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Metformin enhances inhibitive effects of carboplatin on HeLa cell proliferation and increases sensitivity to carboplatin by activating mitochondrial associated apoptosis signaling pathway

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Abstract. – OBJECTIVE: Cervical cancer has become the fourth most common cancer in developing countries. This study aimed to investigate anti-tumor effects of Metformin combining with carboplatin in cervical cell line, HeLa cell.

MATERIALS AND METHODS: Human cervical cancer cell line, HeLa cell, was treated with Metformin (5 mmol/l or 10 mmol/l) or/and carboplatin (25 mg/l or 50 mg/l) at different final concentrations, and divided into 8 groups. 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell viability. Acridine orange/ethidium bromide (AO/EB) staining was used to examine nuclear fragments and cell apoptosis. Annexin V/propidium iodide (PI) staining was employed to detect apoptosis of HeLa cells. Mitochondrial membrane potential of the HeLa cells was evaluated by staining with 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) reagent.

RESULTS: MTT results showed that Metformin combining carboplatin significantly reduced HeLa cell viability compared to that of no-drug treatment group ($p<0.05$). Metformin combining carboplatin significantly increased the amounts of nuclear fragments compared to that of no-drug treatment group ($p<0.05$). The flow cytometry assay results indicated that Metformin combining carboplatin significantly enhanced the apoptotic rates compared to that of no-drug treatment group ($p<0.05$). The JC-1 staining findings illustrated that Metformin combining carboplatin significantly decreased the mitochondrial membrane potential compared to that of no-drug treatment group ($p<0.05$).

CONCLUSIONS: Metformin enhanced the inhibitive effects of carboplatin on HeLa cell proliferation. Metformin increased the sensitivity of HeLa cell to the treatment of Carboplatin by activating mitochondrial-associated apoptosis signaling pathway.

Key Words:
Cervical cancer, Metformin, carboplatin, Apoptosis, Mitochondrial membrane potential.

Introduction

Cervical cancer has become the fourth most common cancer in the developing countries, with over 130000 new cases and over 50000 death cases per year in China[1-3]. The human papilloma virus (HPV) infection is considered to the most common and critical factor for the progression and development of the cervical cancer in clinical, just because over 99% of the cervical cancers illustrate the positive HPV DNA sequences[4]. In clinical, there are many approaches for preventing the cervical cancer, including immunotherapy of cytokines, polyammonia synthesis inhibitor, supplement of trace elements and oral drugs. However, the effects of above approaches are also limited. Although the HPV vaccines have been applied for cervical cancer patients, which could only prevent approximate 70% of the cases[5,6]. In nowadays, the surgery is considered to be the preferred therapy for the early-stage cervical cancer patients, however, which may damage the reproductive functions of females[7]. For the advanced stage or metastatic cervical cancer

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patients, the chemotherapy is also the classical approach[8]. The platinum can effectively kill the tumor cells and remains the first-line anti-cancer drug[9]. The mainstream view holds the view that the cisplatin is the primary chemotherapy drug for the advanced-cervical cancer, however, the response rate of which is only about 20% or less[10]. carboplatin is a kind of platinum analog that characterizes by the reduced toxicity and extensive anti-tumor spectrum compared to the cisplatin[11]. However, the chemotherapeutic drug resistance always induces the recurrence of the tumors in clinical. For the platinum therapy, although the patients initially response to the drug, who undergoing the single-agent platinum therapy usual relapse and become refractory[12,13]. Therefore, it's necessary to discover a novel chemotherapeutic drug for treating cervical cancer. Metformin is frequently used as a anti-diabetic drug in clinical and has been proven to be a effective drug for inhibiting proliferation of many cancer cells[14], such as breast cancer[15], ovarian cancer[16], prostate cancer[17]. The previous studies[18,19] reported that metformin combining with cisplatin could significantly enhance the anti-tumor effects compared to single-agent platinum application for ovarian cancer and esophageal cancer. However, whether the metformin could enhance the anti-tumor effects of carboplatin has not been investigated at home and abroad. Therefore, the present study aimed to investigate the anti-tumor effects of Metformin combining carboplatin in the cervical cell line, HeLa cells.

