for chemotherapy to treat HNSCC either as single agent or in combination.[7,8] Among these, cisplatin is the most efficient agent to treat HNSCC with 14%–41% response rate.[9] Although human head and neck cancer can be treated with cisplatin successfully, a major limitation of cisplatin treatment has been the development of drug-resistance.[9] Multiple mechanisms have been proposed for cisplatin resistance, including reduced intracellular accumulation of the drug, increased levels of glutathione, stimulation of epidermal growth factor receptor (EGFR), up-regulation of anti-apoptotic proteins, and down-regulation of pro-apoptotic proteins.[10-13] Cisplatin resistance is also associated with the altered activation of PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling pathways, or the suppression of tumor-suppressor genes, p53 and phosphatase and tensin homolog (PTEN).[14,15]

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Cordycepin (3'-deoxyadenosine) is a pure substance extracted from *Cordyceps sinensis*, and is considered as an active component in treating tumor cells.[16-18] It has been demonstrated that cordycepin has anti-tumor effect on mouse melanoma and lung carcinoma cells.[19] It is also reported that cordycepin could inhibit the formation of polyadenylate polymerase (PAP) or inactivate mRNA polyadenylation to induce tumor cell apoptosis,[20,21] which is characterized by the cellular rounding-up, cytoplasmic contraction, plasma membrane blebbing, chromatin condensation, and DNA fragmentation.[22]

During the course of apoptosis, the cleavage of caspases (cysteine-dependent aspartate specific protease), such as caspase-3 and-7, could be observed, which will further cleave poly ADP-ribose polymerase (PARP), responsible for DNA repair,[23] and results in the execution of cell death.[24,25] Moreover, it has been shown that stress signals activate the stress-activated protein kinases (SAPK)/Jun terminal kinase (JNK) protein kinases, which will mediate cellular steps in apoptosis of some cell types.[26-28]

Since cordycepin and cisplatin both show anti-tumor effects, an attempt was made to elucidate the combined effect of cisplatin and cordycepin on the tumor cell death of OC3 cell line, a domestic cell line established from a buccal cancer patient with long-term betel nut chewing history in Taiwan in this study.[1,29] Interestingly, a synergistic effect of cordycepin and cisplatin on apoptosis in OC3 was observed. This finding may encourage developing more effective chemotherapy agents with different concomitant administration against betel nut-induced oral cancer.

METHODS

Chemicals

Cordycepin, penicillin-streptomycin, 3-3-[4,5-dimethylthiazol]- 2,5-diphenyltetrazolium bromide, methylthiazoletetrazolium (MTT), dimethyltetrazolium bromide (DMSO), RNase A, and propidium iodine (PI) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum, Dulbecco's modified eagle medium (DMEM) and Keratinocyte-SFM medium were purchased from Gibco Co. (Grand Island, NY, USA). Sodium hydroxide was from Merck Co. (Dermstadt, Germany). 4-(2-hydroxyethyl)-1-piperazin eethanesulfonic acid (HEPES) was purchased from Mallinckrodt Baker, Inc. (Philipsburg, NJ, USA). Sodium bicarbonate, sodium carbonate and sodium chloride were purchased from Riedel der Haen (Seelze, Germany).

Cell Line and Cell Culture

OC3 is a cell line derived from oral epidermal carcinoma, an indigenous oral cancer cell line in Taiwan.[1,29] OC3 cells were maintained in DMEM plus two folds volume of Keratinocyte-SFM mixed medium supplemented with 24 mmol/L $NaHCO_3$, 25 mmol/L HEPES, 100 parts per million (ppm) penicillin, 100 ppm streptomycin and 10% v/v heat-inactivated fetal bovine serum, pH 7.4, incubated in a humidified atmosphere adjusted to 95% air, 5% CO_2 at 37 ℃.[30]

