Effect of artemisinin combined with cisplatin intervention on epithelial–mesenchymal transition, angiogenesis and ATP generation in MGC–803 gastric cancer cell lines

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ARTICLE INFO

Article history:
Received 2 Aug 2016
Received in revised form 16 Aug 2016
Accepted 13 Aug 2016
Available online 24 Aug 2016

Keywords:
Gastric cancer
Artemisinin
Cisplatin
Epithelial–mesenchymal transition
Angiogenesis
Adenotriphos
Reactive oxygen species

ABSTRACT

Objective: To study the effect of artemisinin combined with cisplatin intervention on epithelial-mesenchymal transition, angiogenesis and ATP generation in MGC-803 gastric cancer cell lines. 
Methods: MGC-803 gastric cancer cell lines were cultured and divided into control group, cisplatin group, artemisinin group, cisplatin + artemisinin group, cisplatin + artemisinin + NAC group and NAC group, and after different conditions of treatment, cell viability, apoptosis rate as well as levels of epithelial-mesenchymal transition molecules, angiogenesis molecules and ATP were determined. 
Results: Cell viability, ATP generation as well as VEGFA, VEGFB, VEGFC, N-cadherin and Vimentin levels of cisplatin group, artemisinin group and cisplatin + artemisinin group were lower than those of control group, and early apoptosis rate, late apoptosis rate and E-cadherin levels were higher than those of control group; cell viability, ATP generation as well as VEGFA, VEGFB, VEGFC, N-cadherin and Vimentin levels of cisplatin + artemisinin group were lower than those of cisplatin group and artemisinin group, and early apoptosis rate, late apoptosis rate and E-cadherin level were higher than those of cisplatin group and artemisinin group. E-cadherin level of cisplatin + artemisinin + NAC group was lower than that of cisplatin + artemisinin group, and ATP generation as well as VEGFA, VEGFB, VEGFC, N-cadherin and Vimentin levels were higher than those of cisplatin + artemisinin group. 
Conclusion: Artemisinin combined with cisplatin intervention can synergistically exert the inhibitory effect on epithelial-mesenchymal transition, angiogenesis and ATP generation in MGC-803 gastric cancer cell lines, and the inhibiting effect is partially realized by increasing the generation of reactive oxygen species.

1. Introduction

Gastric cancer is one of the most common gastrointestinal malignant tumors in China, has low early diagnosis rate, has mostly developed to middle-late stage at the time of diagnosis and requires chemotherapy. Cisplatin is the most common chemotherapy drug for clinical treatment of advanced gastric carcinoma, and it can affect the DNA replication in the nuclei and induce cell apoptosis. The most significant disadvantage of cisplatin for advanced gastric cancer chemotherapy, however, is the high incidence of chemotherapy drug resistance, and the drugs with different mechanism of action are also needed for common treatment[1,2]. Artemisinin is the active ingredient separated from Chinese medicine artemisia apiacea, it was used for the treatment of malaria at first, it is found in recent years that the drug has antitumor activity[3,4], and several studies have confirmed that artemisinin has inhibitory effect on the viability of bladder cancer[5], cervical cancer[6] and breast cancer[7]. So it was speculated that artemisinin might also has inhibitory effect on the growth and malignant biological behavior of gastric cancer. In order to verify the above theory, the effect of artemisinin combined with cisplatin intervention on epithelial-mesenchymal transition, angiogenesis and ATP generation in MGC-803 gastric cancer cell lines was analyzed in the following study.
2. Materials and methods

2.1. Experimental materials

MGC-803 gastric cancer cell lines were purchased from ATCC cell bank, cell culture medium and serum were purchased from Gibco Company, artemisinin and N acetylcysteine were purchased from Sigma Company, cisplatin was purchased from Hansoh Pharmaceutical Co., LTD in Suzhou, Annexin-FITC/PI kits were purchased from BD Company, MTS cell viability kits were purchased from Promega Company, ATP detection kits were purchased from Beyotime Company, and enzyme-linked immunosorbent assay kit were purchased from Wuhan Boster Company.