Materials and Methods

Cell Culture

The human cervical cancer cell line, HeLa, was provided by the Changchun Institute of Applied Chemistry, Chinese Academy of Science (Changchun, China). HeLa cells were maintained in the Dulbecco's Modified Eagle Medium (DMEM) F12 (Gibco BRL. Co. Ltd., Grand Island, NY, USA) supplemented with 100 μg/ml streptomycin (Gibco BRL. Co. Ltd., Grand Island, NY, USA), 100 U/ml penicillin (Gibco BRL. Co. Ltd., Grand Island, NY, USA) and 10% fetal bovine serum (FBS, Shanghai Bioleaf Biotech. Co. Ltd., Shanghai, China) at 37°C with 5% CO_2. The present study was approved by the Ethics Committee of Sino-Japanese Friendship Hospital of Jilin University (Changchun, China).

Drug Treatment and Trial Grouping

The HeLa cells were treated by adding the Metformin (5 mmol/l or 10 mmol/l, Sigma-Aldrich, St. Louis, MO, USA) or/and carboplatin (25 mg/l or 50 mg/l, Sigma-Aldrich, St. Louis, MO, USA) at different final concentrations. Therefore, the cells were divided into 8 groups, including Metformin (5 mmol/l) group, Metformin (10 mmol/l) group, carboplatin (25 mg/l) group, carboplatin (50 mg/l) group, Metformin (5 mmol/l) + carboplatin (25 mg/l) group, Metformin (5 mmol/l) + carboplatin (50 mg/l) group, Metformin (10 mmol/l) + carboplatin (25 mg/l) group, Metformin (10 mmol/l) + carboplatin (50 mg/l) group. Meanwhile, the HeLa cell treated without any drugs was designed as the no drug treatment group.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The cultured HeLa cells were digested by using the 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) containing 0.02% ethylene diamine tetraacetic acid (EDTA, Beyotime Biotech. Shanghai, China) at logarithmic-growth phase. The cells were seeded into the 96-well plates (Corning Costar, Corning, NY, USA) and cultured in the Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) for 24 h at 37°C with 5% CO_2. HeLa cells were treated with the Metformin or/and carboplatin and cultured for 24 h. Finally, the MTT (Shanghai Bioleaf Biotech. Co. Ltd., Shanghai, China) was added to HeLa cells and incubated for 4 h at 37°C. Later, the supernatant in the 96-well plates was discarded and 150 μm dimethylsulfoxide (DMSO, Shanghai Yuanye BioSci. Co. Ltd., Shanghai, China) was added to dissolve the formazan products for 10 min. Finally, the optimal density (OD) values of 96-well plates were measured at wavelength of 570 nm by employing an enzyme linked immunosorbent assay (ELISA) plate reader (Synergy 2, Bio-Tek Instruments Inc., Winooski, VT, USA).

Acridine Orange/Ethidium Bromide (AO/EB) Staining

The nuclear fragments and the chromatin-condensation are considered to be the biomarker for the apoptotic cells, which could be reflected by AO/EB staining (SolarBio. Sci. Tech. Co. Ltd., Beijing, China) according to the previous study [20]. Briefly, HeLa cells were seeded into the 6-well plates (5×10^5 cells/well) containing 2 ml

DMEM medium supplementing with 10% fetal bovine serum (FBS) at 37°C for 24 h. Next, HeLa cells were treated with Metformin or/and carboplatin and cultured for another 24 h. Then, the HeLa cells were harvested and centrifuged for 5 min at 1200 r/min and the supernatant was discarded. The remained pellets were re-suspended in the phosphate-buffered saline (PBS, Beyotime Biotech., Shanghai, China). A total of 4 μl ethidium bromide (SolarBio. Sci. Tech. Co. Ltd., Beijing, China) and 4 μl of acridine orange (SolarBio. Sci. Tech. Co. Ltd., Beijing, China) were added into the plates and incubated for 10 min. HeLa cells were centrifuged and the supernatants were discarded. The remained pellets were re-suspended in the PBS solution, and the suspension was seeded on the slide. Finally, the stained cells on the slide were observed by using the fluorescent microscope (Mode: BX53T, Olympus, Tokyo, Japan) with 520 nm emissions and 450 nm excitation.