MTT Assay for Cell Viability

MTT assay was employed to determine cell viability with the treatment of cordycepin and/or cisplatin.[31] OC3 cells were seeded in 96-well plate (Techno Plastic Products AG, Trasadingen, Switzerland) with 1×10^4 cells in 100 μL serum medium among each well. After reaching 70%–80% confluence, cells were treated without or with cordycepin, cisplatin or both agents in various combination of concentrations (10 μmol/L, 100 μmol/L, or 1 mmol/L cordycepin alone; 0.5 μg/mL, 2.5 μg/mL, or 5 μg/mL cisplatin alone; and 2.5 μg/mL cisplatin combined with 10 μmol/L, 100 μmol/L, or 1 mmol/L cordycepin, respectively, for 24 h). MTT was added with final concentration of 0.5 mg/mL, and then incubated for 4 h at 37 ℃. The medium was removed and DMSO (50 μL) was added into each well to dissolve the crystals by shaking the plate weakly for 20 min in dark. The absorbance (optical density, OD) values in each treatment were then determined at λ=590 nm by an enzyme-linked immunosorbent assay (ELISA) microplate reader (VersaMax, MDS Inc., Toronto, Canada).

Morphological Study for Cell Death Appearance

OC3 cells were seeded at a concentration of 6×10^5 cells in a 6 cm Petri dish (Techno Plastic Products AG, Trasadingen, Switzerland) supplemented with 2 mL serum medium. After reaching 70%–80% confluence, cells were treated without or with 100 μmol/L cordycepin only, 2.5 μg/mL cisplatin only, or 100 μmol/L cordycepin combined with 2.5 μg/mL cisplatin for 24 h, respectively. Cell morphology was then observed and recorded under the light microscopy (Olympus CK40, Hamburg, Germany).

Flow Cytometry Analysis for Cell Apoptosis

In order to investigate whether cordycepin and/or cisplatin could induce cell apoptosis, flow cytometric analysis was used with propidium iodine stain to determine DNA fragmentation and the redistribution of cell cycle.[32,33] OC3 cells were seeded in 6 cm Petri dish with 2 mL serum medium, which contained 6×10^5 cells. After reaching 70%–80% confluence, cells were treated without or with 100 μmol/L cordycepin only, 2.5 μg/mL cisplatin only, or 100 μmol/L cordycepin combined with 2.5 μg/mL cisplatin for 24 h, respectively. The treated cells were harvested with trypsin, washed with PBS, and fixed in 75% ethanol for at least 2 h at –20 ℃. After fixation, cells were washed in cold PBS and then collected by centrifugation and stained with PI solution (40 μg/mL PI and 100 μg/mL RNase in PBS). The stained cells were analyzed using a fluorescence activated cell sorter (FACScan, Becton-Dickinson, Mountain View, CA, USA) at λ = 488 nm and analyzed by Cell-Quest™ software (Becton-Dickinson, Mountain View, CA, USA). The DNA content distribution of normal growing cells is characterized by two peaks—G_1/G_0 and G_2/M phase. G_1/G_0 phase indicates that cells are arrested at resting state of cell cycle with most diploid DNA content, while cell DNA content in G_2/M phase increased as a consequence of progressing in cell cycle. Cells in subG_1 phase have least DNA content in cell cycle distribution, called hypodiploid. The hypoploid DNA contents represent the fragmentation of DNA.[32,33]

