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Cytotoxic Activity of Anticancer Drugs on Hepatocellular Carcinoma Cells in Hypoxic-Hyponutritional Culture

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To investigate which anticancer drugs and combination of dual drugs could further promote the inhibition of cell growth *in vitro* against HCC cell line (HepG2) in the hypoxic and hyponutritional culture medium (HHCM) mimicked the different scenarios of transcatheter arterial chemoembolization (TACE). The cells of hepatocellular carcinoma (HCC) treated by TACE suffered various hypoxia and hyponutrition. The cells were treated for 2 hours, 4 hours, 6 hours, and 24 hours, respectively, using 10 drugs including epirubicin (EPI), cisplatin (DDP), mitomycin-C (MMC), oxaliplatin (OXA), hydroxycamptothecin (HCPT), 5-fluorouracil (5-FU), gemcitabine (GEM), docetaxel (DTX), thiotepa (TSPA), and pemetrexed disodium (PEM) in 4 concentrations of HHCM (5%, 10%, 25%, and 50%, respectively) mimicking the scenario of TACE and were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells treated with combinations of dual drugs for 24 hours were also tested. The sensitive drugs with inhibition rates more than 30% were EPI, MMC, HCPT, OXA, and PEM in 4 types of HHCMs. The sensitivity of the cells to treatment with drugs for 24 hours was significantly higher than the sensitivity of the cells to treatment with drugs for 2 hours in 5%, 10%, and 25% HHCM. The sensitivity of the combination of dual drugs was no more than the sensitivity of the single drug with higher sensitivity in 4 concentrations of HHCM. EPI, MMC, HCPT, OXA, and PEM exhibited cytotoxic activity against HepG2 cells in various hypoxia and hyponutrition states. Prolonging the time of exposure could increase the sensitivity of drug, and the combination of dual drugs cannot enhance the cytotoxic effect.

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Hepatocellular carcinoma (HCC) is the cancer with the sixth highest incidence in the world and the third leading cause of cancer-related death.[1] For patients with multinodular HCC and relatively preserved liver function, absence of cancer-related symptoms, and no evidence of vascular invasion or extrahepatic spread at the time of presentation (*i.e.*, those classified as intermediate-stage according to the Barcelona Clinic Liver Cancer (BCLC) staging system[2]), transcatheter arterial chemoembolization (TACE) is the current standard of care.[3]

Hypoxia produces cell death if severe or prolonged, though cancer cells can survive by some genetic and adaptive changes in a mild or short time condition of hypoxia.[4–6] The standard TACE procedure has been established to deliver emulsion of anticancer agent and lipiodol followed by embolization material (*e.g.*, gelatin sponge particles, polyvinyl alcohol (PVA) particles) with or without transarterial chemo-infusion.[3,6,7] The emulsion of lipiodol and anticancer agent is selectively retained within the tumor, which increases the exposure of the neoplastic cells to chemotherapy. Moreover, the embolization material (particles) blocks the tumoral blood supply and limits the washout of drugs, which helps the emulsion to remain inside the tumor.

An unsolved issue is which anticancer drug or which combination of drugs is more effective against HCC. The choice of chemotherapeutic agent remains variable and subject to local practice. Systemic chemotherapy with any of the available agents has marginal antitumor activity with frequent toxicity and no impact on survival.[8] Drug-sensitivity tests for HCC have been performed *in vitro*,[9–11] in which the cells were cultured in the standard assay medium mimicking the scenario of systemic chemotherapy. The chemosensitivity of the HCC cells to anticancer drugs may be different in the conditions of hypoxia and hyponutrition after TACE.

Although more proximal occlusion might be adequate to prevent washout of an emulsion of chemotherapeutic agent and lipiodol, it is difficult to produce all completely nutrition-deprived and hypoxia-induced cell death of a tumor lesion because proximal vessel occlusion results in near-instantaneous recruitment of intraparenchymal collateral flow, and HCCs have a complicated blood supply including the extrahepatic collateral arteries and portal vein.[12–14] Therefore, HCC cells located in different parts of a lesion suffer various degrees of hypoxia and hyponutrition after TACE. For this reason, we used 4 concentrations of hypoxic and hyponutritional culture medium (HHCM), mimicking the scenario of TACE to investigate the sensitivity of anticancer drugs by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Materials and Methods

Drugs and reagents

The 10% fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and the antibiotic solution were purchased from Sijiqing Co (Hangzhou, China). MTT was purchased from Sigma Chemical Company (St Louis, Missouri). Dimethyl sulfoxide (DMSO) was purchased from Sinopharm Chemical Reagent Co (Shanghai, China).