Annexin V/Propidium Iodide (PI) Staining

The HeLa cells were treated in accordance with the protocol of Annexin/propidium staining. In brief, the HeLa cells were re-suspended in PBS and 5 μl Annexin-V/FITC (Dojindo, Kumamoto, Japan) and 5 μl PI (50 μg/ml, Dojindo, Kumamoto, Japan) in dark at room temperature for 15 min. After that, the cells were centrifuged for 5 min at 1000 r/min and the pellets were re-suspended by using PBS buffer. Finally, the percentage of apoptotic cells was evaluated by using the flow cytometry (Beckman Coulter Inc., Brea, CA, USA) within 1 h after the Annexin-V/PI staining.

Mitochondrial Membrane Potential Assay

Twenty-four hours post the metformin or/and carboplatin treatment, the HeLa cells were centrifuged at 3000 r/min for 5 min and the pellets were washed by using PBS. Next, the HeLa cells were stained with 5,5,6,6-tetrachloro-1,1,3,3- tetraethylbenzimidazolylcarbocyanine iodide (JC-1, SolarBio Sci., Tech., Co., Ltd., Beijing, China) at final concentration of 5 μM at 37°C for 20 min. The percentage of the JC-1 staining positive HeLa cells was evaluated by using the flow cytometry (Beckman Coulter Inc., Brea, CA, USA).

Statistical Analysis

Data were represented as mean ± standard deviation (SD and analyzed by using SPSS software (version: 19.0, IBM, Armonk, NY, USA). Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was employed to validate analysis of variance (ANOVA) for comparing measurement data among the multiple groups. A statistical significance was defined when $p<0.05$. Data were obtained from at least six independent experiments.

Results

Metformin Combining Carboplatin Reduced HeLa Viability

The MTT assay results indicated that Metformin combining carboplatin treatment significantly decreased the HeLa cell viability compared to that of single-Metformin or single-carboplatin treatment at 24 h (Figure 1A, $p<0.05$), 48 h (Figure 1B, $p<0.05$) and 72 h (Figure 1C, $p<0.05$). Meanwhile, the Metformin combining carboplatin group exhibited the dosage dependent effects 24 h post the treatment (Figure 1A), however, and never discovered at 48 h (Figure 1B) and 72 h (Figure 1C) post the treatment.

Metformin Combining Carboplatin Increased the Nuclear Fragments

In this study, the AO/EB staining (Figure 2A) was used to evaluate the apoptotic rates of HeLa cells. The AO/EB staining findings showed that both of the single-Metformin treatment and single-carboplatin treatment induced significantly higher apoptotic rates compared to that of no drug treatment group (Table I, Figure 2B, $p<0.05$). Meanwhile, Metformin combining carboplatin treatment significantly enhanced the apoptotic rates compared to that of single-Metformin or single-carboplatin treatment group (Table I, Figure 2B, $p<0.05$).

Metformin Combining carboplatin Enhanced Apoptotic Rates

The Annexin V/PI staining could reflect the cell apoptosis according to the previous study [21]; therefore, we examined the cell apoptosis by using flow cytometry assay (Figure 3A). The results indicated that the apoptotic rates in the single-Metformin treatment and single-carboplatin treatment group were higher significantly compared to that of no drug treatment group (Figure 3B, $p<0.05$). Furthermore, Metformin combining carboplatin treatment significantly increased the apoptotic rates of HeLa cells compared to that of single-Metformin or single-carboplatin treatment group (Figure 3B, $p<0.05$).

