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Medicine

HUMAN BILE IMPAIRS THE VIABILITY OF HEPATOCELLULAR CARCINOMA CELLS IN VITRO.

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ABSTRACT **Background/aims:** Hepatocellular carcinoma is the fifth most common variety of cancer. In over 50% of cases it will spread to the blood vessels, reducing the range of curative treatment options. While vascular invasions occur frequently, biliary invasion is rare and also suggest poor prognosis. This study assesses factors exist in the biliary tree or in the bile capable of preventing cancer cell proliferation. Thus, this experimental in-vitro study was designed to analyze the viability of HCC cells after treatment with human bile.

Methods: HepG2 cells were cultured in Dulbecco's modified Eagle's medium and were divided into 5 groups: one untreated control and four indicated for treatment with different concentrations of human bile (6.25%, 12.5%, 25% and 50%). The Research Ethics Committe of the São José do Rio Preto Medical School approved this protocol (number 830.862). Results were analyzed using the ANOVA and Bonferroni tests. **Results:** In three of the four treated groups there was a significant decrease in cell viability compared with the control group ($P<0.05$) and there was a dose-dependent reduction in cell viability ($P<0.05$).

Conclusion: Human bile has the ability to diminish the viability of human HCC cells.

KEYWORDS : Hepatocellular Carcinoma, Treatment, Human Bile, Cell Viability.

INTRODUCTION

Hepatocellular carcinoma is the fifth most common type of cancer and is the third in terms of total mortality (1). Cirrhosis of any etiology and viral hepatitis are the most common etiologies of HCC, but recent data have shown that in developed countries over half of HCC occurs in patients without viral infection, and that non-alcoholic fatty liver disease (NAFLD) appears to be an important etiological factor (2). Portal vascular macro-invasion by HCC is a common complication of this tumor, whose incidence can vary from 44% to 62.2% (3). The diagnosis of vascular macro-invasion by HCC is performed using dynamic imaging techniqes, and there are no curative treatment options available for this stage of the disease (3,4). Vascular microinvasion is also common, with incidence of between 25% and 63% of cases, depending on the tumor diameter (5). It is also considered a bad prognosis factor, and can only be diagnosed on post-surgical histological assessment (4,5,6). Compared to vascular invasion by HCC, biliary invasion is rarely described and, in most patients, is related to the invasion of the large bile ducts (7,8) while microinvasion of the small bile ducts is less frequently seen (9,10). Reports of biliary invasion are more frequent in Asia than in Western countries, its incidence ranging from 0.53% to 12% (11,12,13,14). HCC with invasion of the biliary tree was identified as icteric hepatoma by Lin in 1975 (15). The first series of icteric hepatoma was described by Kojiro et al (7) in 1982, and the invasion of small bile ducts was found in only 5 of the 24 patients reported. Although considered rare, invasion of the biliary tree by HCC is also associated with poor prognosis (7,12,16). On the other hand, some series showed good survival in these cases (8,14). A restrospective study performed on HCC specimens obtained by hepatectomy showed worse prognosis for patients with microscopic tumor thrombi in the peripheral bile ducts than for those without bile duct tumor invasion (17). Two recent series from Korea, Kim et al (16) and Ha et al (17) showed low five year survival rates of 25% and 50%, respectively, for patients who underwent liver transplantation and had proven HCC invasion of the bile tree of the explanted liver (17,18). Considering the low prevalence of invasive HCC in the biliary tree, this paper hypothesizes that factors could exist in the biliary system, such as in the bile composition or on the biliary tree wall, which could hinder or prevent the proliferation and/or the viability of HCC cells. In order to investigate this hypothesis, an experimental in vitro study was cpnducted with the aim of analyzing the viability of hepatocellular carcinoma HepG2 cells after treatment with a variety of concentrations of human bile.

METHOD

Procedure description and ethical considerations

The HepG2 cells were cultured as described below. The cell line was divided into 5 groups: one untreated control and other four indicated for treatment with different concentrations of human bile. All experimental samples were taken in triplicate. Although bile was collected from one individual multiple organ male donor, the effect

was only tested in cell lines, therefore there was no direct intervention on human health. The study was submited for evaluation and approved by the Reasearch Ethics Committe of the Medical School of São José do Rio Preto, São Paulo, Brazil (FAMERP), under the number 830.862.

Bile extraction

Bile was extracted under sterile conditions from one multiple organ male donor without any known disease in the biliary tree or the gallbladder, and it was stored at -20ºC until the treatment procedure. A signed informed consent was obtained from the family.

Cell Culture

HepG2 cell were cultured at 37ºC in 5% CO_2 in culture flasks containing Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA) and 1% penicilin/ streptomicin (Sigma-Aldrich, St. Louis, MO, USA).

Treatment of cells in vitro

HepG2 cells were divided into five groups: One untreated group was assigned as the control and the other four were indicated for different treatment groups according to different concentrations of bile diluted in DMEM: 6.25%, 12.5%, 25% and 50%. Each individual well of a 6-well plate was inoculated with 2 mL of DMEM containing $1x10^6$ HepG2 cells and different concentrations of bile. Control cells were treated only with DMEM. All the cell groups were treated over a 24 hour period before being submitted to a test of cell viability.