Western Blot for Apoptotic Protein Expression

After reaching 70%–80% confluence, cells were treated without or with 100 μmol/L cordycepin only, 2.5 μg/mL cisplatin only, or 100 μmol/L cordycepin combined with 2.5 μg/mL cisplatin for 24 h, respectively (2.5×10^5) cells. were cultured in 3.5 cm dish. After treatments, cells were rinsed with cold PBS. Then, the cells were harvested by using 30 μL lysis buffer [50 mmol/L Tris-base, 150 mmol/L NaCl, 1% w/v NP40, 0.1% w/v sodium dodecyl sulfate (SDS), 0.5% v/v deoxychloride acid and 1 mmol/L phenylmethy lsulfongl fluoride (PMSF)]. The cell lysate was subjected to centrifugation at $12,000 \times g$ for 20 min at 4 ℃. The supernatant, which contained cell protein, was collected and stored at –20 ℃ until use. The protein concentration was determined by Lowry method.[34] Immunoblot analysis was performed as previously described.[35] Antibody against β-actin was purchased from cell signaling (Beverly, MA, USA). In brief, 20 μg of proteins were solubilized in 1× SDS sample buffer and loaded on a 12.5% w/v sodium dode sulfate polyacrylamide gel electrophoresis (SDS-PAGE) minigels (Mini-Protein Ⅱ system, Bio-Rad, Richmond, CA, USA). Electrophoresis was performed at 100 V for 100 min using standard SDS-PAGE running buffer. The proteins were transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad, Hercules, CA, USA) at 80 mA for 1 h in transfer buffer. The PVDF membrane with transferred protein was incubated in blocking buffer at room temperature for 1 h, and then incubated in fresh blocking buffer containing the primary antibody for 16–18 h at 4 ℃. After washing three times with PBS containing 0.5% Tween-20 for 30 min, the signal in PVDF membrane was detected with 1:4000 dilution of horseradish peroxidase-conjugated secondary antibody, and then visualized by enhanced chemiluminescence (ECL) detection kit (Amersharn-Pharmacia International PLC, Amersham, Bucks, UK). Proteins of interest were quantitated by a computer-assisted image analysis system (Quantity One, Huntington Station, NY, USA). The amount of β-actin (43 kDa) in each lane was also detected as a control to correct the expression of p-JNK, caspase-7 and PARP proteins.

Statistics Analysis

SPSS 13.0 software was used to do the statictical analysis. Each data point in the figures represents the mean ± standard error of mean (SEM) of three independent experiments. Statistically significant differences between treatments and controls were determined by one-way ANOVA and then least significance difference (LSD) comparison procedure. Statistical significance was set at $P<0.05$.

RESULTS

Effect of Cordycepin and/or Cisplatin on OC3 Cell Viability

Results showed that the combined treatment of cordycepin and cisplatin had significantly negative effect on OC3 cell viability with dose-dependent manners (Figure 1, $P<0.05$). Cell surviving rates with 10 μmol/L cordycepin, 100 μmol/L cordycepin and 2.5 μg/mL cisplatin alone treatment were 95%, 70% and 75%, respectively, as cordycepin (10 μmol/L) plus cisplatin (2.5 μg/mL) and cordycepin (100 μmol/L) plus cisplatin (2.5 μg/mL) were 53% and 44%, respectively, which demonstrated

that concomitant administration of cisplatin and cordycepin could significantly induce more cell death with synergistic phenomenon (Figure 1, $P<0.05$).

Due to that 0.5 μg/mL cisplatin treatment has no difference with control treatment, and 5 μg/mL cisplatin treatment has no difference with 2.5 μg/mL cisplatin plus cordycepin (10 μmol/L, 100 μmol/L, and 1 mmol/L) treatments (Figure 1, $P>0.05$), 100 μmol/L cordycepin only, 2.5 μg/mL cisplatin only, or 100 μmol/L cordycepin combined with 2.5 μg/mL cisplatin were used for the following experiments.

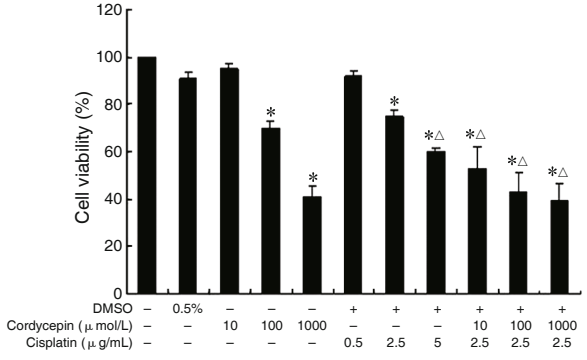

Figure 1. Effect of Cordycepin and Cisplatin on Cell Viability in OC3 Cells (n=3)

Notes: *$P<0.05$, compared with the control (plain medium); △$P<0.05$, compared with cisplatin (0.5 and 2.5 μg/mL) treatment

