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Research Article
Brucine Sensitizes HepG2 Human Liver Cancer Cells to 5-fluorouracil via Fas/FasL Apoptotic Pathway

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Abstract

Background and Objective: The clinical use of chemotherapeutic drugs is normally restricted by severe side effects and drug resistances. Brucine is a natural alkaloid derived from the seeds of *Strychnos nux-vomica* Linn., which has been reported to possess antitumor activity. This study was aimed to investigate the effects of brucine on 5-fluorouracil (5-FU)-induced cell death in human liver carcinoma HepG2 cells. **Materials and Methods:** Cells were treated with 5-FU and brucine alone or in combination and cell viability was measured using MTT method. Apoptotic effects were analyzed by using acridine orange/ethidium bromide (AO/EB) staining and annexin V/PI staining assays. The mRNA expression of Fas/FasL pathway members including Fas, Fas ligand (FasL) and Fas-associated protein with the death domain (FADD) was detected by real-time PCR. Western blot analysis was further conducted to determine the expression of Fas and FasL proteins. **Results:** Combination treatment obviously decreased the viability of HepG2 cells compared to 5-FU treatment alone. Exposure of cells to 5-FU and brucine in combination lead to increased apoptosis as evidenced by staining with AO/EB and annexin V/PI. Real-time PCR assay showed that 5-FU in combination with brucine markedly enhanced the mRNA levels of Fas, FasL and FADD. In addition, the upregulation of Fas and FasL proteins expression was also confirmed by Western blot analysis. **Conclusion:** These findings suggested that brucine could enhance 5-FU-induced the cytotoxicity and apoptosis through Fas/FasL signaling pathway in HepG2 cells. Administration of 5-FU in combination with brucine may be an effective and feasible therapy for human liver carcinoma.

Key words: Brucine, natural alkaloid, 5-FU, combination therapy, antitumor, apoptosis, HepG2 cells, Fas/FasL signaling pathway

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatocellular carcinoma (HCC) remains one of the most prevalent malignancies worldwide[1]. The incidence of this cancer is increasing possibly due to the epidemic of obesity, alcohol-related cirrhosis and chronic viral hepatitis B and C infections[2]. Currently, various methods are available for HCC treatment, including surgery, chemotherapy, radiotherapy and their combination[3]. Despite great progress in prevention and treatment, the clinical outcome is still not satisfactory[4].

5-fluorouracil (5-FU) is a classical chemotherapy agent which has been used for treating HCC for several decades[5]. It can block DNA synthesis by the inhibition of Thymidylate Synthase (TS) that is regulated by cell cycle proteins controlled by phosphorylation, resulting in cytotoxicity and cell death[6,7]. Unfortunately, drug resistance and severe adverse side-effects are major obstacles in cancer chemotherapy[8,9]. Thus, there is an urgent need to develop alternative strategy to treat liver cancer.

Brucine, a natural plant alkaloid, isolated from seeds of *Strychnos nux-vomica* Linn. (Loganiaceae) has been shown to possess analgesic, anti-inflammatory, antitumor and antiangiogenic activities[10,11]. Brucine can induce apoptosis in various cancer cells, including human colon carcinoma LoVo cells, hepatoma SMMC 7221 cells and HepG 2 cells[12-15]. Recently, a therapy combining chemotherapeutic agent and phytochemical has received a great deal of attention because it could not only enhance the treatment effect but also reduce drug toxicity[16]. The aim of the present study was to assess the combined effect of 5-FU and brucine on human hepatoma HepG2 cells *in vitro* and investigate the molecular mechanisms involved.

MATERIALS AND METHODS

Materials: Brucine and 5-fluorouracil were purchased from Aladdin Industrial Corporation (Shanghai, China). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. (St., Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), trypsin, Fetal Bovine Serum (FBS), penicillin and streptomycin were purchased from Invitrogen Life Technologies, Inc. (Grand Island, NY, USA). Acridine Orange (AO) and Ethidium Bromide (EB) were obtained from Amerisco (Solon, Ohio, USA). The annexin V-FITC apoptosis detection kit was supplied by KeyGen technology (Nanjing, China). The RIPA lysis buffer and protease inhibitors were purchased from Vazyme Biotech Co., Ltd. (Jiangsu, China). Antibodies against Fas and FasL were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against GAPDH, anti-rabbit secondary antibodies and the enhanced chemiluminescence (ECL) kit were purchased from CoWin Biotech Co., Ltd. (Beijing, China). All the other reagents used were of analytical grade.