Epirubicin (EPI), mitomycin-C (MMC), oxaliplatin (OXA), hydroxycamptothecin (HCPT), 5-fluorouracil (5-FU), gemcitabine (GEM), and thiotepa (TSPA) were obtained from Pfizer Co (Wuxi, China), Kyowa-Hakko-Kirin Co (Shanhai, China), Sanofi-Aventis Co (Paris, France), Main Luck Pharmaceuticals Co (Shenzhen, China), Jinyao Amino Acid Co (Tianjin, China), Eli Lilly and Company (Indianapolis, Indiana), and Shanghai Xudong Haipu Co (Shanhai, China), respectively. Cisplatin (DDP), docetaxel (DTX), and pemetrexed disodium (PEM) were obtained from Qilu Pharmaceutical Co (Jinan, China).

The testing drug concentrations per well were 10 μg/mL (EPI), 20 μg/mL (OXA), 100 μg/mL (PEM), 200 μg/mL (GEM), 10 μg/mL (DTX), 1 μg/mL (MMC), 3 μg/mL (HCPT), 10 μg/mL (DDP), 100 μg/mL (5-FU), and 2 μg/mL (TSPA), respectively. Their final concentrations were calculated based on common human dose regimens in most TACE procedures.[15–19]

Hypoxic and hyponutritional culture medium

The oxygen- and glucose-deprived medium had been widely used *in vitro*.[20,21] Briefly, the standard assay medium (DMEM supplemented with 10% FBS) was replaced by the glucose-free Earle's

balanced salt solution (EBSS) and cobalt chloride, which were used to mimic the effect of hypoxia by chelation or substitution of iron.[22,23] The HHCM in this study was composed of a standard assay medium and EBSS in various proportions and 100 μmol/L cobalt chloride.[24,25] Concentrations of 5%, 10%, 25%, and 50% HHCM were prepared using the standard assay medium and EBSS in the proportions of 1:19, 1:9, 1:3, and 1:1, respectively.

Groups

The cells treated by a single drug were cultured in 4 concentrations of HHCM, 5%, 10%, 25%, and 50%, for 4 different time periods, 2 hours, 4 hours, 6 hours, and 24 hours, respectively. Therefore, the cells tested were divided into 4 groups in each HHCM, that is, the 2-hour group, 4-hour group, 6-hour group, and 24-hour group, respectively. The combinations of dual drugs involved 7 drugs: EPI, MMC, HCPT, OXA, DDP, GEM, and 5-FU. The cells treated by the combinations of dual drugs were cultured in 4 concentrations of HHCM (5%, 10%, 25%, and 50%) only for 24 hours. That is, the cells treated by the combinations of dual drugs have only a 24-hour group.

Cell line and cell culture

The human hepatocellular carcinoma cell line used in the study was the HepG2 cell line kindly provided by and preserved in the genetics section of Peking University Cancer Hospital. The cell line was maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C in a 5% CO_2 atmosphere with 95% humidity. Cells were passaged twice weekly. All experiments were done with cells in the logarithmic growth phase.

The inhibition rates of cells to single drug and dual drugs were assessed by the MTT assay. Briefly, the cells were seeded in 96-well plates at a density of 2×10^4 cells/well in 100 μL culture medium (standard assay medium) and left to attach to the plate for 24 hours. After 24 hours incubation and attachment, the different concentrations of HHCM (100 μL) with drug (5.5 μL), 5%, 10%, 25%, and 50%, were added in quadruplicate and incubated for an additional 2 hours, 4 hours, 6 hours, and 24 hours, respectively. The incubation time for the combination of dual drugs is only for 24 hours. The culture medium for dual drugs was composed of different concentrations of HHCM (100 μL) and 11 μL of dual drugs (5.5 μL of each drug). The wells of controls contained the same concentration of HHCM (100 μL) without the drugs. The medium was then removed, and fresh medium containing the same concentration of HHCM was added to each well and the cell cultures continued to incubate for 24 hours. After incubation, 5 μL MTT reagent (5 mg/mL) was added, and cell cultures were incubated for 4 hours. A quantity of 100 mL DMSO was added to dissolve the resultant formazan crystals after the medium was removed. The absorbance of the wells was measured in a microplate reader (Bio-Rad model 680, Bio-Rad, Bio-Rad Laboratories, Inc. Hercules, California) at a wavelength of 570 nm.