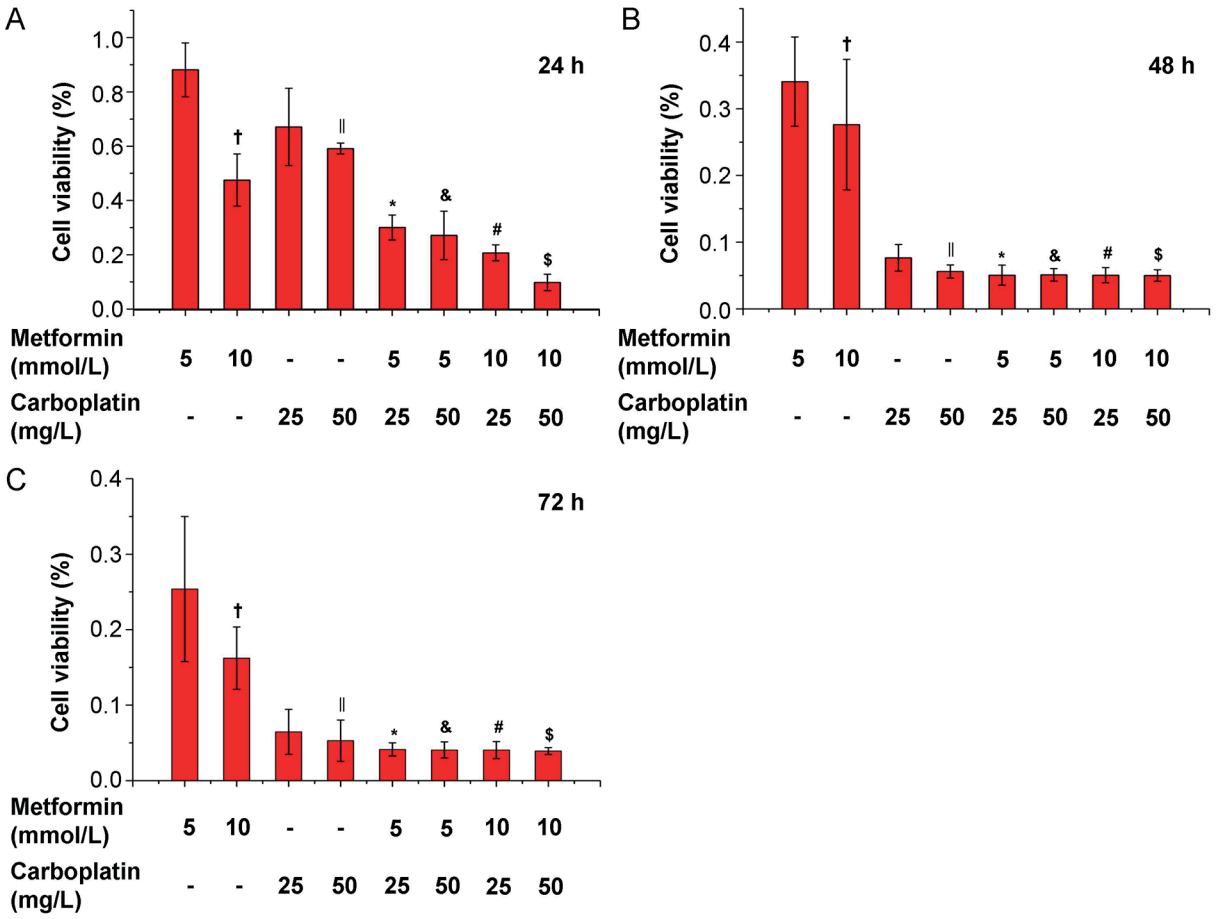

Figure 1. The inhibitive effects of Metformin combining with carboplatin on viability of cervical cancer cell line, HeLa cell. A. Inhibitive effects on HeLa cells at 24 h. B. Inhibitive effects on HeLa cells at 48 h. C. Inhibitive effects on HeLa cells at 72 h. $^{\dagger}p<0.05$ *vs.* 5 mmol/l Metformin, $^{\|}p<0.05$ *vs.* 25 mg/l carboplatin, $^{*}p<0.05$ *vs.* 5 mmol/l Metformin, $^{\&}p<0.05$ *vs.* 5 mmol/l Metformin + 25 mg/l carboplatin, $^{\#}p<0.05$ *vs.* 10 mmol/l Metformin, $^{\$}p<0.05$ *vs.* 10 mmol/l Metformin + 25 mg/l carboplatin.

Table I. Statistical analysis for the AO/EB staining positive and apoptosis Hela cells.