Cell culture and treatments: Human hepatoma HepG2 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Exponentially growing HepG2 cells were seeded into plates and allowed to attach for 24 h before treatment. The stock solutions of 5-FU and brucine were prepared in DMSO and the vehicle was present at final concentration 0.1% DMSO in the medium. The same final concentration of the solvent was used in the corresponding control.

Cell viability assay: Cell viability was determined using the MTT assay as described previously[17]. In brief, the HepG2 cells were seeded into 96-well plates at a density of 1×10^4 cells per well. After overnight growth, the cells were treated with 5-FU or brucine or in combination for 24 h. Then, 10 μL of MTT (5 mg mL^{-1}) was added to each well and the plate was incubated for 4 h at 37°C. Thereafter, the medium was carefully discarded and the formazan crystals were dissolved in 100 μL dimethylsulfoxide (DMSO). The absorbance values at 570 nm were measured using a microplate reader (Model 680, Bio-rad, US). All experiments were performed in triplicate and the results for the absorbance measured in treated cells were calculated as percentages of the absorbance in untreated control cells.

Acridine orange/ethidium bromide (AO/EB) double staining: Analysis of changes in cell morphology was evaluated using AO/EB fluorescence staining[18]. Briefly, HepG2 cells (1×10^5 cells per well) were seeded in a 6-well plate and incubated for 24 h, following by further treatment with 5-FU or brucine or in combination for an additional 24 h. Then the cells were washed twice with PBS and then stained with 1 mL cold PBS containing 40 μL mixture of 1 mg mL^{-1} AO and 1 mg mL^{-1} EB in the dark for 10 min. After two washes with PBS, the stained cells were observed using a fluorescence microscope (Olympus, BX-60, Japan). Each test was performed in triplicate.

Annexin V-FITC/PI double staining study using flow cytometry: Annexin V-propidium iodide double staining assay was done to quantify apoptosis in HepG2 cells using FACscan flow cytometer[19]. In brief, exponentially growing HepG2 cells were seeded in a 6 well plate at 1.5×10^6 cells per well. After cultured for 24 h, cells were exposed to 5-FU or brucine or in combination for 24 h. After the treatment, cells were trypsinized, collected and incubated with 5 µL annexin V-FITC and 10 µL PI in dark for 15 min at 4°C. Cell apoptosis was were analyzed using flow cytometry and Cell Quest software (BD Biosciences, CA, USA) within 1 h. Cells that were annexin V(+)/PI(−) cells (lower right quadrant) were defined as early apoptotic cells and annexin V(+)/PI(+) (upper right quadrant) as late apoptotic cells. Each test was performed in triplicate:

$$\text{Apoptotic rate (\%)} = \frac{\text{No. of apoptotic cells}}{\text{No. of total cells observed}}\times100$$

RNA extraction and real-time quantitative PCR: The mRNA levels of Fas, Fas ligand (FasL) and FADD were determined by real-time quantitative PCR. Total RNA from cells in each group was isolated by using trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Real time-PCR were performed with 40 ng RNA using iScript™ one-step RT-PCR kit with SYBR® green (Bio-rad) as previously described[20], using the following primers:

Fas (forward: 5 -TGCCATAAGCCCTGTCCTC-3 and reverse: 5-ACGCAGTCTGGTTCATCCC-3), FasL (forward: 5-CCAGCTTG CCTCCTCTTGAG-3 and reverse: 5-TCCTGTAGAGGCTGAGGT GTCA-3), FADD (forward: 5-AGGTGGAGAACTGGGATTTGA-3 and reverse: 5-CTCCAAGGAAATGGGACAAA-3) and GAPDH (forward: 5-TGGGTGTGAACCATGAGAAGT-3 and reverse: 5-TGAGTCCTTCCACGATACCAA-3).

