Metformin (MET) is a conventional first-line drug for type 2 diabetes treatment, and its pharmacological effect is mainly through the enhancement of insulin sensitivity, thereby reducing the blood glucose level of patient. Researches in recent years indicate that MET might have a certain protective effect for cases of diabetes complicated with liver cancer,[1–4] which has a potential inhibitory effect on hepatocellular carcinoma occurrence and development, but whether it could influence the growth and function of human hepatocellular carcinoma cells is not yet clear.[5, 6]. The purpose of this paper was to observe the effect of MET on the proliferation and apoptosis of human hepatoma HepG2 cells. Through discussing the effect of MET on the expression of cyclin D1 and reactive oxygen species (ROS) generation of human hepatocellular carcinoma cell, which might involved in the mechanism of cell apoptosis, we might explore the anti-cancer action of metformin.

Materials and methods

Cell line

Human hepatoma cell line HepG2 was kept by the Institute of Basic Medical Sciences China. Cells were cultured in DMEM complete medium with 10% fetal calf serum, at 37 °C in the presence of 5% CO₂. Cells were passaged when they reached 80% confluence.

Main reagents

Metformin hydrochloride: Sigma Company, USA; Cisplatin: Qilu Pharmaceutical Company, China; Trypsin (Trypsin, 1:250), MTT, and DMSO: Amresco Company, USA; DMEM and fetal bovine serum: America Gibco products; Annexin V-FITC/PI apoptosis detection kit, and cellular whole protein extraction kit: Beijing Soledad Bao Technology Co., Ltd. products (China); Rabbit anti human cyclin D1 polyclonal antibody, and HRP labeled goat anti rabbit IgG antibody: Shanghai SANGON Biological Engineering Ltd., China.
Major equipments and supplies
CO₂ incubator: Thermo Company, USA; Inverted optical microscope, and IX-70 inverted fluorescence microscope: Olympus Company, Japan; Nitrocellulose membrane electrophoretic transfer system: Bio-Rad Company, USA; Flow cytometry: BD FACSCalibur America.

MTT assay of the effect of MET on the HepG2 proliferation
HepG2 cells at the logarithmic growth phase were harvested. The cell suspension was adjusted to 5 × 10⁵/mL, inoculated in 96 well plate with 100 µL per well and incubated at 5% CO₂, 37 °C for 24 h. Then the cells were cultured with 20 µL drugs diluted in different concentrations for the designated time point (24, 48 and 72 h). At the end, 20 µL MTT (5 mg/mL) was added to each well and the samples were incubated at 37 °C for 4 h. After that, the culture medium was discarded and 150 µL DMSO was added to dissolve the formazan crystals completely for 15 min at room temperature. Absorbance (A value) at 570 nm wavelength was measured with a micro-plate ELISA reader. The inhibition rate (%) = (1 – Adrug / Acontrol ) × 100%.

The test was divided into 6 groups; group A: control group; groups B, C, and D: metformin (2, 10, and 50 mM); group E: cisplatin (5 µg/mL); group F: metformin (10 mM) + cisplatin (5 µg/mL).

Detection of cellular apoptosis by flow cytometry
HepG2 cells at the logarithmic growth phase were harvested and seeded in 6 well culture plate with 3 mL of 1 × 10⁶/mL inoculum density and cultured in DMEM medium with 10% fetal bovine serum for 24 h. Then the cells were treated with 100 µL drug and cultured in serum-free DMEM medium for 24 h. After drug treatment, cells were collected and washed 3 times with cold PBS. Then the cells were resuspended and mixed with 5 µL Annexin V-FITC and 10 µL PI (20 µg/mL) at room temperature and kept in darkness at room temperature for 15 min. Adding 400 µL binding buffer, the samples were analyzed by flow cytometry within 1 h.

The expression of cyclin D1 assayed by Western blot
The HepG2 cells were obtained by the same as described in the section of flow cytometry. After drug treatment, cells were washed 3 times with cold PBS, the total protein was extracted and kept at -20 °C. The protein was quantificated by BCA method. Then 60 µg protein sample was loaded and separated by polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane through a semidytransfer cell. The membrane was blocked with 5% skim milk and incubated with a 1:1000 dilution of rabbit anti-human cyclin D1 polyclonal antibody at 4 °C overnight. After being washed 3 times with TBST, the membrane was incubated with 1:2000 dilution of HRP labeled goat anti-rabbit IgG at room temperature for 1 h. After being washed 3 times with TBST, the membrane was detected by ECL.

Reactive oxygen species (ROS) measured by ROS-DHE fluorescence probe
The HepG2 cells were obtained by the same as described in the section of flow cytometry. After drug treatment, the cells were washed two times with PBS. Then 2 mL of ROS-DHE fluorescent probe working solution was slowly added to the well along the wall and cultured at 37 °C for 20 min. Discarding the culture medium with staining liquid, the cells were washed two times with DMEM (serum-free). The 500 µL DMEM (serum-free) preheated to 37 °C was added to the well along wall carefully. ROS was detected by inverted fluorescence microscope at excitation wavelength of 540 nm and emission wavelength of 590 nm.

Statistical analysis
All of the data were analyzed by SPSS 18.0. Counting data were expressed as x ± s, with between-group t-test, and P < 0.05 was considered statistically significant.