The relative inhibition rate of tumor cells for drugs was calculated using the following formula: inhibition rate (%) $= (1 - \mathrm{OD}_{\text{drug exposure}}/\mathrm{OD}_{\text{control}}) \times 100$. The results were generated from 3 independent experiments; each experiment was performed in quadruplicate. The anticancer activity was regarded as sensitive when the tumor inhibitory rate was greater than or equal to 30%, and the inhibitory rate greater than 50% was considered as highly sensitive.

Statistical analysis

Statistical analysis was performed using a statistical software package (SPSS 17.0, SPSS Inc, Chicago, Illinois). The inhibition rate of cells was expressed as the mean ± SD from 3 independent experiments performed in quadruplicate. Differences between groups were tested using paired Student's t test. Results were considered significant at 95% confidence interval ($P < 0.05$). All statistical tests were 2-sided.

Results

In 50% HHCM, the drugs 5-FU, DTX, GEM, and TSPA were not sensitive. Inhibition rates greater than 30% were found with EPI, MMC, and HCPT in all 4 time groups and PEM, OXA, and DDP in the 6-hour and 24-hour groups. For each of 6 sensitive drugs, there was no significant difference in sensitivity among the different groups. The highest inhibition rates of the sensitive drugs in all groups were as follows in descending order: EPI, MMC, HCPT, DDP, PEM, and OXA (Fig. 1). The sensitivity of EPI was similar to that of MMC and significantly higher than that of HCPT, DDP, PEM, and OXA ($P =$ 0.019, 0.08, 0.01, and 0.039, respectively). There were no significant differences in sensitivity among the drugs MMC, HCPT, DDP, PEM, and OXA. Com-

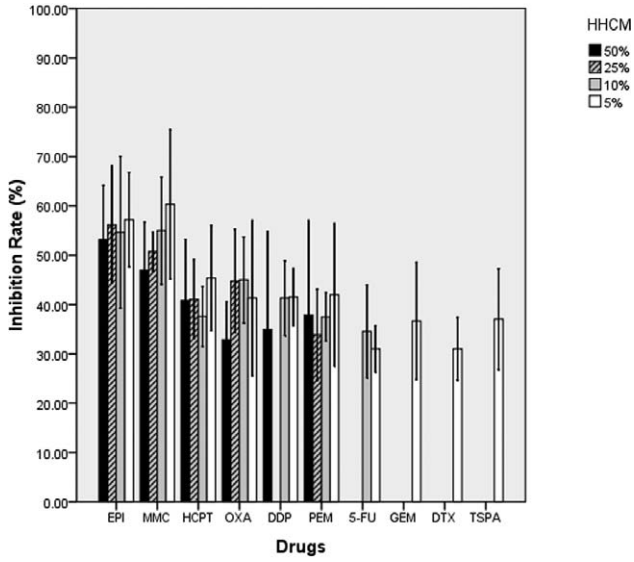

Fig. 1 The highest inhibition rate of the single sensitive drug in all time groups in 50%, 25%, 10%, and 5% HHCM, respectively.

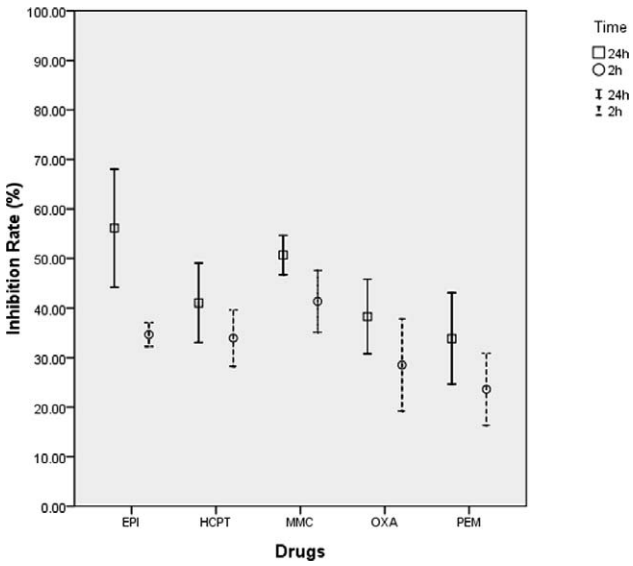

Fig. 3 The inhibition rate of the sensitive drugs in the 2-hour and 24-hour groups in 25% HHCM. The inhibition rate of the 24-hour group was significantly higher than that of the 2-hour group for the 5 drugs.

