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Brief Report

Comparison of Cytotoxic Activity of Bile on HepG2 and CCRF-CEM Cell Lines: An in Vitro Study

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Abstract

The aim of this study was to examine the effect of crude bile on the human HepG2 and CCRF-CEM cell lines. Cells were exposed to different dilutions of bile. Antiproliferative effects were determined by the cytotoxic MTT assay. Cells undergoing apoptosis were identified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Bile administration resulted in dose-dependent cytotoxicity in both HepG2 and CCRF-CEM cell lines. Incubated cells exhibited morphologic features of apoptosis. Bile has significant cytotoxic activity in HepG2 and CCRF-CEM cancer cells via induction of apoptosis. The mechanism of apoptosis needs to be further evaluated. It may have clinical utility in the treatment of cancer after in vivo confirmation of activity.

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Keywords ● Bile ●Neoplasms ● Cytotoxicity

Introduction

Bile or gall is a bitter-tasting, dark green to yellowish brown fluid, produced by the liver of most vertebrates. Bile acids, the major organic solutes in bile, are made by the cytochrome P450-mediated oxidation of cholesterol. These acids are subsequently excreted via bile into the small intestine where they aid solubilization and absorption of lipids.[1,2] Bile acids also control hepatic glucose homeostasis, thermogenesis, energy homeostasis, and inflammatory responses.[3] The primary bile acids, cholic acid and chenodeoxycholic acid (CDCA), are directly synthesized from cholesterol by hepatocytes. Most bile acids are conjugated with glycine or taurine to decrease toxicity and increase solubility for secretion into bile. Almost 95% of total bile acids are re-absorbed in the ileum and excreted into portal blood circulation and returned to the liver. The remaining 5% of bile acids that escape the enterohepatic circulation, enter the colon where enteric bacteria modify the bile acid side chain. Therefore, secondary hydrophobic bile acids are formed, namely, deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA).[4]

There are controversies about the cytotoxic or cytoprotective effects of different bile acids. Epidemiological studies have shown a strong relationship between elevated fecal bile acids and increased risk of colon cancer.[5] Others have shown that bile acids inhibit cell growth and induce apoptosis.[5] Bile salts seem to play a role in neoplastic development in Barrett's metaplasia via high up-regulation of COX-2, CDX-2 and down-regulation of DNA repair enzymes.[6,7] Another study evaluating the effect of bile acids on ovarian cancer cells showed that cholic acid and ursodeoxy cholic acid (UDCA) had only minimal cytotoxic effect even at maximum

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concentrations. In contrast, DCA and CDCA had a significant dose-dependent cytotoxic effect on morphological features of apoptosis.[8]

At physiological concentration in serum, deoxy cholic acid induces survival and migration of breast cancer cells.[9] In practice, UDCA is used as a treatment of primary biliary cirrhosis and to dissolve cholesterol gallstones.[10,11] UDCA is a major primary bile acid in some species of bears. Dried bear bile has been used in traditional Chinese medicine as a treatment of liver disorders.[11]

In Turkish ethnic people who lived in Fars province, southern Iran, dried fox bile is believed to eradicate the malignant cells in humans. We aimed to examine the apoptotic and growth inhibitory effects of fox bile on hepatocellular and acute lymphoblastic leukemia cell lines. These lines were selected because the behavior and treatment of lymphoma and carcinoma cell lines are different.

Materials and Methods

This experimental study was performed under the supervision of the Animal Care Committee of Iran Veterinary Organization. A wild fox was hunted alive and bile was obtained from its gall bladder under aseptic conditions in the Comparative Medicine Research Center at Shiraz University of Medical Sciences, Shiraz, southern Iran. During the postoperative period, the animal was maintained under controlled environmental conditions (ambient temperature of 21±2°C, relative humidity of 65-70%, and a balanced diet with free access to food and water).

Cell Culture

Two human cell lines, HepG2 (NCBI Code: C158) and CCRF-CEM (NCBI Code: C105), were purchased from the National Cell Bank of Pasteur Institute (Tehran, Iran). CCRF-CEM is a non-adherent lymphoblastoid cell line and HepG2 are adherent epithelial-like cells derived from liver tissue.

Viability

The cells (1×10^5) were seeded, in triplicate, 24 hours prior to treatment. A fresh two-fold serial dilution of complete bile is prepared in order to treat the cells. After treatment, the number of viable cells was estimated by trypan blue exclusion test.