	Total cell number (n)	Apoptosis cell number	Percentage (%)
No drug treatment	300	20	6.67
5 mmol/l	300	86	28.67
10 mmol/l	300	133	44.33†
25 mg/l	300	111	37.00
50 mg/l	300	92	30.67§
5 mmol/l+25 mg/l	300	123	41.00*
5 mmol/l+50 mg/l	300	108	36.00$^{\&}$
10 mmol/l+25 mg/l	300	139	46.33$^{\#}$
10 mmol/l+50 mg/l	300	135	45.00$^{\$}$

†p<0.05 *vs.* 5 mmol/l Metformin, §$p<0.05$ *vs.* 25 mg/l Carboplatin, *$p<0.05$ vs. 5 mmol/l Metformin, &$p<0.05$ vs. 5 mmol/l Metformin + 25 mg/l Carboplatin, #$p<0.05$ vs. 10 mmol/l Metformin, \$$p<0.05$ *vs.* 10 mmol/l Metformin + 25 mg/l Carboplatin.

Metformin Combining carboplatin Decreased Mitochondrial Membrane Potential

Mitochondrial membrane potential could reflect the status of apoptosis[22]; therefore, we evaluated the mitochondrial membrane potential (Figure 4A) in this study. The results indicated that mitochondrial membrane potential in single-Metformin and single-carboplatin treatment group were lower significantly compared to that

in no drug treatment group (Figure 4B, $p<0.05$). Moreover, Metformin combining carboplatin treatment significantly reduced the mitochondrial membrane potential of cells compared to that of single-Metformin or single-carboplatin treatment group (Figure 34, $p<0.05$).

Discussion

In recent years, although plenty of time and money have been cost, the progress of novel strategy for gynecologic oncology is also limited. Metformin is a first-line drug for treating the type 2 diabetes in clinical, due to it's inexpensive, less side effects and specific anti-diabetic functions[23]. Nowadays, Metformin is considered to be an anti-tumor drug with best applicable potential for the gynecologic oncology field[24,25]. In year 2005, Evans et al[26] reported for the first time that Metformin could significantly reduce the cancer incidence of diabetes. Meanwhile, the other *in vivo* or *in vitro* study also found that Metformin inhibits the proliferation of tumor cells, such as oral squamous carcinoma[27], ovarian cancer[28], breast cancer[29]. We also proved that Metformin combining with carboplatin significantly enhanced the inhibitive effects of carboplatin on the HeLa cell proliferation. This data suggests that the double agents combined application merits the anti-tumor effects. Apoptosis, also named as "programmed cell death", is a critical and natural process for maintaining the normal cell growth and

