Reversal effect of Fe$_3$O$_4$-magnetic nanoparticles on multi-drug resistance of ovarian carcinoma cells and its correlation with apoptosis-associated genes

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[Abstract] Background and Objective: Resistance to cisplatin (DDP) remains a major obstacle for the successful treatment of ovarian cancer. This study was to determine the reversal effect of Fe$_3$O$_4$-magnetic nanoparticles (MNs) on DDP-resistance of ovarian cancer cell line SKOV3/DDP, and to explore its correlation with apoptosis-associated genes. Methods: SKOV3/DDP cells were divided into the DDP group, the Fe$_3$O$_4$-MNPs group, the combination (DDP plus Fe$_3$O$_4$-MNPs) group, and the control group. Cell proliferation was determined by MTT assay. Cell apoptosis was analyzed by flow cytometry (FCM). Intracellular DDP level was detected by inductively coupled plasma atomic emission spectrometry (ICP-AES). The expressions of apoptosis-associated genes, Bcl-2 and survivin were detected by reverse transcription-polymerase chain reaction (RT-PCR). Results: Fe$_3$O$_4$-MNPs reversed DDP-resistance of SKOV3/DDP cells by 2.259 folds. The cell apoptosis rate and the intracellular DDP level were significantly higher in the combination group than in the DDP group ($P<0.05$). Moreover, the mRNA levels of Bcl-2 and survivin were significantly lower in the combination group than in the DDP group ($P<0.05$). Conclusions: Fe$_3$O$_4$-MNPs can reverse the DDP resistance of ovarian carcinoma SKOV3/DDP cells, and the effect may be ascribed to the down-regulation of Bcl-2 and survivin expression.

Key words: Fe$_3$O$_4$-magnetic nanoparticles, ovarian neoplasm, SKOV3/DDP cells, multi-drug resistance, reverse, apoptosis-associated genes

Multidrug resistance (MDR) is characterized as resistance induced by single drug, which leads to cross-resistance to other drugs with different chemical structures and mechanisms. MDR has been proposed as the most important self-protection mechanism for tumor cells to avoid cytotoxic injury caused by chemotherapy. It is a common drug-resistance pattern of tumor cells, and is the major cause of chemotherapy failure and recurrence of ovarian cancer. Combined chemotherapy based on cisplatin (DDP) is usually used to treat ovarian cancer. DDP suppress the proliferation of cancer cells by inducing cell apoptosis. Recently, the application of magnetic nanoparticles (MNPs) loaded with chemotherapeutic drug is a new strategy for target therapy of solid tumors. In our previous studies, we found that daunorubicin (DNR)-loaded Nano-Fe$_3$O$_4$ and Nano-Au can effectively suppress the growth of K562/A02 cells, while the same amount of Nano-Fe$_3$O$_4$ and Nano-Au have no obvious toxicity to K562/A02 cells. This study was to explore the effects of Fe$_3$O$_4$-MNPs on the expression of apoptosis-associated genes, proliferation, and drug-resistance of human ovarian cancer drug-resistant cell line SKOV3/DDP.)

Methods and Materials

Materials

Fe$_3$O$_4$-MNPs were offered by State Key Laboratory of Bioelectronics, Southeast University. Before application, Fe$_3$O$_4$-MNPs were well distributed in freshly prepared RPMI-1640 medium with 10% fetal bovine serum (FBS). DDP was purchased from Qilu Pharmaceutical Co. Ltd.; FBS was from...
Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.; Trizol and Taq polymerase were from Takara BioMed Co. Ltd. (USA and Japan, respectively); reverse transcription kit was purchased from Promega Co. (USA). Oligo primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services, Co., Ltd. Macroplate reader (model-550) was purchased from Bio-Rad Company (Japan); gene amplifier (PTC-100) was from MJ Research Company (USA); flow cytometry (FACSL Calibur) was from BD Company; inductively coupled plasma atomic emission spectrometry (ICP-AES, Optima-5300) was from PE Company (USA); UV spectrometry was from Peking Tanon Company.

**Cell lines and culture conditions**

SKOV3/DDP cells, purchased from Peking Cancer Hospital, were cultured in RPMI-1640 containing 10% FBS at 37°C in a 5% CO2 incubator with saturated humidity, and passages every 2–3 days. SKOV3/DDP cells at logarithmic growth phase were divided into DDP group, Fe3O4-MNPs group, combination (DDP-loaded Fe3O4-MNPs) group and control group.