*Cell Growth Inhibition Assay (*MTT Assay*)*

Cell growth inhibition was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (Sigma, Germany) assay.[12] Briefly, 1×10^5 cells/well were seeded in each well and exposed to serial dilution of bile (in triplicate) and incubated at 37°C in a 5% CO_2 incubator for 24 hours. Before the assay, the pH of the plates was checked. Then, MTT was added and incubation was continued for a further 4 hours at 37°C. The produced formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the optical density (OD) was measured at 570 nm. The reference wavelength was 690 nm. The mean optical density (OD±SD) was calculated for each group. Non-treated cultures contained the solvent but not the bile. The values were the mean of three different experiments and the growth inhibition was estimated as the reduction of values from a DMSO control. The percentage of inhibition of cells exposed to the various treatments was obtained as follows:

1– (OD observed/OD control)×100=% inhibition

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

Individual apoptotic cell death was observed using an in situ cell detection kit, AP (Roche, Mannheim, Germany) according to the manufacturer's instructions. TUNEL preferentially labels apoptosis in comparison with necrosis. This can be done by enzymatic in situ labeling of apoptosis induced DNA strand breaks. DNA polymerase as well as terminal deoxynucleotidyl transferase (TdT) has been used for the incorporation of labeled nucleotides to DNA strand breaks in situ. After Adding substrate solution, the samples were analyzed by light microscopy. Apoptotic cells were identified under a light microscope (Olympus, Japan) by dark blue nuclei.

Results

In this experimental study, we tested the cytotoxicity of bile on the HCC cell line using the MTT assay. When HepG2 and CCRF-CEM cells were exposed to serial concentrations of bile, there was clear evidence of dose-dependent cytotoxicity and cell proliferation inhibition (figure 1).

The reduction in the rate of cell proliferation is more obvious in HepG2 than CCRF-CEM. Before assay, the pH of all plates was neutral. Therefore, the cell proliferation inhibition was not related to a change in pH. To evaluate whether the growth inhibition by the compounds against HepG2 cells was mediated through apoptotic process, we performed TUNEL assay. Exposures to a high concentration of bile led to morphological changes consistent with apoptosis. These changes included extensive cytoplasmic vacuolization and development of nuclear irregularity and fragmentation (figure 2 A and B).

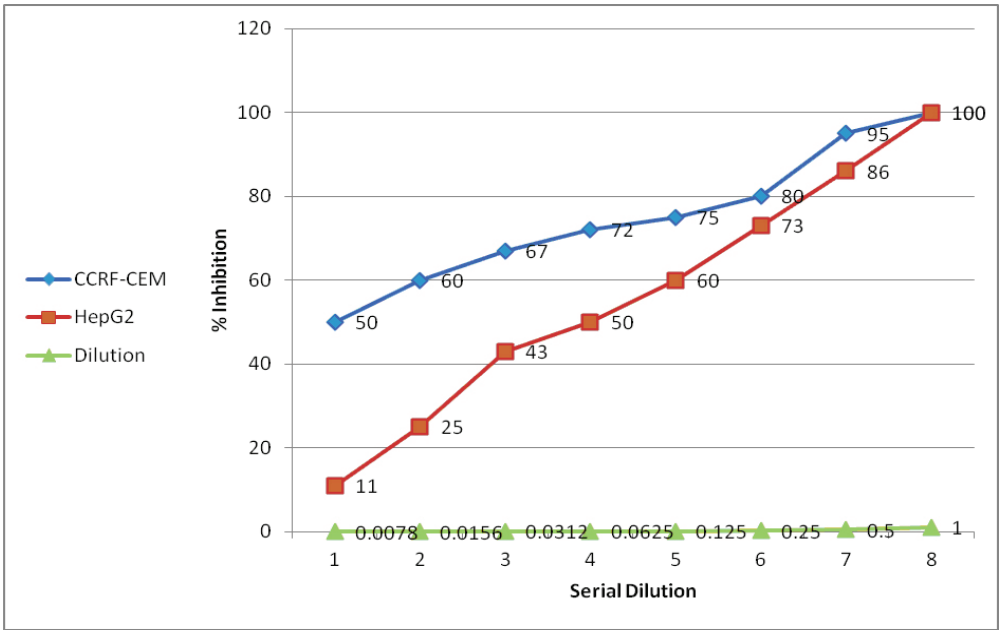
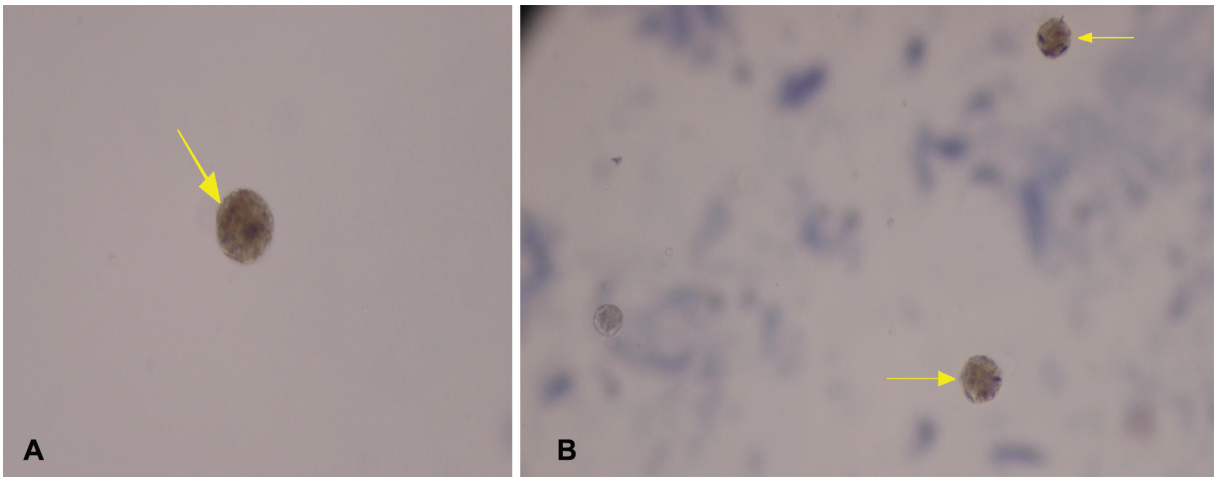