Results
Inhibitory effect of MET on the proliferation of HepG2 cells
With MET at different concentrations (0, 2, 10, and 50 µmol/L) and different schedule times (24, 48, and 72 h) treatment of HepG2 cells, cell proliferation was inhibited, the results were shown in Table 1. Compared with the control group, the difference was significant (P < 0.01). The results showed that MET inhibited the proliferation of HepG2 cells with a dose and time dependent manner. Compared with mono-component group, MET combined with cisplatin could reduce the dosage of cisplatin and in-

Table 1 The inhibitory effect of MET on the proliferation of HepG2 cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>B</td>
<td>16.1 ± 2.4*</td>
<td>23.9 ± 3.4*</td>
<td>35.4 ± 4.1*</td>
</tr>
<tr>
<td>C</td>
<td>31.5 ± 3.3*</td>
<td>50.8 ± 4.9*</td>
<td>62.7 ± 4.7*</td>
</tr>
<tr>
<td>D</td>
<td>50.2 ± 4.0*</td>
<td>62.7 ± 5.3*</td>
<td>71.6 ± 5.0*</td>
</tr>
<tr>
<td>E</td>
<td>52.3 ± 5.3*</td>
<td>65.4 ± 4.8*</td>
<td>76.7 ± 6.6*</td>
</tr>
<tr>
<td>F</td>
<td>65.1 ± 4.6*</td>
<td>70.1 ± 5.9*</td>
<td>81.6 ± 6.5*</td>
</tr>
</tbody>
</table>

Note: A: control group; (B–D) metformin (2, 10, and 50 mM) groups; E: cisplatin (5 µg/mL) group; F: metformin (10 mM) + cisplatin (5 µg/mL) group. * P < 0.01 vs control group.
crease the inhibitory effect on HepG2 cells, showed that MET had a synergistic effect with cisplatin.

**Effects of MET on cell apoptosis of HepG2 cells**

Different concentrations of MET were incubated with HepG2 cells for 24 h, with the increase of MET concentration, cell apoptosis rate increased. As shown in Fig. 1, the cell apoptosis rates in groups A–F were 0.81%, 3.94%, 5.23%, 6.54%, 7.45% and 8.01% respectively. The results showed that the cell apoptosis rate of MET groups and cisplatin group was higher than that of the control group ($P < 0.05$).

**Effect of MET on the expression of cyclin D1 protein**

Different concentrations of MET were incubated with HepG2 cells for 24 h, as shown in Fig. 2, with the increase of MET concentration, the expression of cyclin D1 decreased. The results showed that the expression of cyclin D1 of MET groups and cisplatin group was lower than that of the control group ($P < 0.05$).

**Effect of MET on ROS generated by HepG2 cells**

Fluorescence microscopy could directly observe the effect of MET on reactive oxygen species (ROS) generated by HepG2 cells. With the increase of MET concentration, more red fluorescence generated, showed an increase in cell ROS content, as shown in Fig. 3.

**Discussion**

Polesel et al.\(^7\) found that there was a significant increase in hepatocellular carcinoma risk in patients with diabetes, which was 3.5 times of non diabetes mellitus (DM) patients. Davila et al.\(^8\) surveyed the prevalence of hepatocellular carcinoma cases in DM group (43%) was significantly higher than that of the control group (19%). These showed that there was closely related to the occurrence of hepatic cell carcinoma with DM. Compared with insulin and sulfonylurea treatment group, MET treatment group of malignant tumors in patients with type 2 diabetes, the morbidity and mortality were significantly decreased. Further *in vitro* studies have found, MET had...
an inhibition effect on the cell proliferation of various malignant tumors [9–12]. Therefore, elucidation of the inhibition of MET on human hepatocellular carcinoma cells and its mechanism, might provide new ideas for prevention of hepatocellular carcinoma.

The proliferation, differentiation and apoptosis of cells maintain the balance of body tissue growth. When apoptosis is inhibited, the cell death rate decreases, resulting body growth imbalance. The advantage in growth eventually induces carcinogenicity. The inhibition of cell apoptosis may contribute to tumor genesis, and enhancement of tumor cell apoptosis can prevent tumor genesis. The experiment observed the inhibitory effect of MET on the proliferation of HepG2 cells, it was found that MET inhibited the proliferation of HepG2 cells with a dose and time dependent manner. Flow cytometry results further confirmed that MET could induce the apoptosis of HepG2 cells in a dose dependent.

MET effect on the cancer cells apoptosis may be through a variety of ways, including participating in regulation of the apoptosis gene of cancer cell, regulating protein expression, arresting cell cycle, interfering the way of signal transduction, etc. Cyclin D1 is a cell cycle protein, a marker of cell proliferation, which has become one of the targets for tumor therapy. Cyclin D1 is an important positive regulator of the cell cycle, its site of action is the G1/S checkpoint. High expression of cyclin D1, closely related to the occurrence and development of certain tumors. We studied the effect of MET on cyclin D1 expression, MET could decrease the expression of cyclin D1, arrest tumor cells in the phase G, inhibit the proliferation of tumor cells. We also studied the effect of MET on the apoptosis inhibiting gene NFκB and apoptosis activation gene P53 (see our other reported results).

Reactive oxygen species (ROS) is a series of active oxygen free radicals produced in course of the cells oxygen metabolism, the change of ROS level in cells can cause signal transduction pathway associated with tumor cell proliferation or apoptosis, the increased of intracellular levels of ROS can induce cell apoptosis. We studied the effects of MET on intracellular ROS, found that MET could promote the formation of ROS in HepG2 cells. It suggests that MET may promote ROS production by HepG2 cells, induce apoptosis of HepG2 cells. But because of the complex way of cell apoptosis, the anti-tumor mechanism of MET still need further studies [10, 13–14].

In summary, this study found that MET could inhibit the proliferation and induce apoptosis of HepG2 cells, which can provide experimental basis for clinical treatment of the application of MET in hepatocellular carcinoma.

Conflicts of interest
The authors indicated no potential conflicts of interest.
References


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