**MTT assay**

**Effect of DDP on proliferation of SKOV3/DDP cells**  SKOV3/DDP cells (5×10^4/mL) were seeded into 96-well plates by 200 μL/well. After 24-hour culture when most cells attached to the culture flask, the culture medium was refreshed. SKOV3/DDP cells were cultured with 10-60 μmol/L (double dilution) DDP (DDP groups) or without DDP (control group). After 48-hour incubation, 20 μL MTT solution (5 mg/mL) was added into each well for 4-hour cell culture, then the supernatant was removed, and 200 μL dimethyl sulfoxide (DMSO) was added in each well to dissolve crystals. The absorbance value at 540 nm (A540) was measured using a microplate reader. Each assay was repeated three times to calculate the mean value. The inhibition rate of cell proliferation was determined as follows: (1-A540 of DDP group/A540 of control group)×100%.

**Effect of DDP and Fe3O4-MNPs on proliferation of SKOV3/DDP cells**  Cells were cultured as above. SKOV3/DDP cells in combination groups were cultured with 25 μg/mL Fe3O4-MNPs and 5, 10, 20, 30, 40 μmol/L DDP, respectively; those in control group were cultured in medium. Each assay was repeated three times to calculate the mean value. The reversal index of MDR was calculated as the mean 50% inhibition concentration (IC50) of anti-cancer drug/the mean IC50 of anti-cancer drug combined reversal drug.

**Apoptosis detected by flow cytometry**

Cells were grouped as stated and cultured with 20 μmol/L DDP and 25 μg/mL Fe3O4-MNPs alone or in combination, washed three times with cold PBS (the supernatant was removed), fixed in EP tube by 2 mL of 70% cold ethanol, then washed twice with PBS (the supernatant was removed). Cell precipitation was added with 150 μL RNase (0.01%, mV) and cultured at 4°C in dark for 30 min, then added with 100 μL propidine iodide (100 μg/mL) and incubated in dark for 30 min. After filtered by 300-mesh nylon filters, the cells were detected by Becton-Dickinson FACS Calibur flow cytometry: 1×10^4 cells were obtained by CellQuest software and apoptosis rate was calculated by ModFit software.

**Intracellular DDP accumulation determined by ICP-AES**

The cells in above groups were harvested, washed three times with PBS, and centrifuged by 3 000 r/min for 30 min. The pellet were resuspended in 1 mL of PBS and decomposed by 5 mL concentrated nitric acid to a final volume of 25 mL. Finally, the intracellular DDP content was determined by ICPAES (Optima-5300).

**Transcription of survivin and bcl-2 detected by semi-quantitative RT-PCR**

Cells were grouped and treated as stated in subsection 1.2 and treated accordingly. Total RNA was extracted according to the manufacturer’s instructions. The purity and concentration of RNA were determined by UV spectrometry. Reverse transcription-polymerase chain reaction (RT-PCR) was performed according to TaKaRa kit protocol. cDNA was synthesized from 2 μg of total cellular RNA and the reaction volume was 20 μL; 5 μL-synthesized cDNA was used as template and amplified by using PTC-100 gene amplifier. The PCR reaction system contained 5 μL 10 xPCR buffer, 1.5 μL MgCl2 (50 mmol/L), 1 μL dNTP mixture (10 mmol/L), 1 μL upstream primer and 1 μL downstream primer (10 pmol/L), and 0.3 μL DNA polymerase (5 U/μL), and added with distilled water to a final volume of 50 μL. The primers for β-actin (forward: 5’-TATGACTTGGTGCTTACC-3’; reverse: 5’-CTTTCCACGGTTCAGT-3’), survivin (forward: 5’-CAGGCAACCGCATCCT-3’; reverse: 5’-CCAGGTTAATTTCTTCAAACT-3’), and bcl-2 (forward: 5’-GAGGAAACAGGGTGCGATA-3’; reverse: 5’-CCACCGAATCTGAGGCG-3’) were used. The lengths of amplified products were 155 bp, 255 bp and 452 bp, respectively. The amplification conditions were pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, reannealing at 55°C for 45 s and elongation at 72°C for 1 min, followed by extension at 72°C for 10 min. The mixture of 10 μL reaction products and 2 μL loading buffer was electrophoresed on 1.5% agarose gels and PCR fragments were visualized by UV-2000 illumination. β-actin was used as internal control. The experiment was repeated three times.