paring the combination of dual drugs with each of the drugs alone, the inhibition rate of dual drugs was not significantly greater than that of the single drug, with higher sensitivity in 50% HHCM after the cells were treated for 24 hours (Fig. 2).

In 25% HHCM, the drugs DDP, 5-FU, GEM, DTX, and TSPA did not display an effective cytotoxic effect on HepG2 cells. The inhibition rates greater than 30% were found with EPI, MMC, and HCPT in all time groups; with OXA in the 4-hour, 6-hour, and 24-hour groups; and with PEM in the 24-hour group

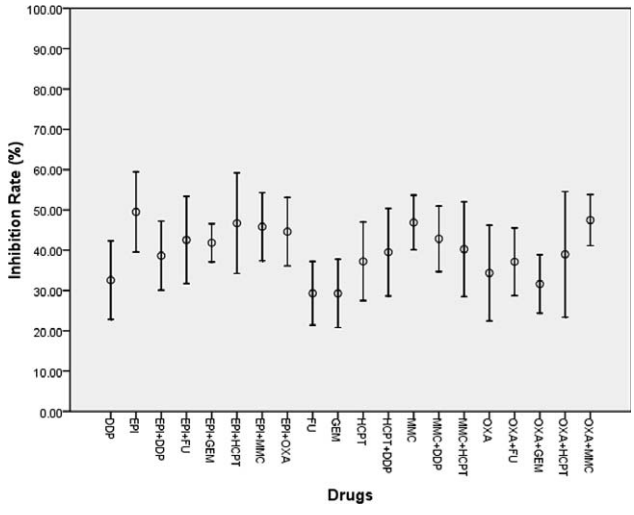

Fig. 2 The inhibition rate of single drug and dual drugs in 50% HHCM.

only. The inhibition rates of 5 sensitive drugs in the 24-hour group was more significant than that of those in the 2-hour group (Fig. 3). The highest inhibition rates of the sensitive drugs in all groups were as follows in descending order: EPI, MMC, OXA, HCPT, and PEM (Fig. 1). The sensitivity of EPI was similar to that of MMC, and they showed high sensitivity. The sensitivity of EPI was significantly higher than that of OXA, HCPT, and PEM ($P = 0.001$, 0.024, and 0.012, respectively). The sensitivity of MMC was significantly higher than that of HCPT and PEM ($P = 0.038$ and 0.022, respectively). The sensitivity of OXA was significantly higher than that of PEM ($P = 0.049$). The inhibition rate of dual drugs was similar to that of the single drug, with higher sensitivity in 25% HHCM after the cells were treated for 24 hours (Fig. 4).

In 10% HHCM, the drugs GEM, DTX, and TSPA were not sensitive. Inhibition rates greater than 30% were found with EPI and PEM in all 4 groups; with MMC and HCPT in the 4-hour, 6-hour, and 24-hour groups; with OXA and DDP in the 6-hour and 24-hour groups; and with 5-FU in the 24-hour group. The sensitivity of the 7 drugs in the 24-hour group was significantly higher than that of those in the 2-hour group (Fig. 5). The highest inhibition rates of the sensitive drugs in all groups were as follows in descending order: MMC, EPI, OXA, DDP, HCPT, PEM, and 5-FU (Fig. 1). MMC and EPI were highly