The amount of target mRNA was determined using the comparative threshold (C_t) method by normalizing target mRNA C_t values to those for GAPDH (ΔC_t). Statistical analysis of real-time PCR data was performed using ΔC_t values[21]. Each genes analysis was performed in triplicate.

Protein extraction and western blot analysis: After each treatment, cells were collected, lysed on ice in cell lysis buffer (Beyotime Inst., Biotech, China) and subjected to centrifugation at 4°C with 12,000 rpm for 15 min. The protein concentration in the supernatant was determined using the BCA assay (Beyotime Inst., Biotech, China). Equal amounts of protein (40 µg) were subjected to 12% SDS-polyacrylamide gel electrophoresis along with protein molecular weight standards and then transferred to nitrocellulose membranes. The membranes were blocked with tris-buffered saline plus tween-20 (TBS-T) containing 5% non-fat milk for 1.5 h and incubated with TBS-T containing specific primary antibodies including anti-Fas, anti-FasL and anti-GAPDH overnight at 4°C. Then, appropriate secondary horseradish-peroxidase labeled antibodies were incubated for 1 h at 37°C. The immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (CoWin Biotech Co., China) and exposed to gel imaging system. The band density analysis was performed using ImageJ software. Band intensities were normalized to GAPDH.

Statistical analysis: Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* correction for multiple comparisons using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as the Mean±SEM. Significant differences were considered with values of $p<0.05$.

RESULTS

Effect of brucine on 5-FU induced cell viability: First, the viability of the brucine-treated HepG2 cells was evaluated using the MTT assay. Figure 1a shows the brucine induced a decrease in cell viability in a dose-dependent manner. Treatment with brucine at the concentration of 100 µg mL^{-1} had a moderately inhibitory effect and about 20% of cell growth inhibition ratio was achieved.

To investigate whether brucine promotes 5-FU induced death of HepG2 cells, the cells were treated with 5-FU in the presence and absence of brucine. Treatment of HepG2 cells with 10, 20, 40, 80 and 100 µg mL^{-1} of 5-FU alone decreased the cell viability by 9.13±3.62, 17.21±6.53, 24.03±6.92, 32.97±5.80 and 46.38±5.26%, respectively (Fig. 1b). However, after co-administration of 5-FU with brucine (100 µg mL^{-1}), the viability of HepG2 cells was significantly reduced in a concentration-dependent manner of 5-FU, showing 1.63-fold decrease at 40 µg mL^{-1} of 5-FU. It indicated that combination treatment led to higher inhibition rate than 5-FU alone in HepG2 cells after 24 h exposure and 100 µg mL^{-1} of brucine and 40 µg mL^{-1} of 5-FU were used in the subsequent experiments.

Effect of combination treatment on cell morphological changes: To determine whether the cell death is associated with apoptosis, AO/EB double staining was used to detect the morphological changes in HepG2 cells. The AO, a membrane-permeable dye can stain both normal and apoptotic cells, whereas EB stains only cells that have lost membrane integrity. Therefore, AO can emit a green