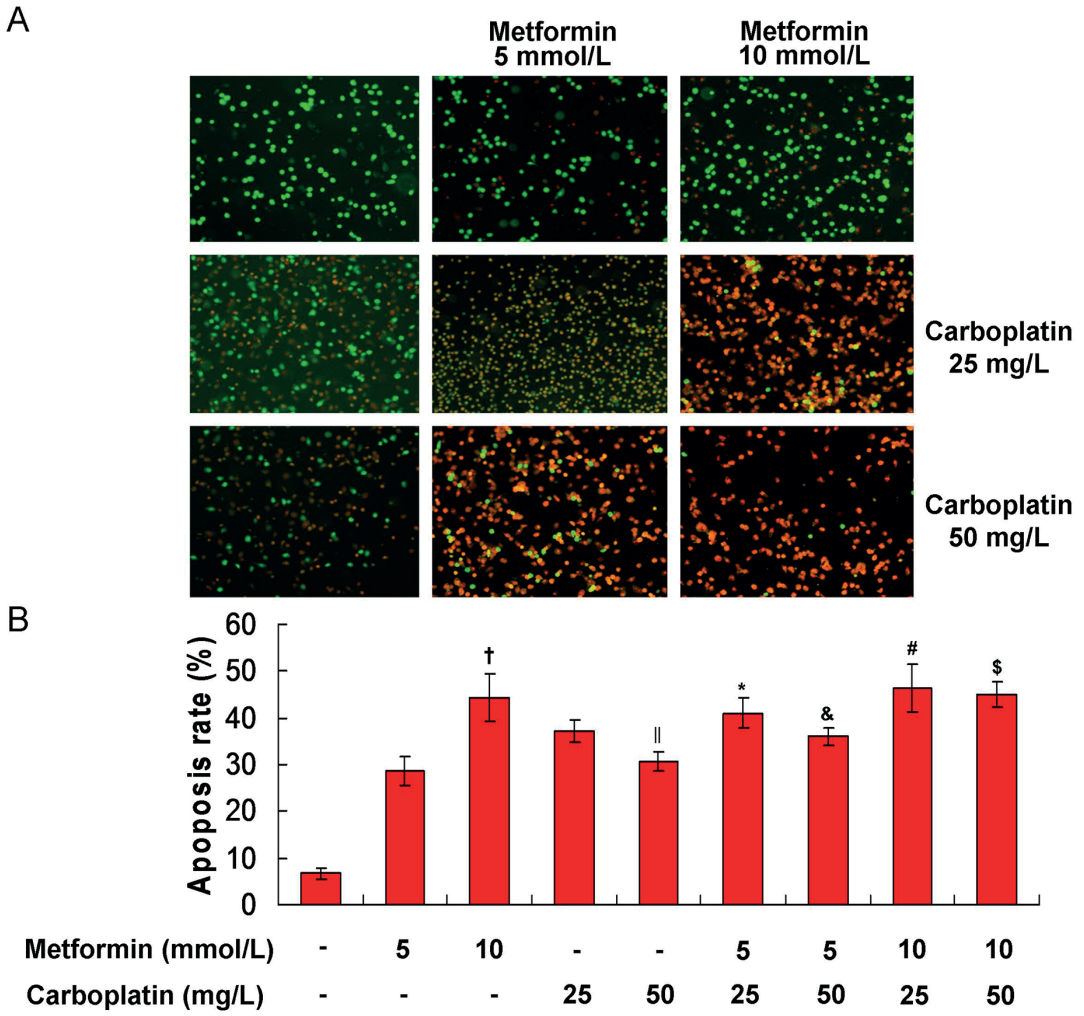

Figure 2. Evaluation for the formation of nuclear fragments in HeLa cells by using AO/EB staining. ***A,*** AO/EB staining for the HeLa cells undergoing Metformin or/and carboplatin treatment. ***B,*** Statistical analysis for the amounts of AO/EB staining positive HeLa cells. $^{\dagger}p<0.05$ *vs.* 5 mmol/l Metformin, $^{\|}p<0.05$ *vs.* 25 mg/l carboplatin, $^{*}p<0.05$ *vs.* 5 mmol/l Metformin, $^{\&}p<0.05$ *vs.* 5 mmol/l Metformin + 25 mg/l carboplatin, $^{\#}p<0.05$ *vs.* 10 mmol/l Metformin, $^{\$}p<0.05$ *vs.* 10 mmol/l Metformin + 25 mg/l carboplatin.