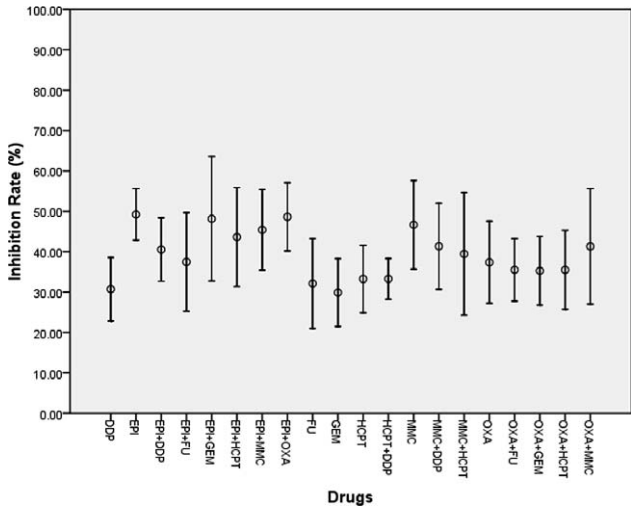

Fig. 4 The inhibition rate of single drug and dual drugs in 25% HHCM.

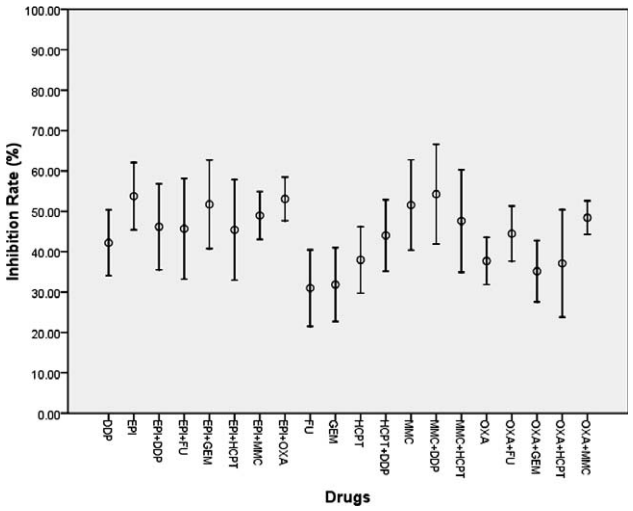

Fig. 6 The inhibition rate of single drug and dual drugs in 10% HHCM.

sensitive. The sensitivity of MMC was similar to that of EPI, and their sensitivities were significantly higher than those of the 5 other drugs. OXA showed significantly higher sensitivity than DDP, HCPT, and PEM ($P = 0.007$, 0.009, and 0.025, respectively). The inhibition rate of dual drugs in 10% HHCM was not significantly higher than that of the single drugs, with higher sensitivity after the cells were treated for 24 hours (Fig. 6).

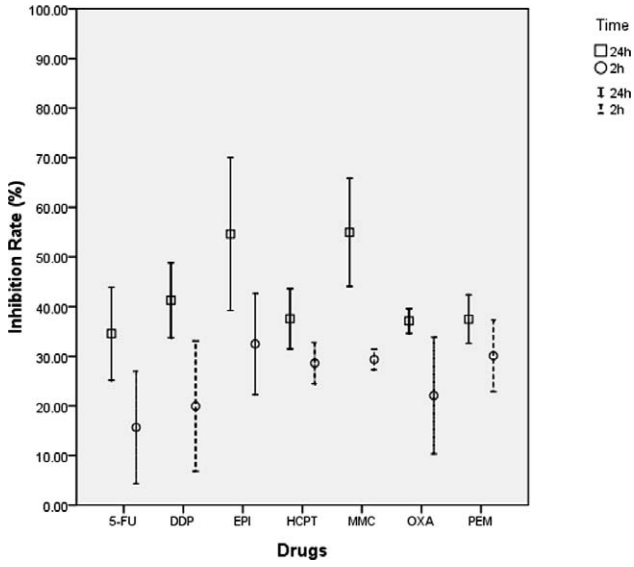

Fig. 5 The inhibition rate of the sensitive drugs in the 2-hour and 24-hour groups in 10% HHCM. The inhibition rate of the 24-hour group was significantly higher than that of the 2-hour group for the 7 drugs.