Effect of Cordycepin and/or Cisplatin on Morphological Changes in OC3 Cells

OC3 cells were treated with 100 μmol/L cordycepin only, 2.5 μg/mL cisplatin only, or 100 μmol/L cordycepin combined with 2.5 μg/mL cisplatin for 24 h, respectively, and morphological changes were examined under a light microscopy. In control and DMSO treatments, cells showed polygonal shape with healthy appearances, blurred outline and firm attachment, which is normal cell growth phenomenon (Figures 2A and 2B). After 24 h treatment with 100 μmol/L cordycepin, cells appeared rounded-up phenomenon but still adhered to the ground matrix (Figure 2C). After 24 h treatment with 2.5 μg/mL cisplatin, many cells rounded-up with some floating in medium (Figure 2D). A concomitant administration of 2.5 μg/mL cisplatin and 100 μmol/L cordycepin for 24 h resulted in more loss of cell attachment to ground matrix, more appearance of membrane blebbings, and much more floating cells (Figure 2E). These phenomena suggested that concomitant administration of both cisplatin and cordycepin induced apoptotic cell death in OC3 cell line, which was more effective than cisplatin or cordycepin only treatments.

Tendency and Analysis of Cell Cycle under Cordycepin and/or Cisplatin Influence in OC3 Cells

Previous results illustrated that cordycepin and/or cisplatin would induce cell death in OC3 cells. Flow cytometry analysis was used to determine whether DNA fragmentation occurred and if there was any change in cell cycle progression. The distribution of PI stained OC3 cells in control (Figure 3A), DMSO (Figure 3B), 100 μmol/L cordycepin only (Figure 3C), 2.5 μg/mL cisplatin only (Figure 3D), and 100 μmol/L cordycepin combined with 2.5 μg/mL cisplatin for 24 h (Figure 3E) were illustrated, respectively. Statistical analysis from 3 independent experiments of Figures 3A, 3B, 3C, 3D, and 3E regarding the change of subG_1, G_1, and G_2/M phases of cell cycle in percentages was

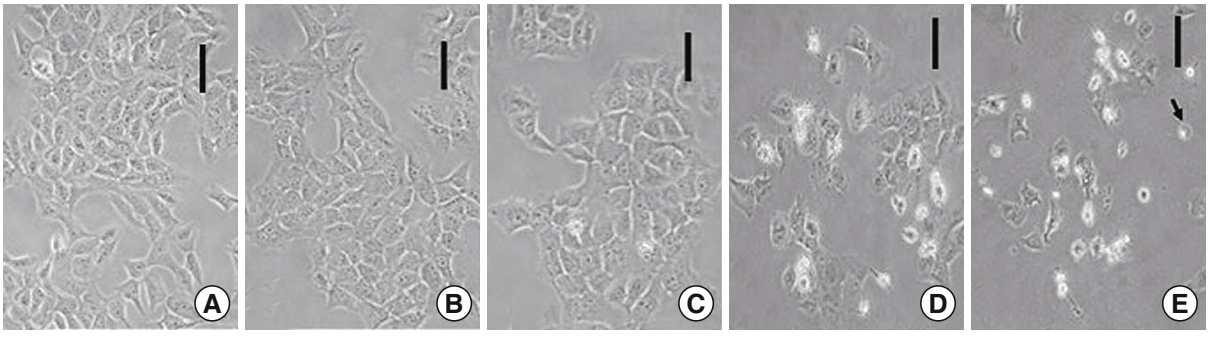

Figure 2. Effect of Cisplatin, Cordycepin and Combined Treatment of Cordycepin and Cisplatin on Morphological Changes in OC3 Cells

Notes: A: OC3 cells were treated in plain medium, B: medium with DMSO (0.5%), C: medium with 100 μmol/L cordycepin, D: medium with 2.5 μg/mL cisplatin, and E: medium with 100 μmol/L cordycepin plus 2.5 μg/mL cisplatin for 24 h. Morphological changes of cells were examined under light microscopy (bar: 0.1 mm; arrow: rounded cells). Rounded-up cells were readily observed indicated by arrow in cisplatin and cordycepin treatment for 24 h. Experiments were performed three times with similar results