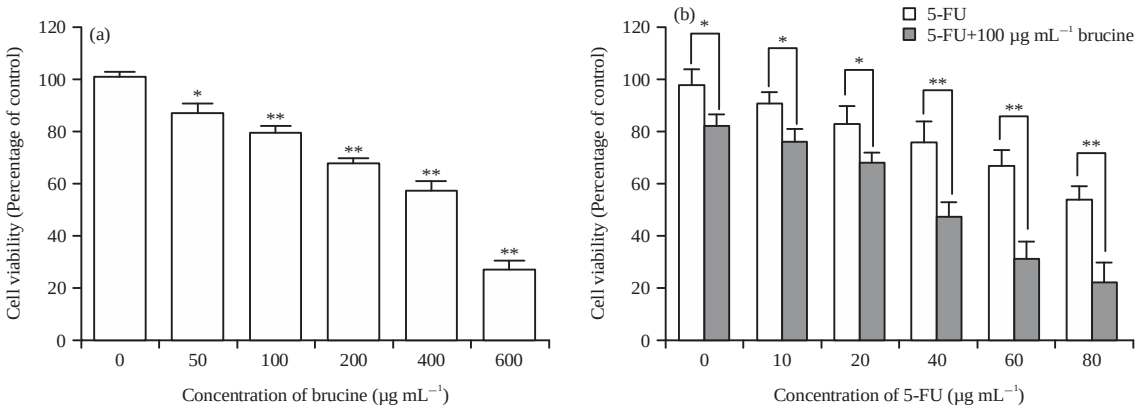

Fig. 1(a-b): Effects of the combination of 5-FU and brucine on the growth of HepG2 cells. (a) Cells were treated with brucine with various concentrations (0, 50, 100, 200, 400 and 600 μg mL^{-1}) for 24 h. Data represent the Mean±SEM of at least three independent experiments. *p<0.05, **p<0.01 compared with control and (b) HepG2 cells were treated with 5-FU alone or combination of 5-FU and 100 μg mL^{-1} brucine for 24 h. Data represent the Mean±SEM of at least three independent experiments. *p<0.05, **p<0.01 compared with the cells treated 5-FU alone

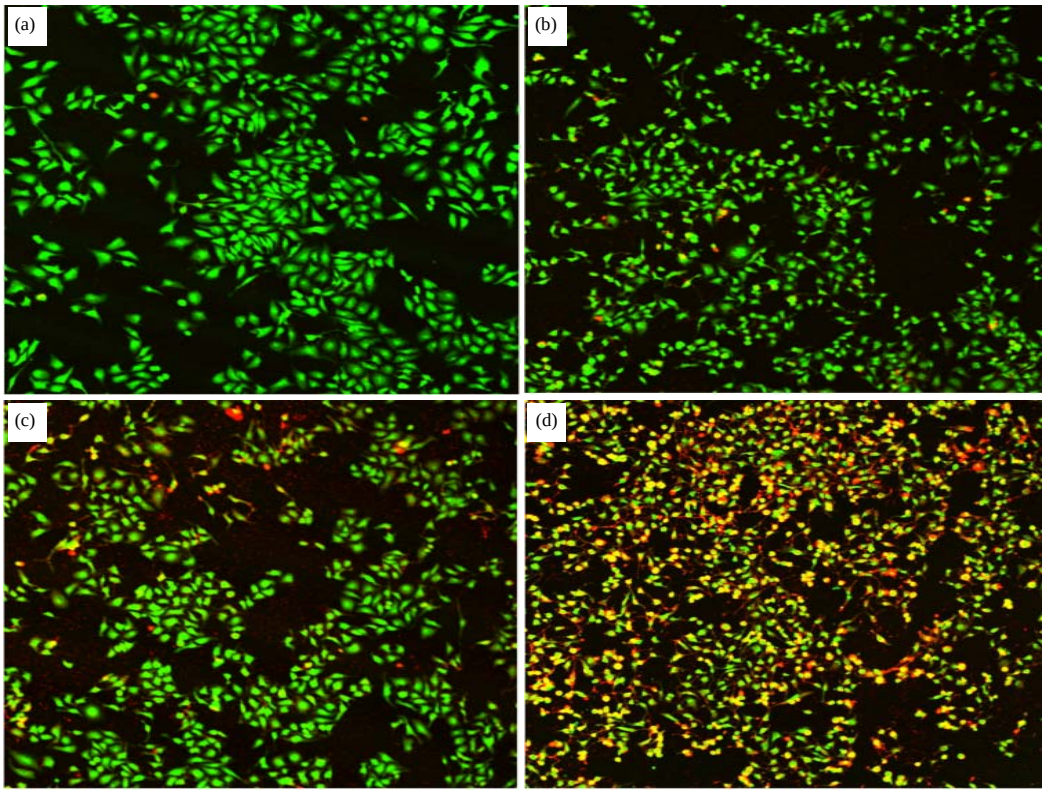

Fig. 2(a-d): Effects of the combination of 5-FU and brucine on cell morphological changes. (a) Control HepG2 cells were treated with (b) Brucine, (c) 5-FU and (d) Brucine+5-FU in combination for 24 h and observed by AO/EB double staining with fluorescent microscope (×200). Normal cell are in spindle shape with the nuclei stained green uniformly and early phase and late phase apoptotic cells present condensed green or depressed orange nuclei separately

fluorescence if it passes through the complete cell membrane and embeds in nuclear DNA, while EB can mark nuclear DNA of damaged cells and emit a red/orange fluorescence. Figure 2 shows the uniformly green live cells with normal morphology were observed in the control group. After treated by brucine, a small fraction of naturally apoptotic cells