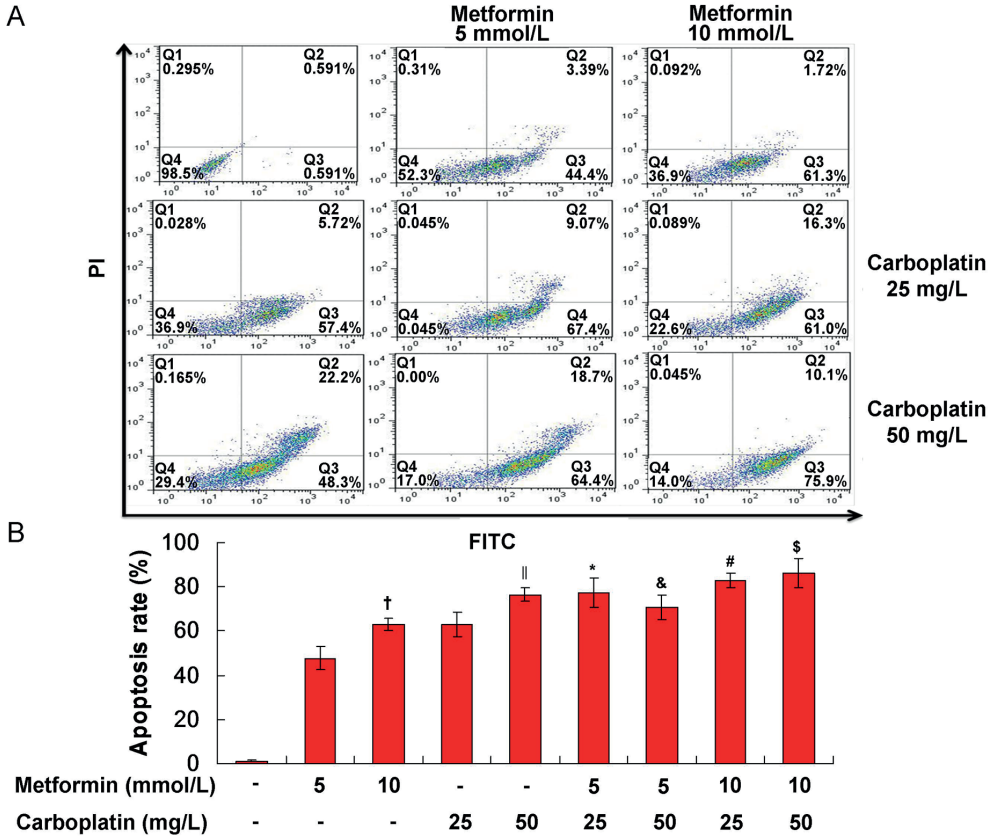

Figure 3. Cell apoptosis examination of HeLa cells by using Annexin V/PI staining. A. Annexin V/PI staining of HeLa cells undergoing Metformin or/and carboplatin treatment. B. Statistical analysis for the Annexin V/PI staining positive HeLa cells. $^{\dagger}p<0.05$ *vs.* 5 mmol/l Metformin, $^{\dagger}p<0.05$ *vs.* 25 mg/l carboplatin, $^{*}p<0.05$ *vs.* 5 mmol/l Metformin, $^{\&}p<0.05$ *vs.* 5 mmol/l Metformin + 25 mg/l carboplatin, $^{\#}p<0.05$ *vs.* 10 mmol/l Metformin, $^{\$}p<0.05$ *vs.* 10 mmol/l Metformin + 25 mg/l carboplatin.

eliminating the cell injury[30]. There are mainly two pathways mediating the apoptosis, including mitochondrial pathway mediated apoptosis and death-receptor pathway mediated apoptosis. In this study, we investigated the roles of mitochondrial pathway in the apoptotic process of HeLa cells. Researches[31,32] reported that Metformin could induce apoptosis and inhibit proliferation in both *in vivo* and *in intro* levels. Actually, Metformin plays the anti-tumor roles through triggering the adenosine 5'-monophosphate (AMP)-activated protein (MAPK)-mammalian target of rapamycin (mTOR) signaling pathway, and then induces the mitochondrial pathway-dependent cell apoptosis[33]. However, roles of mitochondrial pathway-dependent cell apoptosis have never been clarified till now. Therefore, we investigated the mitochondrial pathway associated apoptosis in HeLa cells by using AO/EB staining, Annexin V/PI staining and mitochondrial membrane potential assay, respectively. AO/EB staining and Annexin V/PI staining results showed that Metformin combining carboplatin increased the apoptotic rates. While the mitochondrial membrane potential assay results indicated that the Metformin combining carboplatin decreased mitochondrial membrane potential. All of these findings suggest that Metformin combining carboplatin treatment induced the formation of nuclear fragments and the induction of mitochondrial membrane potential, both of which represent the mitochondrial pathway mediated cell apoptosis. Moreover, Metformin, as an inhibitor for the mitochondrial respiratory chain complex 1, causes the accumulation of the reactive oxygen species (ROS) and subsequently induces cell aging and cell apoptosis[34,35]. Actually, the decreased mitochondrial membrane potential caused by Metformin combining carboplatin also induces the HeLa cell apoptosis by promoting the release of cytochrome C from inner-space of mitochondria to the cytoplasm. Then, cytochrome C induces the