In 5% HHCM, all 10 drugs were sensitive. The highest inhibition rates of the sensitive drugs in all groups were as follows in descending order: MMC, EPI, HCPT, PEM, DDP, OXA, TSPA, GEM, 5-FU, and DTX (Fig. 1). The inhibition rates greater than 30% were found with EPI, MMC, and HCPT in all 4 groups; with PEM in the 4-hour and 24-hour groups; and with OXA in the 6-hour and 24-hour groups. DDP, TSPA, GEM, 5-FU, and DTX only showed sensitivity in the 24-hour group. The sensitivities of 10 drugs in the 24- hour group were significantly higher than those in the 2-hour group (Fig. 7). MMC and EPI were highly sensitive. There was no significant difference in sensitivity among HCPT, PEM, DDP, and OXA. There was also no significant difference in sensitivity among 5-FU, GEM, DTX, and TSPA. The inhibition rate of a combination of dual drugs in 5% HHCM was also not significantly higher than that of the single drug with higher sensitivity after the cells were treated for 24 hours (Fig. 8).

The highest inhibition rates of each of the sensitive drugs (MMC, EPI, HCPT, PEM, DDP, OXA, and 5-FU) in all groups were not significantly different among different concentrations of HHCM (Fig. 1). But TSPA, GEM, and DTX were only sensitive in 5% HHCM.

Discussion

There is a lack of consensus regarding the use and type of chemotherapy agent, as well as the optimum methods of delivery of the agents in TACE.[15,26]

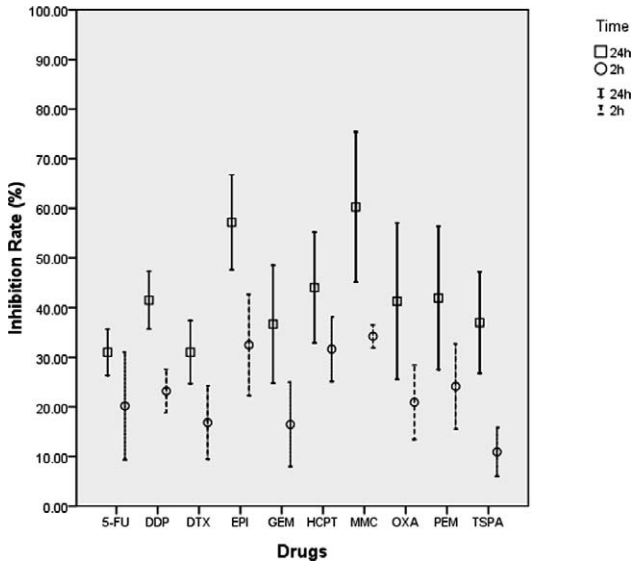

Fig. 7 The inhibition rate of all 10 drugs in the 2-hour and 24-hour groups in 5% HHCM. The inhibition rate of the 24-hour group was significantly higher than that of the 2-hour group for all drugs.

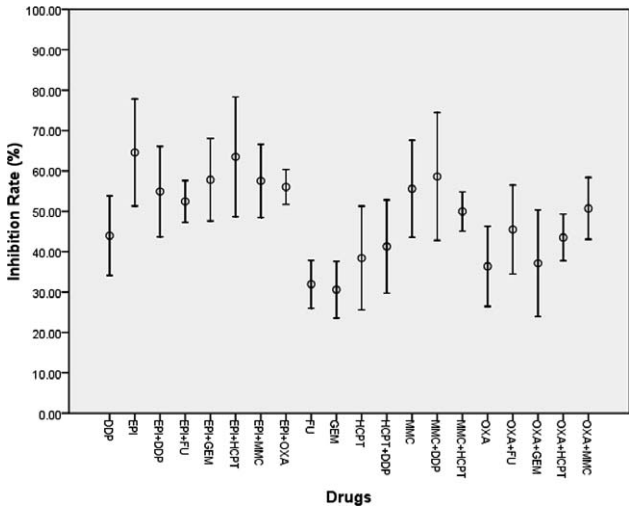

Fig. 8 The inhibition rate of single drug and dual drugs in 5% HHCM.

Drug-sensitivity tests have been successfully used in assay-guided chemotherapy for certain cancers, including breast cancer, ovarian cancer, melanoma, and gastric cancer.[27–30] The predictive information of a drug-sensitivity test mimicking the scenario of TACE may help interventional radiologists choose sensitive chemotherapy agents, while eliminating ineffective agents used in chemotherapy regimens for TACE. Even in patients who show no good tumor response after one TACE session, a change to another sensitive drug could be considered as an alternative to further TACE cycles of therapy. MTT assay has been most widely used in different cancers and is sensitive, accurate, and efficient in the *in vitro* evaluation of anticancer or immunologic agents prior to preclinical and clinical testing. In this study, HHCM was used without drug instead of the standard medium as a control to observe whether the anticancer drugs can further inhibit cell growth in addition to hypoxia caused by embolization. Cells were treated by a combination of dual drugs for 24 hours in order to test whether there is an antagonistic or synergistic cytotoxic effect compared with both drugs acting alone.