2.2. Experimental methods

2.2.1. Cell culture methods

MGC-803 cell lines were recovered and then cultured in the medium containing 10% serum, digested and sub-cultured by routine 0.125% trypsin, the sub-cultured cells were inoculated in culture bottles and different specifications of culture plates, the cells in the culture bottles were used for continuous sub-culture, and the cells in culture plates were used for drug treatment.

2.2.2. Cell grouping and treatment methods

Cells in culture plates grew to the density of 80%-90% and then received drug treatment, and they were divided into control group, cisplatin group, artemisinin group, cisplatin + artemisinin group, cisplatin + artemisinin + NAC group and NAC group who received different conditions of treatment, specifically as follows: control group were treated with culture medium without serum, cisplatin group were treated with serum-free medium containing 10 mg/L cisplatin, artemisinin group were treated with serum-free medium containing 50 μmol/L artemisinin, cisplatin + artemisinin group were treated with serum-free medium containing containing 10 mg/L cisplatin and 50 μmol/L artemisinin, cisplatin + artemisinin + NAC group were treated with serum-free medium containing 10 mg/L cisplatin, 50 μmol/L artemisinin and 10 μmol/L NAC, and NAC group were treated with serum-free medium containing 10 μmol/L NAC.

2.2.3. Cell viability and apoptosis detection

To detect cell viability, cell were inoculated in 96-well culture plates and treated with different conditions for 24 h, then MTS test fluid 20 μL was directly added in the culture wells, cells were incubated for 4 h, and then absorbance A at 450 nm wavelength was read from automatic microplate reader and used as cell viability value. To detect cell apoptosis, cells were inoculated in 12-well culture plates and treated with different conditions for 24 h, then operation was performed in accordance with Annexin V/FITC and PI staining kits, and then early apoptosis rate and late apoptosis rate of cells were detected on flow cytometer.

2.2.4. ATP generation detection

To detect ATP generation, cells were inoculated in 12-well culture plates and treated with different conditions for 24 h, culture medium was discarded and then ATP detection kits were used to determine ATP generation in cells, cells were split at first to get cell suspension, then reaction substrates were added to determine fluorescence value, and standard fluorescence value was referred to calculate ATP levels in sample under test; same batch of cell suspension was collected, BCA kits were used to determine total protein content, and then the ATP content corresponding to each mg total protein was calculated and in terms of nmol/mg total protein.

2.2.5 Enzyme–linked immunosorbent assay

To detect E-cadherin, N-cadherin and Vimentin levels in cells as well as VEGFA, VEGFB and VEGFC levels in culture medium, cells were inoculated in 24-well culture plates and treated with different conditions for 24 h, then the culture medium and cells were collected respectively, and enzyme-linked immunosorbent assay kits were used to determine E-cadherin, N-cadherin, Vimentin, VEGFA, VEGFB and VEGFC levels; same batch of cells were collected, BCA kits were used to determine total protein content, and then target protein content corresponding to each mg total protein was calculated and in terms of ng/mg total protein.

2.3. Statistical methods

SPSS 20.0 software was used to input and analyze data, measurement data analysis was by variance analysis, pair-wise comparison was by LSD-t test, and \( P<0.05 \) indicated statistical significance in differences.