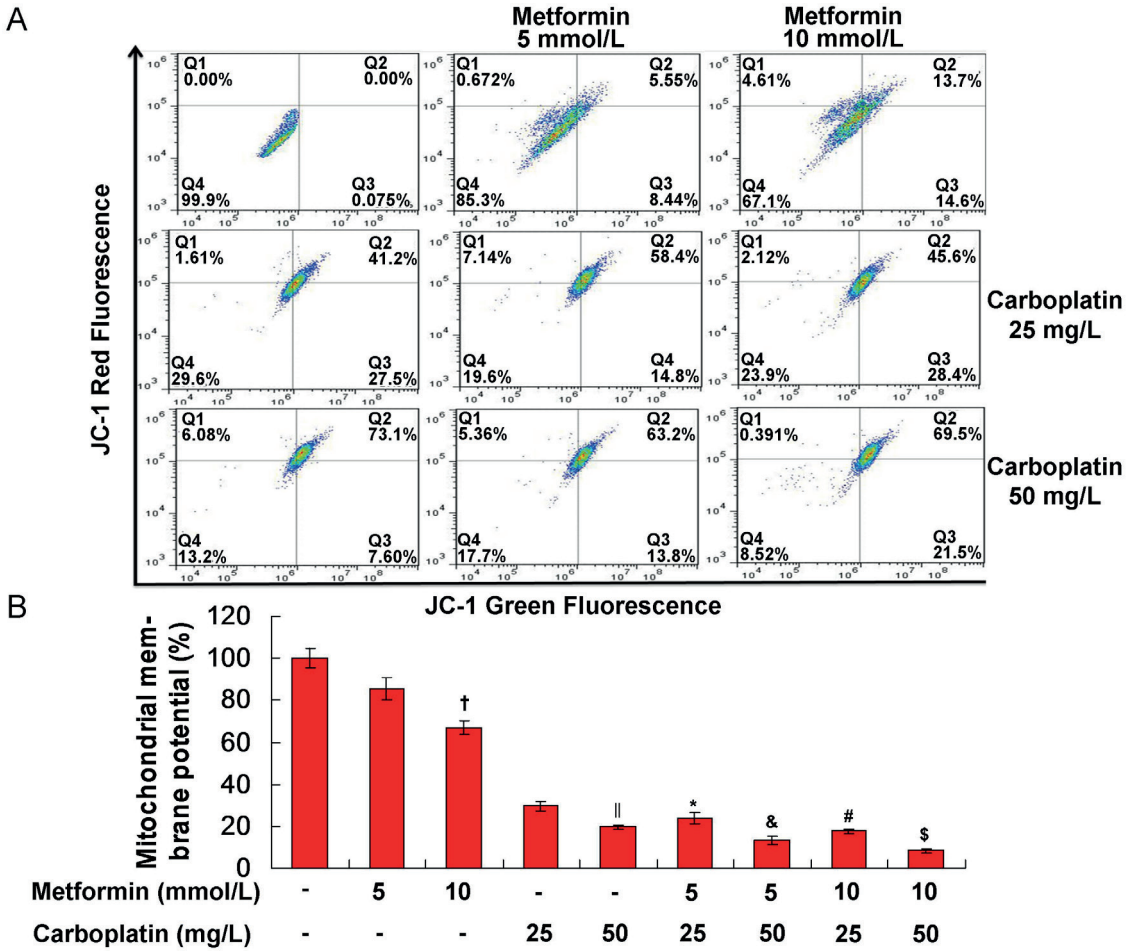

Figure 4. Mitochondrial membrane potential evaluation with JC-1 staining method. ***A,*** Images of the JC-1 staining for mitochondrial membrane potential. ***B,*** Statistical analysis for JC-1 staining HeLa cells. $^{\dagger}p<0.05$ *vs.* 5 mmol/l Metformin, $^{\|}p<0.05$ *vs.* 25 mg/l carboplatin, $^{*}p<0.05$ *vs.* 5 mmol/l Metformin, $^{\&}p<0.05$ *vs.* 5 mmol/l Metformin + 25 mg/l carboplatin, $^{\#}p<0.05$ *vs.* 10 mmol/l Metformin, $^{\$}p<0.05$ *vs.* 10 mmol/l Metformin + 25 mg/l carboplatin.

cascaded activation of caspase-8, caspase-9 and caspase-3, and finally causes apoptosis[36,37]. Although this study received a few interesting results, there were also some limitations. Firstly, the effects of Metformin combining carpoblatin on cell apoptosis only conducted in HeLa cells, but not in the animal models. Secondarily, the potential mechanism for Metformin combining Carpoblatin caused HeLa cell apoptosis has not been clarified. Thirdly, the other strategies for combining drugs have not been investigated.

Conclusions

We demonstrated that metformin enhanced the inhibitive effects of carboplatin on HeLa cell proliferation. Metformin increased the sensitivity of HeLa cell to the treatment of Carpoblatin by activating the mitochondrial-associated apoptosis-signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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