**Statistical analysis**

One-way analysis of variance was performed with SPSS11.5 software. A value of P<0.05 was considered significant.

**Results**

**The effects of DDP and Fe3O4-MNPs on proliferation and MDR of SKOV3/DDP cells**

The IC50 of DDP for SKOV3/DDP cells was 39.31 μmol/L, while the IC50 of DDP-loaded Fe3O4-MNPs was 17.4 μmol/L. The inhibitory effects of DDP alone or in combination with Fe3O4-MNPs on the proliferation of SKOV3/DDP cells were significantly enhanced as the drug concentration increased (P<0.05) (Fig. 1). Moreover, DDP-loaded Fe3O4-MNPs reversed drug-resistance of SKOV3/DDP cells by 2.259 folds.
Cell apoptosis

When cultured with DDP and DDP-loaded Fe$_3$O$_4$-MNPs for 48 h, the apoptosis rates of SKOV3/DPD cells were enhanced as compared with control; it was significantly higher in combination group than in DDP group (42.1±5.6)% vs. (26.9±4.1)%, P<0.05) (Fig. 2).

**Intracellular DDP accumulation in SKOV3/DPD cells**

When cultured with DDP and DDP-loaded Fe$_3$O$_4$-MNPs for 48 h, the intracellular DDP concentration of SKOV3/DPD cells was increased; it was significantly higher in combination group than in DDP group [(0.074±0.006) μmol/L vs. (0.057±0.003) μmol/L, P<0.05].

**The transcription of bcl-2 and survivin mRNA in SKOV3/DPD cells**

When cultured for 48 h, no obvious down-regulation of survivin and bcl-2 mRNA expression in SKOV3/DPD cells was seen in Fe$_3$O$_4$-MNPs group as compared with control group (P>0.05); while significant down-regulation was seen in DDP group and combination group (P<0.05), and the difference between DDP group and combination group was significant (P<0.05) (Fig. 3).

**Discussion**

Satisfactory cytoreductive surgery and chemotherapy are the main treatments for ovarian cancer, but their effects are affected by primary or secondary drug-resistance. DDP is the first-line chemotherapeutic drug for ovarian cancer. As a cell cycle nonspecific agent, DDP exerts its anti-tumor activity through forming inter-strand/intra-strand crosslink with DNA molecule or inhibiting RNA transcription, thereby, arresting tumor cells at G2 phase and inducing cell apoptosis. In the mechanisms of DDP-resistance, the in vivo distribution and metabolism of DDP and combination group (P<0.05), and the difference between DDP group and combination group was significant (P<0.05) (Fig. 3).

**Figure 1** Inhibitory effects of 48-hour treatment of cisplatin (DDP) and DDP plus Fe$_3$O$_4$-magnetic nanoparticles (MNPs) on proliferation of SKOV3/DPD cells

**Figure 2** Effects of 48-hour treatment of DDP, Fe$_3$O$_4$-MNPs, or both on apoptosis of SKOV3/DPD cells

**Figure 3** The expression of survivin and Bcl-2 mRNA in SKOV3/DPD cells after 48-hour treatment of DDP, Fe$_3$O$_4$-MNPs, or both

Lane M, marker; lane 1, SKOV3/DPD cells treated with 20 μmol/L DDP and 25 μg/mL Fe$_3$O$_4$-MNPs; lane 2, SKOV3/DPD cells treated with 20 μmol/L DDP; lane 3, SKOV3/DPD cells treated with 25 μg/mL Fe$_3$O$_4$-MNPs; lane 4, control SKOV3/CDDP cells.

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affect the effective plasma drug concentration, which may be responsible for the decline of DDP-sensitivity.\(^5\) Li et al.\(^6\) found that the trend of intracellular drug accumulation have increased when the concentration gradient between intracellular and extracellular DDP is enlarged along with the increase of dose, suggesting that low sensitivity of ovarian cancer cells to DDP may due to the obstacle for DDP to enter tumor cells. Moreover, the decline of drug accumulation is commonly seen in DDP-resistant cell lines.\(^7\) however, the mechanism is uncertain which may involves declined absorption, active efflux, or both.