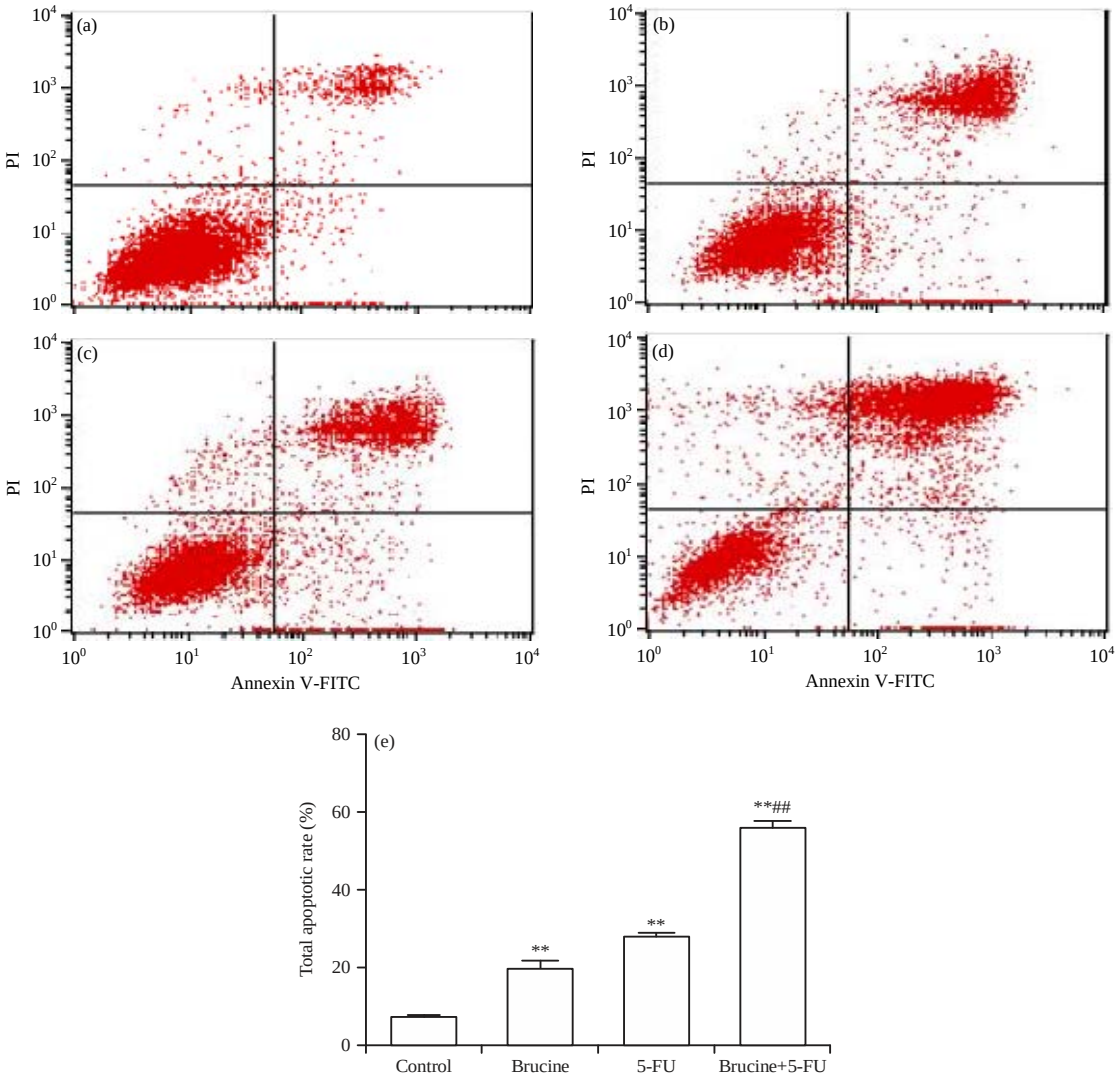

Fig. 3(a-e): Effects of the combination of brucine and 5-FU on cell apoptosis. Representative images of FACS analysis of cell death upon single or joint exposure to brucine and 5-FU for 24 h using the FITC-conjugated annexin V and PI staining, (a) Control, (b) Brucine, (c) 5-FU, (d) Burcine+5-FU and (e) Total apoptotic rates shown as histograms after treatment. Data are presented as Mean±SEM (n = 3). **p<0.01 compared with the control group, ##p<0.01 compared with 5-FU group

presented condensed green or depressed orange nuclei, whereas, orange/red apoptotic cells with fragmented chromatin and apoptotic bodies occurred in 5-FU group. In contrast, the combination of brucine and 5-FU obviously enhanced these morphological changes and increased the cell apoptosis in HepG2 cells compared with 5-FU alone.

Effect of combination treatment on cell apoptosis: To further quantify cell apoptosis, HepG2 cells were stained with annexin V-FITC and PI and then analyzed. Figure 3a-e shows, the ratios of apoptotic cells, as evidenced by annexin V-positivity in the control and brucine groups were 7.13±0.48 and 19.69±3.25%, respectively, whereas treatment with 40 μg mL^{-1} of 5-FU for 24 h increased this ratio to 27.74±1.76%. It is noteworthy that the percentage of total apoptotic cells after co-incubation with brucine and 5-FU was 55.49±3.47%, significantly higher than that of the 5-FU group ($p<0.01$), indicating that combination of two agents dramatically promoted apoptosis of HepG2 cells.

Effect of combination treatment on genes expression of Fas/FasL apoptotic pathway: To explore whether the apoptosis occurred via Fas/FasL pathway, the mRNA expression of Fas, FasL and FADD was examined by real-time