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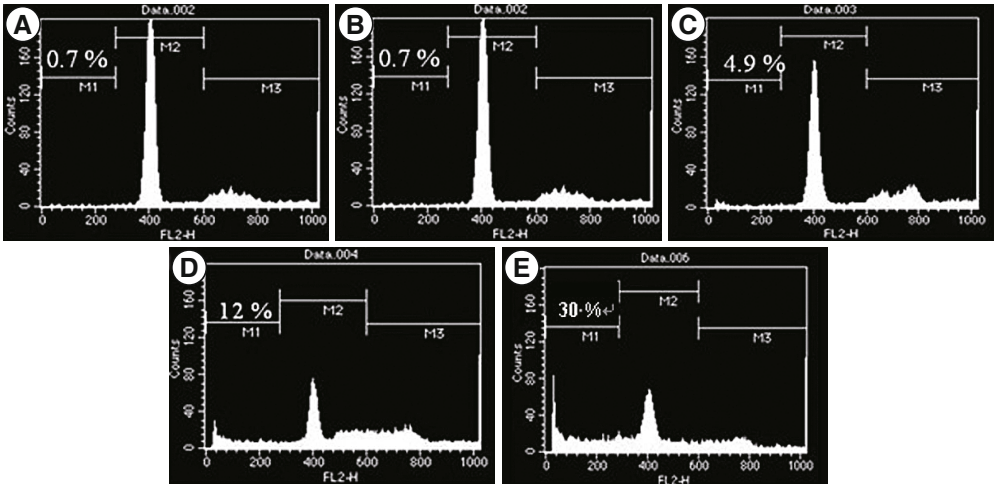

Figure 3. Effect of Cisplatin, Cordycepin and Combined Treatment of Cordycepin and Cisplatin on SubG$_1$ Cell Cycle Phase in OC3 Cells

Notes: A: The histogram plot of flow cytometry analysis in OC3 cells treated in plain medium, B: medium with DMSO (0.5%), C: medium with 100 μmol/L cordycepin only, D: medium with 2.5 μg/mL cisplatin only, and E: medium with 100 μmol/L cordycepin plus 2.5 μg/mL cisplatin for 24 h were illustrated. After the treatments, cells were fixed, stained with PI, and analyzed of cell cycle progression by flow cytometry as described in methods. M_1=SubG$_1$ (cells with less than normal amount of DNA content); M_2=G$_1$ (cells in G$_1$ cell cycle phase); M_3=G$_2$/M (cells in G$_2$/M cell cycle phase)

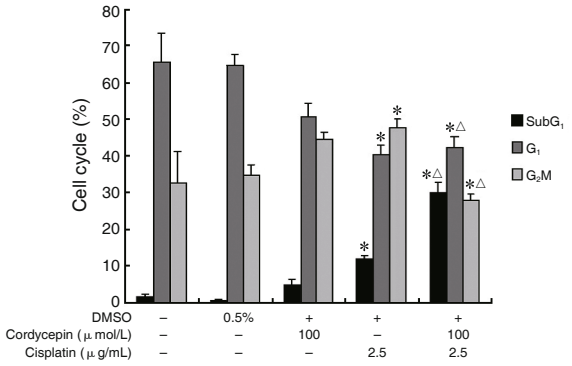

Figure 4. Quantification in Percentage among SubG$_1$, G$_1$ and G$_2$/M Phases Cell Number (*n*=3)

Notes: $^*P$<0.05, compared with control (plain medium) in each cell cycle phase (subG$_1$, G$_1$ or G$_2$/M); $^\triangle P$<0.05, compared with cisplatin (2.5 μg/mmol/L) treatment in subG$_1$ and G$_2$/M cell cycle phases

analyzed, and illustrated in Figure 4.