Figure 1: The figure shows the effect of bile on percent growth inhibition in HepG2 and CCRF-CEM cell lines by MTT assay.



Figures 2A & 2B: Apoptotic HepG2 cells as demonstrated by TUNEL assay which shows dark staining brown nucleus (×400) (yellow arrows).

Apoptosis can be distinguished from necrosis by a set of characteristic morphological hallmarks, e.g. chromatin condensation, nuclear fragmentation, cell shrinkage, plasma membrane blebbing, and the presence of apoptotic bodies. These changes were not observed in low concentrations of bile.

Discussion

Cancer treatment has a chronic course, and the affected patients suffer from the side effects of chemotherapy. In order to palliate symptoms and maintain the patients' quality of life, novel treatments with lower toxicity are welcome. Naturally occurring compounds with cytotoxic activity such as bile acids/salts offer attractive therapeutic options. The cytotoxic effects of bile acids on various cancers were previously evaluated. Martinez and colleagues studied the effect of four bile acids, cholic acid, CDCA, DCA and UDCA on colon carcinoma cell lines.[11] They found that CDCA or DCA caused morphological changes of apoptosis, whereas UDCA inhibited cell proliferation but did not induce apoptosis. Cholic acid had no discernible effect on colon cancer cells. [11] The DCA induced apoptosis via a protein kinase C-dependent signaling pathway. Im and colleagues evaluated the effects of synthetic derivatives of UDCA and CDCA on cervical cancer cell line and suggested that apoptosis occurred via NF-κB regulation of apoptotic genes such as Bax.[13] DCA and CDCA had a significant dose-dependent cytotoxic effect on ovarian cancer cells with morphological features of apoptosis.[8]

Kim and their colleagues studied different human cancer cells such as breast, prostate, cervix, brain, colon, and human T cell leukemia and found that the synthetic bile acid induced growth inhibition and apoptosis. Induction and

up-regulation of Bax and activation of caspases pathways were involved in this process.[14]

Choi and co-workers studied the effects UDCA, CDCA and also the synthetic derivatives of UDCA and CDCA on the proliferation of human prostate carcinoma cells.[15] They found that CDCA and UDCA had no effects on the growth of malignant cells, but the synthetic derivatives showed a weak to completely inhibitory activity on tumoral cells. They believed that the proliferation-inhibitory effect arrested the cell cycle progression at the G1 phase and induced apoptosis.[15] We found a similar result that the cytotoxic effect of crude bile was mediated by apoptosis. However, the effects of different natural or synthetic bile acids were not evaluated in our study.

On the other hand, the bile acids were considered as carcinogen in the gastrointestinal system. In Barrett's epithelial cells, DCA induced reactive oxygen/nitrogen species (ROS/RNS) production, which caused genotoxic injury, induced the activation of the NF-κB pathway and ultimately enabled cells with DNA damage to resist apoptosis.[7]

How specific bile acids promote neoplasia is yet unknown. The effects of different bile acids are not similar and the combination of bile subtype with appropriate pH and exposure time are critical.[6,7] PH can alter bile acid activity. Glycine-conjugated bile acids are involved in neoplastic development at acidic pH (pH~4), and unconjugated bile acids are involved in neoplastic development at a more neutral pH (~6).[7] DCA and LCA had tumorigenic effects, whereas UDCA has been efficiently used as a cytoprotective agent.[10] Ursodeoxycholic acid inhibits mitogenic signaling and suppresses cell proliferation in colonic tumorigenesis. UDCA protects cells from p53-mediated apoptosis by promoting its degradation via the Mdm-2-ubiquitin-proteasome pathway.[16] Therefore, the bile and bile acids had broad spectrum activity from carcinogenesis to cytotoxic effect on cancer cells. This finding was dependent on type of bile acid, exposure time and environmental pH.

Conclusion

Bile has dose-dependent cytotoxic effects on HepG2 and CCRF-CEM cell lines. DCA and CDCA are supposed to be responsible for this effect. Furthermore; the observed cytotoxicity appears to be mediated via apoptosis and bile might be applicable to the treatment of various human cancers.

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Conflict of Interest: None declared

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