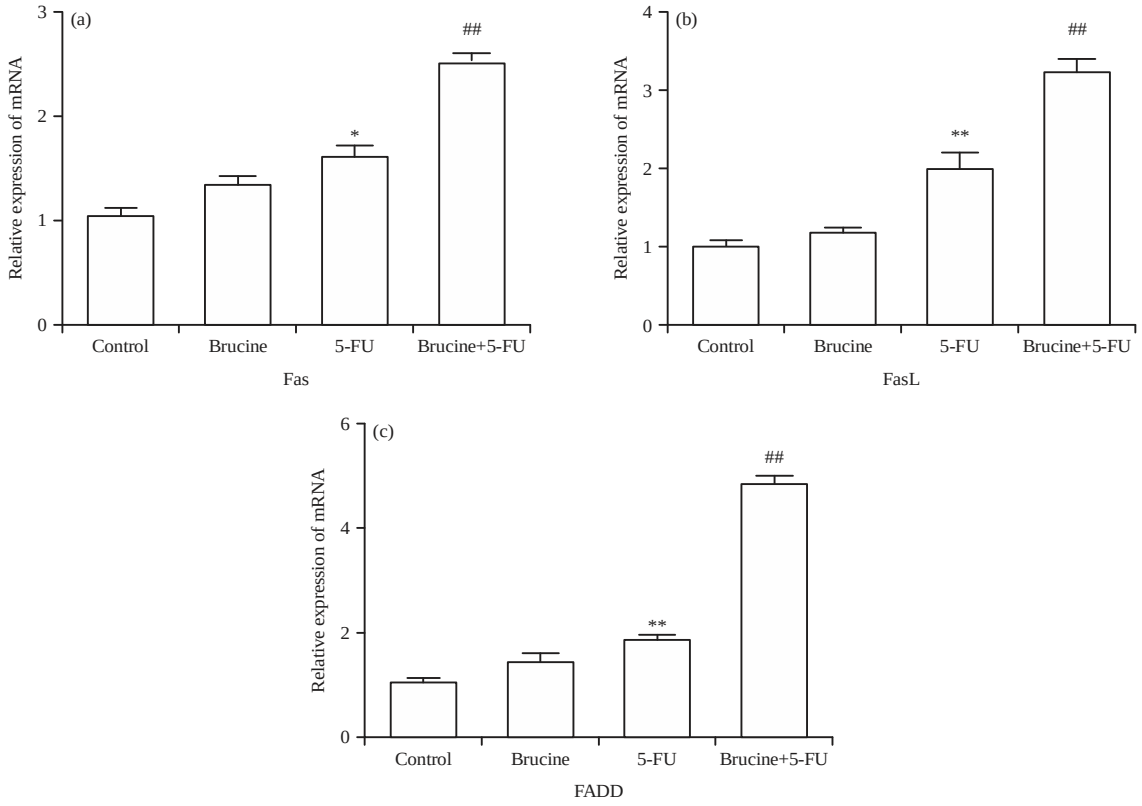

Fig. 4(a-c): Effect of the combination of brucine and 5-FU on genes expression of Fas/FasL apoptotic pathway. (a) Fas mRNA expression, (b) FasL mRNA expression and (c) FADD mRNA expression. The mRNA levels were detected by real-time PCR analysis and GADPH was as internal reference to normalized genes signal. Each value represents the Mean±SEM (n = 3), *p<0.01, **p<0.01 compared with the control group, ##p<0.01 compared with 5-FU group

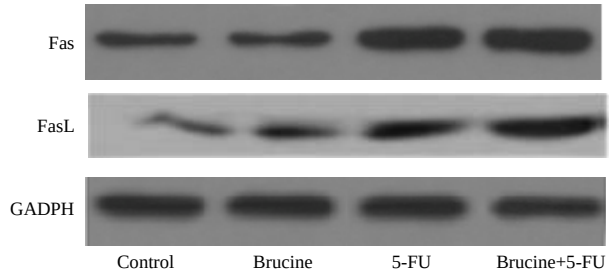

Fig. 5: Effect of the combination of brucine and 5-FU on Fas and FasL proteins expression. Western blot was employed to determine the proteins expression of Fas and FasL with GADPH as loading control. The immunoblots shown are from one experiment representative of three that gave similar results

PCR method. Compared to the control group, expression levels of Fas, FasL and FADD in burcine group were higher, however, no statistical differences were observed (Fig. 4a-c). While 5-FU treatment for 24 h markedly upregulated these genes expression ($p<0.05$). Moreover, HepG2 cells exposed to 5-FU and brucine for 24 h showed significantly increased mRNA expression levels for Fas receptor, Fas ligand and FADD, equivalent to 1.56, 1.62 and 2.60 folds, respectively, when compared to cells treated with 5-FU alone.

Effect of combination treatment on Fas and FasL proteins expression: To further confirm the results, Western blot analysis was employed to compare changes in the Fas and FasL protein levels. Figure 5 shows the expression of Fas and FasL was similar in brucine and control