Around 60%–70% cells in control and DMSO treatments distributed in G$_1$ phase (Figures 3A, 3B and Figure 4). However, the percentage of G$_1$ phase decreased to 40%–50% as cells were treated with cordycepin only, cisplatin only, and cordycepin plus cisplatin (Figures 3C, 3D, and 3E and Figure 4). The percentage of G$_2$/M phase cells increased from 30%–40% in control groups to 50% in cordycepin or cisplatin only groups (Figure 3A, 3C, and 3D and Figure 4). Interestingly, the percentage of G$_2$/M phase cells in cordycepin plus cisplatin group dropped back to 30% (Figure 3E and Figure 4). Moreover, the percentage of subG$_1$ phase cells in control groups was 1%–2% (Figures 3A, 3B and Figure 4), and it significantly increased to 5% in cordycepin alone group and 12% in cisplatin alone group, respectively (Figures 3C and 3D and Figure 4, P<0.05). Specifically, the percentage of subG$_1$ phase cells in cordycepin and cisplatin co-treatment group significantly increased to 30% (Figure 3E and Figure 4, P<0.05). These findings suggest that there was a synergistically apoptotic effect of cordycepin and cisplatin co-treatment on OC3 cells based on subG$_1$ phase cell proportion changes.

Effects of Cisplatin and/or Cordycepin Treatments on p-JNK, Caspase-7, and PARP in OC3 Cells

Previous results illustrated that cisplatin and cordycepin would cause cell apoptosis in OC3 cells. It has been shown that JNK, caspase, and PARP pathways may play important roles in apoptosis among tumor cells activated by chemotherapy agents.[23,36,37] Thus, the expressions of p-JNK, caspase-7, and PARP protein in OC3 cells were further investigated. The expression of p-JNK in OC3 cells under control and DMSO treatments was low (Figures 5A and 5B). However, the expression of p-JNK increased by cordycepin (100 μmol/L), cisplatin (2.5 μg/mL), and cordycepin (100 μmol/L) and cisplatin (2.5 μg/mL) co-treatment in OC3 cells. Cisplatin (2.5 μg/mL) significantly induced p-JNK expression as compared to control

(Figures 5A and 5B, *P*<0.05). Moreover, cordycepin and cisplatin co-treatment induced more p-JNK expression compared to cordycepin or cisplatin alone treatments (Figures 5A and 5B, *P*<0.05).

The expression of caspase-7 in OC3 cells under control and DMSO treatments was very low (Figures 5A and 5C). However, the expression of caspase-7 increased by cordycepin (100 μmol/L), cisplatin (2.5 μg/mL), and cordycepin (100 μmol/L) and cisplatin (2.5 μg/mL) co-treatment in OC3 cells. Cisplatin (2.5 μg/mL) significantly induced caspase-7 expression compared to control (Figures 5A and 5C, *P*<0.05). Moreover, cordycepin and cisplatin co-treatment induced much more caspase-7 expression compared to cordycepin or cisplatin alone treatments (Figures 5A and 5C, *P*<0.05).

There was no fragmentation of PARP (89 kDa) in OC3 cells under control and DMSO treatments (Figures 5A and 5D). Fragmentation of PARP was slightly increased by 100 μmol/L cordycepin treatment, and significantly increased by 2.5 μg/mL cisplatin (Figures 5A and 5D, *P*<0.05). Interestingly, co-treatment of cisplatin (2.5 μg/mL) and cordycepin (100 μmol/L) triggered almost complete cleavage of PARP in OC3 cells, which was significant difference compared to cordycepin or cisplatin alone treatments (Figures 5A and 5D, *P*<0.05). The results of Western blot showed that cisplatin and cordycepin co-treatment-induced apoptosis was accompanied by caspase-7 and PARP activations.