The aim of the agent used in TACE is expected to augment antitumor efficacy with milder side effects. The reinforced cytotoxic effect may be helpful in preventing tumor recurrence by treating the peripheral part of the tumor where complete ischemia is impossible to achieve. Our data suggest that long-time drug exposure could increase the sensitivity of the cells to drugs in the context of ischemia. Therefore, the drug should be infused through the catheter/microcatheter by a mechanical infusion pump set at a fixed rate for a long time or used in the emulsion with lipiodol. The intraoperative bolus arterial infusion is not recommended in the procedure of TACE. The inhibition rates of each drug were not significantly different among 4 concentrations of HHCM, which indicates that it is unlikely that more hypoxia and hyponutrition would enhance the sensitivity of HepG2 cells to the agents. EPI, MMC, HCPT, OXA, and PEM were sensitive in all 4 concentrations of HHCM. Among them, MMC and EPI were highly sensitive. Our study also demonstrated that the inhibition rate of the combination of dual drugs is similar to the single drug in that combination, with higher sensitivity. There is neither additive nor synergistic cytotoxic effect compared with each drug alone. Therefore, to prevent potentially adverse effects, the combination of dual drugs mixed with lipiodol should be avoided.

The drugs 5-FU, DTX, GEM, and TSPA were not sensitive in the 25% and 50% HHCM, which indicates that these 4 drugs cannot further improve the therapeutic effect in mild hypoxia. The drugs 5-FU and GEM have been applied to the clinical TACE practice, and some drug sensitivity tests *in vitro* have demonstrated their sensitivity,[9,10,17–19] but our result is converse. The lower inhibition rate ob-

served in the experimental setting let us hypothesize that the environment of hypoxia and hyponutrition shifts more cells into a quiescent state. The increase in quiescent cells might result in decreased sensitivity of the drugs that only preferentially kill cycling cells.[31] It is noted that TSPA, GEM, and DTX were slightly sensitive only in the 24-hour group in 5% HHCM but not in any other groups. The sensitivity of the cells to these 3 drugs is probably due in part to the context of extremely severe hypoxia and hyponutrition. With severe hypoxia and hyponutrition, cell death can occur through specific cellular mechanisms.[8,32] But it is unclear how severe hypoxia and hyponutrition affect the sensitivity of these 3 drugs.

There are several limitations to our study. First, we tested only one cell line using one method of drug-sensitivity assay, thus the bias by artifacts related to assay- and cell line–specific effects is unavoidable. Second, the complexity of both the vascular supply of the tumor and its hemodynamics result in HCC cells located in different regions suffering various degrees of hypoxia and various concentrations of drug in the TACE procedure. In addition, most patients with HCC presented with underlying liver disease (usually cirrhosis). These conditions are difficult to mimic *in vitro*. Third, the internal environment of a perfused liver is different from an isolated HCC cell line. In a mild or short duration hypoxic environment *in vivo*, cancer cells undergo genetic and adaptive changes (*e.g.*, a shift from aerobic to anaerobic metabolism angiogenesis, growth-factor signaling, immortalization, genetic instability), which allow them to survive and even proliferate, although the exact change within the tumor microenvironment remains unclear.[4,5,25] Thus, results of the *in vitro* study cannot be directly extrapolated to the *in vivo* situation. Future studies regarding the cytotoxicity should expand to multiple cell lines and unravel the molecular biology of HCC cell in TACE.

In conclusion, the lists of sensitive drugs were different in different contexts of hypoxia and hyponutrition. Five drugs, EPI, MMC, HCPT, OXA, and PEM, exhibited cytotoxic activity against HepG2 cell line in 4 concentrations of HHCM. Prolonging the time of exposure could increase the sensitivity of the drug in the context of severe ischemia. The combination of dual drugs may not enhance the cytotoxic effect of the emulsion of lipiodol toward the cell line compared with single drug.

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