groups, whereas, 5-FU obviously enhanced their expression. In contrast, the protein levels of Fas and FasL were significantly increased by treatment with 5-FU plus brucine compared to the levels in the cells treated with 5-FU alone. Taken together, these findings suggested that brucine could promote the Fas/FasL apoptotic pathway induced by 5-FU.

DISCUSSION

Recently, combination with agents that sensitize cancer cells to conventional chemotherapy drugs has gained considerable attention in efforts to improve their therapeutic outcomes[22]. Brucine is a naturally occurring dihydroindole-type alkaloid from the seeds of *Strychnos nux-vomica* L.[23]. It has been demonstrated to exhibit anticancer activity and induce apoptosis in a wide variety of cancer cells[24,25]. Furthermore, Serasanambati *et al.*[16,26] reported that brucine in combination with gemcitabine showed supra-additive anticancer effects in human breast cancer MDA MB-231 cells and MCF-7 cells. They found that only brucine either alone or in combination, but not gemcitabine was effective in inhibiting the expression of NF-κB subunit (p65) in MCF-7 cells. Therefore, brucine is regarded as a promising candidate for a new type of chemotherapeutic agent for cancer treatment. It would be interesting to explore whether treatment with brucine alone or in combination with an anticancer drug can enhance antitumor activity and reduce dose. In the present study, it was investigated that the effects of combining 5-FU, a well-known anticancer agent, with brucine in human liver carcinoma HepG2 cells and evaluated the probable mechanism of action.