3. Results

3.1. Effect of cisplatin and artemisinin on cell viability and apoptosis rate

Variance analysis showed that cell viability, early apoptosis rate and late apoptosis rate of four groups were significantly different \( (P<0.05) \); pair-wise comparison by LSD-t test showed that cell viability of cisplatin group, artemisinin group and cisplatin + artemisinin group were lower than that of control group while early apoptosis rate and late apoptosis rate were higher than those of control group; cell viability of cisplatin + artemisinin group was lower than those of cisplatin group and artemisinin group while early apoptosis rate and late apoptosis rate were higher than those of cisplatin group and artemisinin group, and differences of pair-wise comparison were significantly different \( (P<0.05) \).
Cisplatin has higher incidence of drug resistance rate and more toxic and side reactions when it is used for advanced gastric cancer chemotheraphy, which can affect the effect of chemotherapy. Artemisinin is the active ingredient extracted from artemisia apiacea, it mainly contains dihydroartemisinin, artemether, arteether and other derivatives, and it was used for the treatment of malaria at first. In recent years, a number of studies have confirmed that artemisinin has inhibitory effect on cell viability of bladder cancer[5], cervical cancer[6], breast cancer[7] and other malignant tumors. So it was speculated that artemisinin might also have inhibitory effect on the growth and malignant biological behavior of gastric cancer. In order to validate the speculation, cell growth conditions were tested after cisplatin and artemisinin treatment at first in the study, the tested indexes included cell viability and apoptosis rate, the comparison and analysis between different treatment conditions showed that artemisinin and cisplatin treatment alone and combined treatment

### Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell viability (450 nm)</th>
<th>Cell apoptosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.02±0.14†</td>
<td>5.82±0.77‡</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.53±0.09†</td>
<td>18.49±3.14‡</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>0.69±0.08†</td>
<td>13.82±2.38‡</td>
</tr>
<tr>
<td>Cisplatin + artemisinin</td>
<td>0.31±0.05§#&amp;</td>
<td>28.97±4.59§#&amp;</td>
</tr>
</tbody>
</table>

*: compared with control group, P<0.05; †: compared with cisplatin group, P<0.05; ‡: compared with artemisinin group, P<0.05.

### Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>ATP generation(nmol/mg total protein)</th>
<th>VEGFA (ng/mg total protein)</th>
<th>VEGFB (ng/mg total protein)</th>
<th>VEGFC (ng/mg total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.4±2.95</td>
<td>68.39±9.14</td>
<td>30.24±4.94</td>
<td>43.22±6.78</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>22.1±2.79</td>
<td>34.4±5.92†</td>
<td>19.3±2.76</td>
<td>22.6±3.52‡</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>25.2±3.14†</td>
<td>30.2±6.14†</td>
<td>20.4±3.14§</td>
<td>21.4±2.95</td>
</tr>
<tr>
<td>Cisplatin + artemisinin</td>
<td>13.2±2.39§#&amp;</td>
<td>16.3±2.91§#&amp;</td>
<td>12.8±2.85§#&amp;</td>
<td>14.1±2.14§#&amp;</td>
</tr>
</tbody>
</table>

*: compared with control group, P<0.05; †: compared with cisplatin group, P<0.05; ‡: compared with artemisinin group, P<0.05.

### Table 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Epithelial molecules</th>
<th>Mesenchymal molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E-cadherin</td>
<td>N-cadherin</td>
</tr>
<tr>
<td>Control</td>
<td>14.8±1.78</td>
<td>11.3±1.57</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>20.2±3.25†</td>
<td>7.9±0.93†</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>19.3±2.58†</td>
<td>7.1±0.89†</td>
</tr>
<tr>
<td>Cisplatin + artemisinin</td>
<td>31.2±4.69§#&amp;</td>
<td>4.5±0.63§#&amp;</td>
</tr>
</tbody>
</table>

*: compared with control group, P<0.05; †: compared with cisplatin group, P<0.05; ‡: compared with artemisinin group, P<0.05.

### 3.2. Effect of cisplatin and artemisinin on ATP generation and VEGF levels in cells

Variance analysis showed that ATP generation as well as VEGFA, VEGFB and VEGFC levels of four groups were significantly different (P<0.05); pair-wise comparison by LSD-t test showed that ATP generation as well as VEGFA, VEGFB and VEGFC levels of cisplatin group, artemisinin group and cisplatin + artemisinin group were lower than those of control group; ATP generation as well as VEGFA, VEGFB and VEGFC levels of cisplatin + artemisinin group were lower than those of cisplatin group and artemisinin group, and differences of pair-wise comparison were significantly different (P<0.05).