Cell Viability Assay

Cells were incubated at a concentration of $1x10^6$/well in 6-well plates in 2 mL DMEM supplemented with 10% FBS. Bile was added to the cells at different concentrations (6.25%, 12.5%, 25% and 50%), and the cells were incubated for 24 hours. Following incubation, the culture medium cell viability was measured through a count. The HepG2 cells were removed, and the cells were disaggregated by the action of tripsin, cells from each group were counted in a Neubauer chamber after trypan blue staining. Cell viability was calculated for all the groups, comparing the number of viable cells to the control group, and the results were expressed in percentages.

Statical Analysis

The averages of viable cells were compared among the different treatment groups and the untreated control group using the ANOVA test, followed by the Bonferroni test. All values were expressed as the average ± standard deviation (SD). $P<0.05$ was considered statistically significant. All analyses were performed using GraphPad PRISM4 software.

RESULTS

Test of cell viability by MTT assay

The number of viable cells was estimated by counting in a Neubauer chamber after trypan blue staining. The test for cell viability in three of the four treated groups showed a significant decrease in cell viability compared with the control group ($P<0.05$). Furthermore, there was a dose-dependent reduction in cell viability ($P<0.05$; Figure 1) after treatment with bile at different concentrations. It is noteworthy that the concentration of 6.25% did not affect cell viability ($P>0.05$; Figure 1).

DISCUSSION

This paper is the first to describe human bile in human HCC cell culture, and it demonstrates that bile decreases cell viability in vitro: the higher the bile concentration, the more significant the action. When bile was diluted to 6.25%, there was no significant difference from the control. When comparing 12.5%, 25% and 50% dilutions,however, it was observed that cell viability gradually decreased, getting closer to zero when bile was used at higher concentrations (fig. 1), which supports the hyphotesis that bile, or some factor in the biliary tree enviroment, as yet unidentified, has a deleterious effect on HCC cell viability.

HCC invasion of the biliary tree is not often observed in routine clinical practice and thus has been little studied; publications are usually small series (7,8,10). Some questions have been raised in such articles: 1) Wheter the thrombi found in the big ducts are really biliary invasions. Such a question has been asked due to the ease with which the thrombi are removed after hepatectomy, as they are always free without any adherence to or infiltration in the duct walls. 2) Wheter these thrombi are of HCC originating from within the biliary tree. There is evidence that hepatic progenitor cells present within the Hering canals may produce HCC, forming thrombi inside the biliary tree (19,20). As recently demonstrated in an animal model by Holczbauer (21), HCC may originate in different cells, such as adult hepatocytes, hepatic progenitors, fetal hepatoblasts, and mature hepatocytes. If HCC rarely invades the biliary tree (8,11,17), it is possible that there is a factor in it or the bile prevents the viability of HCC cells, considering that the tumors frequently do not invade the ducts and that these structures are anatomically linked, as demonstrated by Rappaport (22) (1973). Zhao (2014) (23) used bear bile on human hepatocellular carcinoma cells and demonstrated that the bile induced apoptosis in these cells. It could be melatonin that generates this action, considering that it is found in high levels in the bile (24) and that in experimental models it decreases angiogenesis (25,26). Therefore, this experiment demonstrated that human bile has the capacity to decrease the viability of human hepatocellular carcinoma cells. This implies that some factor exists in the bile that decreases HCC cell viability. Further studies are required to reveal the molecular mechanisms involved in the anti-carcinogenic effect.

List of abbreviations:

HCC - Hepatocellular Carcinoma
NASH - Nonalcoholic steatohepatitis
DMEM - Dulbecco's modified Eagle's medium
ANOVA - Analysis of variance
NAFLD - Non-alcoholic fatty liver disease
FAMERP - Medical School of São José do Rio Preto
FBS - Fetal bovine serum
SD - Standard deviation
MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide

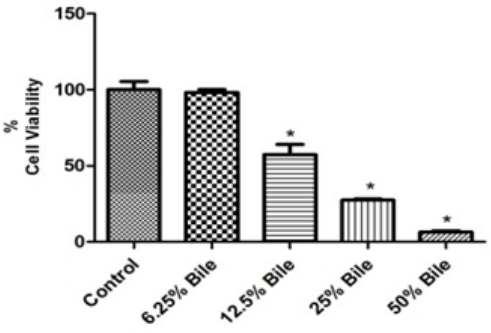

Figure 1. Dose-dependent reduction in cell viability after treatment with bile at concentrations of 6.25, 12.5, 25 and 50% diluted in DMEM for 24h. A) Bile at 6.25%. B) Bile at 12.5%. C) Bile at 25%. D) Bile at 50%. E) Statistical analysis. *P<0.05. Statistical significance compared to control group was determined by ANOVA.

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