DISCUSSION

Continuous proliferation is one of the most typical properties of cancer cells.[38] It is generally recognized that the suppressor genes responsible for controlling cell proliferation lose their functions in transformed cells.[39] These transformed cells in culture also possess capacity of proliferation. It is assumed that if a chemical is capable of inhibiting transformed cells progressing in cell cycle, it could potentially become a valuable anti-tumor agent. The combination of different chemicals might be more effective in treating cancer cells, because many studies have demonstrated that combination of different chemicals do have synergistically apoptotic effects on tumors.[40,41]

In the present study, co-administration of cisplatin

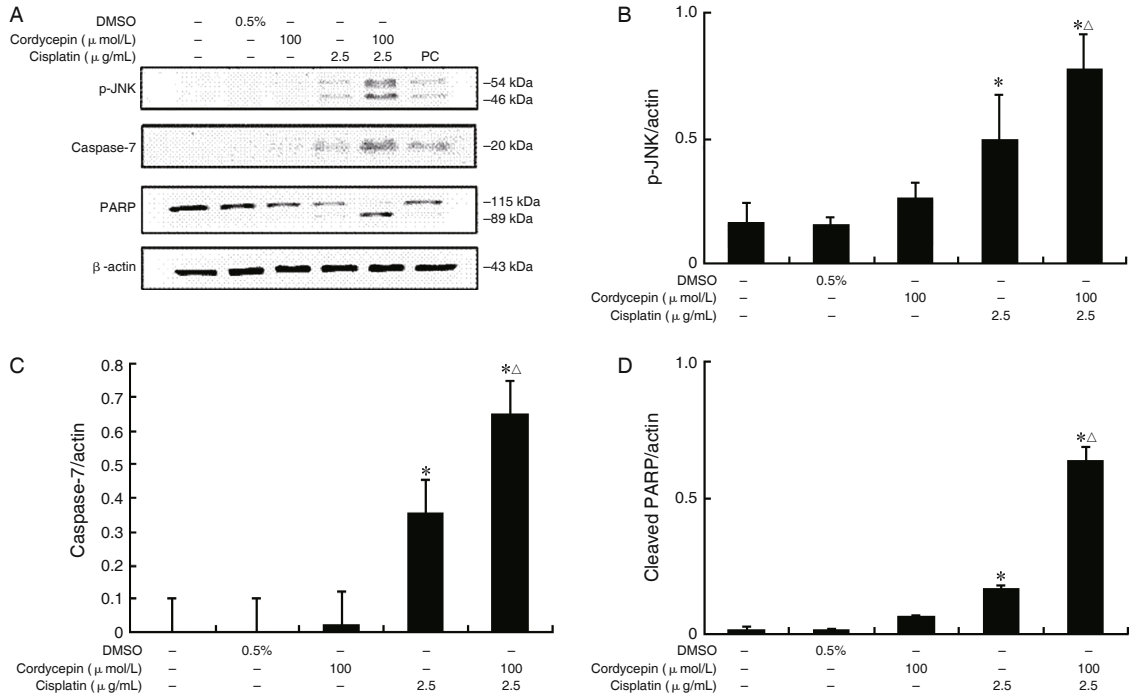

Figure 5. Effect of Cisplatin, Cordycepin and Combined Treatment of Cordycepin and Cisplatin on the Protein Expression of p-JNK, Caspase-7, and PARP in OC3 Cells (*n*=3)

Notes: A: p-JNK (54/46 kDa), caspase-7 (20 kDa), and PARP (89 kDa) specific bands were detected by Western blot; B: The integrated optical densities (IOD) of p-JNK; C: caspase-7, and D: PARP proteins after normalization with β-actin (43 kDa) in each lane using PDI image system were demonstrated. ***P*<0.05, compared with control; $^{\triangle}$*P*<0.05, compared with cisplatin (2.5 μg/mL) only and 100 μmol/L cordycepin only treatments

and cordycepin induced more OC3 cell rounded-up with blebbing membrane, and further reduced OC3 cell viability with apparent DNA fragmentation. The induction of DNA fragmentation phenomenon by cisplatin and cordycepin correlated with the increase of subG$_1$ phase cell number, which confirms that cisplatin and cordycepin could induce more apoptosis in OC3 cells.