### 3.3. Effect of cisplatin and artemisinin on epithelial–mesenchymal transition marker molecules in cells

E-cadherin level of cisplatin + artemisinin group was higher than that of control group, and ATP generation as well as VEGFA, VEGFB, VEGFC, N-cadherin and Vimentin levels were lower than those of control group; E-cadherin level of cisplatin + artemisinin + NAC group was lower than that of cisplatin + artemisinin group, and ATP generation as well as VEGFA, VEGFB, VEGFC, N-cadherin and Vimentin levels were higher than those of cisplatin + artemisinin group.

### 3.4. Effect of combined ROS inhibitor treatment on ATP generation, VEGF levels and epithelial–mesenchymal transition

E-cadherin level of cisplatin + artemisinin + NAC group was lower than that of cisplatin + artemisinin group, while N-cadherin and Vimentin levels were lower than those of control group; E-cadherin level of cisplatin + artemisinin + NAC group was higher than that of control group, and ATP generation as well as VEGFA, VEGFB, VEGFC, N-cadherin and Vimentin levels were lower than those of control group; E-cadherin level of cisplatin + artemisinin + NAC group was lower than that of cisplatin + artemisinin + NAC group, and ATP generation as well as VEGFA, VEGFB, VEGFC, N-cadherin and Vimentin levels were higher than those of cisplatin + artemisinin + NAC group.

### 4. Discussion

Cisplatin has higher incidence of drug resistance rate and more
could all reduce the cell viability and increase the apoptosis rate, and the effect of artemisinin combined with cisplatin treatment on cell viability and apoptosis was more significant than those of single treatment. Thus it indicated that both cisplatin and artemisinin could inhibit the growth of gastric cancer cells, there was synergistic effect between the two, and the combined treatment of the two drugs had more significant inhibition on the growth of gastric cancer.

Epithelial-mesenchymal transition (EMT) is the common pathological feature of gastric cancer, colorectal cancer, lung cancer, ovarian cancer and other malignant tumors, specifically refers to the epithelial cell transition to mesenchymal cells under the stimulation of some pathological factors and is accompanied by the changes in the expression of a variety of genes. The polarity of mesenchymal cells is weak and the intercellular adhesion is loose, and in the process of EMT, cancer cells will get strong movement performance and migrate to the distant. E-cadherin is a marker molecule of epithelial cells located in cell membrane, the extracellular fragments can form dimers with same molecules on the surface of another epithelial cell, and intracellular fragments can form complex with catenin, thus forming adhesion junction between cells; N-cadherin and Vimentin are the marker molecules of mesenchymal cells that can reduce intercellular adhesion and between cells; N-cadherin and Vimentin can form complex with catenin, thus forming adhesion junction extracellular fragments can form dimers with same molecules on marker molecule of epithelial cells located in cell membrane, the is loose, and in the process of EMT, cancer cells will get strong polarity of mesenchymal cells is weak and the intercellular adhesion