Firstly, MTT assay was employed to determine the effects of brucine and 5-FU or their combination on the growth of HepG2 cells. Our data revealed that brucine sensitized 5-FU to inhibit HepG2 cells proliferation *in vitro*. The combination of 5-FU and brucine exhibited stronger anticancer efficiency than 5-FU alone (Fig. 1b). In addition, AO/EB double stained method indicated that combination therapy showed more obvious morphologically apoptotic characters (Fig. 2). Following this, the FACS analysis to confirm the occurrence of apoptosis was applied. Figure 3 indicated that this combination of 5-FU and brucine significantly increased the population of annexin V-positive cells (apoptotic cells) in the right quadrants of the flow cytometry graphs. These results agreed with the data from the above cytotoxicity assay, suggesting that the combination-induced anticancer effect was mediated by the induction of apoptosis in HepG2 cells.

Chemotherapy drug 5-FU is extensively used to treat solid tumors such as liver, breast, colorectum and brain cancer[27]. Recently, there has been growing interest in the development of combination therapies of 5-FU with other agents to overcome the drug resistance and increase efficacy. Sun *et al.*[28] found that epigallocatechin gallate (EGCG) could significantly enhance 5-FU antitumor activity in MCF7 cells by increasing apoptosis and decreasing the cell viability and the greatly improved resistance efficacy might be through regulating the expression of Bcl-xL. Another report by Xu *et al.*[29] showed that luteolin derived from *Reseda odorata* L. could synergize the antitumor effects of 5-FU on hepatocellular carcinoma HepG2 and Bel7402 cells, which might be related to apoptosis and regulation of 5-FU metabolism. Moreover, Gao *et al.*[30] demonstrated that 5-FU and docosahexaenoic acid (DHA) alone or in combination could markedly suppress the proliferation of human gastric cancer AGS cells in a significant time and dose-dependent manner via involving interference with energy production. It has been well reported that 5-FU exerts its cytotoxicity through activation of both extrinsic and intrinsic pathways of apoptosis[31]. Here, we focused on the effect of combination of 5-FU and brucine on the extrinsic apoptosis pathway. In the extrinsic pathway, which is also known as the "Death receptor pathway", apoptosis is triggered by the ligand-induced activation of death[32]. Death receptors are members of the TNF receptor gene super family, which consists of more than 20 proteins with a wide range of biological functions including the regulation of cell death and survival, differentiation or immune regulation[33,34]. In general, the death receptors mainly comprise Fas (CD95/APO-1), TNF receptor 1 (TNFR1), the two agonistic-receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5)[35]. Among them, the molecular events that are activated by FasL to trigger Fas-mediated apoptosis are well characterized. Upon FasL stimulation, the activated receptor then transmits the apoptotic signal to the cytoplasm by recruiting the adapter molecule Fas-Associated Death Domain (FADD) and procaspase-8 via its cytoplasmic death domain to form the Death-Inducing Signalling Complex (DISC) and in turn activates effector caspases such as caspase-3 in causing cell death[36-38]. Present data indicated that combination therapy with 5-FU and brucine increased expression of genes associated with the Fas-signaling cascade including Fas receptor, Fas ligand and FADD compared to either agent alone. Additionally, the Fas and FasL proteins expression in response to 5-FU in combination with brucine was confirmed to be up-regulated by Western blot. Collectively, these findings indicate that the combination of 5-FU and brucine is a promising therapeutic strategy to induce apoptosis in the HepG2 cells by activating Fas/FasL pathway. The results are in agreement with the previous reports which showed that colon, pancreatic and leukemia cell lines were sensitived to Fas ligand after treatment with a wide range of chemotherapeutic drugs[39-41].

CONCLUSION

In conclusion, the present study provided evidence of the synergistic anticancer effect of 5-FU and brucine in human hepatocellular carcinoma HepG2 cell line. Combination treatment of 5-FU and brucine for 24 h significantly increased the anticancer efficacy, which might be related with apoptosis by activating the Fas/FasL pathway. This finding is encouraging for the development of advantageous treatment regimens for hepatocellular carcinoma and further experimental studies are needed.

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