In morphological change and cell viability studies, cisplatin and/or cordycepin treatment did induce cell death with more loss of cell attachment to ground matrix, more appearance of membrane blebbings, and much more floating cells. The percentage of cell death induced by the combination of both agents was about 55%, which was equal to the addition of 30% (by 100 μmol/L cordycepin) and 25% (by 2.5 μg/mL cisplatin). In fact, it has been demonstrated that the lower doses of cordycepin causes poly(A) changes to reduce the proliferation of NIH3T3 fibroblasts, and the higher doses of cordycepin inhibits cell attachment with a reduction of focal adhesions.[42] Thus, our observations are comparable to their findings.

In cell cycle analysis, cisplatin plus cordycepin co-treatment induced 30% subG$_1$ phase cell number, which the effect was almost double as compared to the addition of 5% (by 100 μmol/L cordycepin) and 12% (by 2.5 μg/mL cisplatin), suggesting a synergistic effect of cordycepin and cisplatin on apoptosis in OC3 cells. It has been shown that combination of agents could induce more anti-tumor effectiveness.[43-45] Thus, our observation is not unprecedented.

In cell cycle analysis, the proportion of cells arrested at subG$_1$ phase increased in cordycepin alone treatment, cisplatin alone treatment, and cisplatin plus cordycepin co-treatment. In contrast, the proportion of cells arrested at G$_1$ significantly reduced. Moreover, proportion of cells arrested at G$_2$/M phase increased in cisplatin or cordycepin only treatments, but significantly decreased in cisplatin plus cordycepin co-treatment. This phenomenon inferred that a considerable portion of G$_1$ phase cells in cisplatin or cordycepin only treatments progressed toward G$_2$/M phase. Once progressed into G$_2$/M phase, cells became insensitive to cisplatin or cordycepin. This incident might coincide with the possible development of cisplatin resistance after chemotherapy,[9] indicating that cells might change under anti-tumor agent pressure and became even more proliferative. However, co-treatment of cisplatin and cordycepin significantly increased subG$_1$ phase cells and reduced G$_2$/M phase cells, suggesting that both agents could work more effectively to induce OC3 cell DNA fragmentation, and then apoptosis.

It has been illustrated that anti-tumor agents could activate caspase-7 expression and then cleave PARP, which would further obstruct DNA repair and result in the execution of cell apoptosis.[23-25] Moreover, the activation of SAPK/JNK protein kinases, mediating cellular steps in apoptosis of some cell types,[26-28,46] could activate the expression of caspase-7 and PARP cleavage.[47] Furthermore, many works have demonstrated that JNK is required for cordycepin-mediated induction of G$_2$/M cell cycle arrest via p21 WAF1 expression in human colon and bladder cancer cells.[48,49] In the present study, co-treatment of cordycepin and cisplatin significantly induced more expression of p-JNK and caspase-7, and the cleavage of PARP compared to cisplatin or cordycepin alone treatments. When we further analyzed the differences among those proteins, there was an additive effect in p-JNK expression in co-treatment of cordycepin and cisplatin. Moreover, synergistic effect on the expression of caspase-7 and the cleavage of PARP protein could be observed in co-treatment of cordycepin and cisplatin compared to cisplatin or cordycepin alone treatments. It has been demonstrated that the combinations of several antitumor drugs such as rapamycin, carboplatin and paclitaxel; IP6 and paclitaxel; COX-2 and EGFR tyrosine kinase inhibitors; and cisplatin and protein kinase C (PKC) inhibitor could lead to additive and/or synergistic effects in head and neck tumor cells through different cellular mechanisms.[44,45,50] Thus, JNK, caspase, and PARP pathways may play important roles in apoptosis of tumor cells activated by chemotherapy agents, and our observation is parallel to those observations.

In conclusion, cisplatin and cordycepin possess synergistically apoptotic effect by activating the expression of p-JNK, caspase-7, and PARP pathway in human OC3 oral cancer cell line. Cordycepin could induce synergistic effect with cisplatin, which highly suggest that combination of cordycepin and cisplatin might be a good potential anti-tumor drug compared to single agent for chemotherapy. Certainly, more cell lines used in the study plus *in vivo* experiments will further replenish the outcome.

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