definite effect on promoting angiogenesis and lymphangiogenesis[12], different molecular subtypes have different functions, VEGFA and VEGFB have strong angiogenesis-promoting effect, and VEGFC has strong lymphangiogenesis-promoting effect[13,14]. Local gastric cancer angiogenesis and lymphangiogenesis can not only provide the necessary materials for ATP generation by mitochondrial oxidation respiration, but can also provide path for cancer cell metastasis through blood vessels and lymph vessels. Therefore, inhibiting ATP generation as well as the synthesis and secretion of VEGF molecules is an important target for inhibiting gastric cancer cell growth. In the study, ATP generation and VEGF levels in cells were analyzed of after artemisinin and cisplatin treatment, and results show that artemisinin and cisplatin treatment alone and combined treatment had more significant effect on ATP and VEGF than single treatment. Thus it indicated that both cisplatin and artemisinin could inhibit the ATP generation as well as epithelial-mesenchymal transition, angiogenesis and ATP generation, and a variety of chemotherapy drugs can affect the generation of ROS in cells to regulate the biological behavior of cells[15,16]. In the study, it was meant to explore whether cisplatin combined with artemisinin treatment increased the generation of ROS to influence the epithelial-mesenchymal transition, angiogenesis and ATP generation of gastric cancer cells. N-acetylcysteine (NAC) is the antagonist of reactive oxygen species (ROS) are the important components that regulate the cell growth and influence the epithelial-mesenchymal transition, angiogenesis and ATP generation, and a variety of chemotherapy drugs can affect the generation of ROS in cells to regulate the biological behavior of cells[15,16]. In the study, it was meant to explore whether cisplatin combined with artemisinin treatment increased the generation of ROS to influence the epithelial-mesenchymal transition, angiogenesis and ATP generation of gastric cancer cells. N-acetylcysteine (NAC) is the antagonist of reactive oxygen species and can antagonize intercellular ROS function, and after the combination application of NAC with cisplatin and

### Table 4.
Effect of combined ROS inhibitor treatment on ATP generation, VEGF levels and epithelial-mesenchymal transition.

<table>
<thead>
<tr>
<th>Group</th>
<th>ATP generation (nmol/mg total protein)</th>
<th>VEGFA (ng/mg total protein)</th>
<th>VEGFB (ng/mg total protein)</th>
<th>VEGFC (ng/mg total protein)</th>
<th>E-cadherin (ng/mg total protein)</th>
<th>N-cadherin (ng/mg total protein)</th>
<th>Vimentin (ng/mg total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.42±5.96</td>
<td>68.39±9.14</td>
<td>30.24±4.94</td>
<td>43.22±6.78</td>
<td>14.82±1.78</td>
<td>11.33±1.57</td>
<td>17.74±2.41</td>
</tr>
<tr>
<td>Cisplatin + artemisinin</td>
<td>13.26±2.39</td>
<td>16.38±2.93</td>
<td>12.28±2.85</td>
<td>14.12±2.14</td>
<td>31.25±4.69</td>
<td>4.52±0.63</td>
<td>8.39±1.17</td>
</tr>
<tr>
<td>Cisplatin + artemisinin + NAC</td>
<td>25.33±4.63</td>
<td>52.12±7.68</td>
<td>26.69±4.13</td>
<td>34.52±5.52</td>
<td>19.33±2.63</td>
<td>9.14±1.26</td>
<td>13.59±1.94</td>
</tr>
<tr>
<td>NAC</td>
<td>34.28±5.24</td>
<td>64.46±9.74</td>
<td>31.42±5.14</td>
<td>42.84±7.14</td>
<td>15.51±1.49</td>
<td>10.49±2.03</td>
<td>16.28±2.15</td>
</tr>
</tbody>
</table>

* compared with control group, P<0.05; # compared with cisplatin + artemisinin group, P<0.05.
artemisinin, it was found in the study that ATP, VEGFA, VEGFB, VEGFC, N-cadherin and Vimentin levels of cisplatin + artemisinin + NAC group were higher than those of cisplatin + artemisinin group, and E-cadherin level was lower than that of cisplatin + artemisinin group. However, the above indicators of cisplatin + artemisinin + NAC group were still different from those of control group. It showed that reactive oxygen species antagonist NAC could partially reverse the regulating effect of cisplatin + artemisinin on epithelial-mesenchymal transition, angiogenesis and ATP generation, and the regulating effect of cisplatin + artemisinin on gastric cancer cells was realized by ROS.

To sum up, artemisinin combined with cisplatin intervention can synergistically exert the inhibitory effect on epithelial-mesenchymal transition, angiogenesis and ATP generation in MGC-803 gastric cancer cell lines, and the inhibiting effect is partially realized by increasing the generation of reactive oxygen species.

References