MDR reversal has become an important research topic on cancer chemotherapeutic drugs. It is believed that any approaches to elevate intracellular effective drug concentration are feasible to overcome MDR.\(^4\) Li et al.\(^6\) found that the responsiveness of cancer cells to apoptosis is one of the critical factors that affecting the efficacy of chemotherapy, and resistance may occur when cell apoptosis is suppressed. Thereby, screening effective chemosensitizer for improving the efficacy on DDP-resistant ovarian cancer is the key to improve the prognosis.

Recently, the application of MNPs loaded with chemotherapeutic drugs is a new strategy for the target therapy of solid tumor.\(^2\) Fe\(_3\)O\(_4\)-MNPs is easy to reach target location due to its super magnetism. The surface hydroxyls on nanoparticles possess large specific surface absorption and are easy to couple with specific target molecules, which lead to a high drug-loading rate. In this study, we used Fe\(_3\)O\(_4\)-MNPs in combination with different concentrations of DDP to treat SKOV3/DDP cells and found that Fe\(_3\)O\(_4\)-MNPs reversed MDR by 2.259 folds, suggesting that Fe\(_3\)O\(_4\)-MNPs can promote the inhibitory effect of DDP on the proliferation of SKOV3/DDP cells; we also found that intracellular DDP concentration was higher in combination groups than in DDP group, suggesting that Fe\(_3\)O\(_4\)-MNPs increased intracellular DDP concentration in SKOV3/DDP cells and promoted its antitumor effect. The results of flow cytometry showed that the apoptosis rates of SKOV3/DDP cells were higher in combination groups than in DDP group, suggesting that Fe\(_3\)O\(_4\)-MNPs loaded with DDP have improved ability to induce tumor cell apoptosis.

The mechanism about how Fe\(_3\)O\(_4\)-MNPs elevate intracellular effective DDP concentration is unclear. It is suggested that Fe\(_3\)O\(_4\)-MNPs possess high surface activity to make the particles aggregate together and pack the drug conveniently, concentrate DDP in cells and enhance the toxicity of DDP to SKOV3/DDP cells with no need to increase DDP dose, hence, reverse MDR. However, the specific molecular mechanism needs to be further investigated.

Survivin, a newly identified anti-apoptotic gene, locates on chromosome 17q25. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has complicated functions including inhibiting cell apoptosis, promoting cell transformation and taking part in cell mitosis, angiogenesis, and MDR. Survivin is expressed in all common malignant tumors, but not in normal tissues. Zhang et al.\(^8\) found that survivin was expressed in SKOV3/DDP cells, but not in SKOV3 cells. Bcl-2, an inhibitor of apoptosis protein, mainly locates on mitochondrial membrane. It is closely related to the drug-resistance of ovarian cancer. Li et al.\(^6\) found that the expression of Bcl-2 was higher in SKOV3/DDP cells than in SKOV3 cells. Li et al.\(^6\) also observed that the expression of Bcl-2 was increased with the enhancement of acquired drug-resistance induced by chemotherapeutic drugs in ovarian cancer cells, and ovarian cancer cells with Bcl-2 overexpression were resistant to chemotherapeutic drugs; meanwhile, Bcl-2 overexpression could be decreased by increasing dose of drugs or prolonging administration. In this study, Fe\(_3\)O\(_4\)-MNPs showed no direct activity to down-regulate the expression of either survivin or bcl-2 in SKOV3/DDP cells, while DDP-loaded Fe\(_3\)O\(_4\)-MNPs down-regulated their expression. It may be due to that Fe\(_3\)O\(_4\)-MNPs increased intracellular DDP concentration.

In conclusion, DDP-loaded Fe\(_3\)O\(_4\)-MNPs can reverse DDP-resistance in SKOV3/DDP cells; it is supposed that the reversal of DDP-resistance of SKOV3/DDP cells depends on elevating intracellular drug concentration and down-regulating the expression of anti-apoptotic genes Bcl-2 and survivin to induce apoptosis. But how to elevate intracellular DDP concentration in DDP-resistant cell lines remains to be further investigated.

References

