Long-term intervention of taurocholic acid over-expressing in cholestatic liver disease inhibits the growth of hepatoma cells

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Abstract: Bile acids usually build up in patients with cholestatic liver disease. It was found that the concentration of taurocholic acid (TCA), one of the taurine conjugates of primary bile acids in serum, was elevated the most. While the role played by TCA in the disease is unclear, there is concern whether TCA contributes to the development of hepatocarcinoma from cholestasis. In the present study, the cell viability, flow cytometry, real-time polymerase chain reaction, intracellular ROS measurement, and intracellular Ca2+ measurement were used to investigate the effects of TCA on THLE-2 and HepG2 cells. The results showed that TCA is capable of inhibiting HepG2 cell growth whereas it has relatively little or no impact on that of THLE-2 cells until later stages of 16-day treatment. The growth inhibition is a result of cell apoptosis induced by the increase of Ca2+ and ROS level, and also associated with the increased expression of c-Myc, CEBPα, TNF-α, ICAM-1, VCAM-1, CXCL-2, and Egr-1. HepG2 growth inhibition could contribute to the research on the treatment methods of patients already with hepatocarcinoma.

Key words: Taurocholic acid; HepG2 cells; THLE-2 cells; Apoptosis; Hepatocarcinoma.

Introduction

Liver cancer has been the second most common cause of death from cancer worldwide, whose ratio of mortality to incidence is as high as 95% according to latest data evaluation of GLOBOCAN (1). Therefore, the causes of liver cancer and exploration of new treatment methods have attracted much attention. The main risk factors, like hepatitis B and C virus infection, alcohol intake and ingestion of the fungal metabolite aflatoxin B1, for developing liver cancer are well known. Meanwhile, patients with cholestatic liver disease tend to develop liver cancer (2, 3). However, the mechanisms of cholestatic liver diseases transforming into liver cancer remain unclear. Cholestasis is characterized by accumulation of bile acids and inflammation, causing hepatocellular damage (4). The composition of bile acids is complicated and may play a key role in the development of liver carcinogenesis from cholestatic liver diseases (5). Taurocholic acid (TCA) belongs to conjugated bile acids with taurine. Research indicated that the concentration of taurine and glycine conjugates of primary bile acids in serum was elevated in cholestatic liver cirrhosis patients when compared to non-cholestatic donors, with the elevation of TCA the most obvious (6, 7). Therefore, TCA can be used as an important marker for the diagnosis of cholestatic diseases. Studies have been conducted to investigate the relationship of TCA with the development of liver cancer. It was found that HepG2 (one of the most used human hepatoma cell lines) cell viability showed no significant difference after TCA exposure at 6 and 24 h in comparison with the untreated control (4, 8). The current research is mainly focused on the short-term exposure of TCA to hepatocellular carcinoma cells, and the results indicated that there was no significant change in the activity of hepatocellular carcinoma cells after a short-term exposure of TCA. Moreover, little molecular analysis was conducted to explore the underlying mechanisms, and there was no further investigation for the effects of long-term exposure of TCA on hepatoma cell proliferation.

In the current research, the normal hepatocytes (THLE-2 cell line) and hepatocarcinoma cells (HepG2 cell line) were exposed to long-term intervention of TCA under the concentration in human cholestatic serum sample in vitro, and their growth characteristics and the underlying mechanisms were investigated.

The results showed that under the concentration in human cholestatic serum samples, TCA is capable of inhibiting HepG2 cell growth by inducing apoptosis, whereas it has relatively little or no impact on that of THLE-2 cells until later term intervention. Furthermore, the molecular analyses suggested that TCA-induced growth inhibition were influenced by the expression change of c-Myc, CEBPα, TNF-α, ICAM-1, VCAM-1, CXCL-2, and Egr-1. Apoptotic analysis by flow cytometry, intercellular ROS and Ca2+ measurements further indicated that...
the HepG2 cell inhibition was caused by apoptosis. These findings imply that TCA may be helpful for the development of new treatment methods against liver cancer in the future while long-term effects of TCA on THLE-2 cells may contribute to their unregulated growth, which leads to abnormality or even tumorous change.

Materials and Methods

Cell culture and reagents

THLE-2 and HepG2 cell lines were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were placed in 75 cm² U-shaped canted neck cell culture flask (Corning, USA), fed with RPMI 1640 and MEM/EBSS Hyclone respectively containing 10% fetal bovine serum and 1% Penicillin-Streptomycin (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China), and cultured in 37 °C incubator with 5% CO₂. The culture medium was changed every 2 days. Cells were collected and cryopreserved until further use when the confluency reached up to 80-90%. Taurocholic acid sodium salt hydrate (TCA) was purchased from Sigma-Aldrich, USA.

Measurement of cell viability

The THLE-2 and HepG2 cells in the logarithmic phase were digested, centrifuged and re-seeded into a six-well plate at a density of 2×10⁵ cells per well. Cells were treated with 50 µM TCA (cholestasis serum concentration) for 16 days, and cells were counted and analyzed every 4 days. Three wells were set in each group. The culture medium was changed every 2 days.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from the cell samples using RNAiso Plus kit (Takara Clontech, Japan), following manufacturer’s recommendations. RNA quantity and quality were determined with the NanoDrop C 2000 UV-Vis Spectrophotometer (Thermo Scientific, Braun-schweig, Germany). After RNA was reverse-transcribed into cDNA, each cDNA pool was stored at -20 °C until further analysis. Specific oligonucleotide primer pairs were purchased from Sangon Biotech (Shanghai, China) and used for real-time PCR. The sequences of the primers used are listed in Table 1. Real-time PCR analysis was performed on Roche LightCycler® 96 (Roche Diagnostics, Mannheim, Germany) using SYBR green I as the fluorophore (Takara Clontech, Japan). Each sample was run in triplicate. β-actin was used as the internal control. The 2-ΔΔCt method was used for data analysis.

Flow cytometry assay

Annexin V Fluorescein (FITC) and Propidium Iodide (PI) double staining assay (DoJindo Chemical Technology Co. Ltd, Shanghai, China) was applied for the apoptosis assay. After TCA treatments, cells were harvested, washed twice by cold PBS, centrifuged at 1500 rpm for 5 min, re-suspended in 100 µL of 1×Annexin V binding buffer solution with a cell density of 1×10⁶ cells/mL, and incubated with 5 µL of Annexin V FITC and 5 µL of PI for 15 min at room temperature in the dark. Then 400 µL of 1×Annexin V binding buffer solution was added and after one hour incubation, samples were analyzed by BD FACSCalibur flow cytometer (Becton-Dick Medical Devices Co. Ltd, Shanghai, China) to identify apoptotic cells. Data was analyzed using CellQuest Pro software (BD Medical Devices).

Intracellular reactive oxygen species measurement

The intracellular reactive oxygen species (ROS) concentration was measured using DCFH-DA fluorescent indicators according to operation manual (Nanjing built bioengineering institute, China). In brief, cells were incubated with 20 µM DCFH-DA in PBS buffer at 37°C for 30 min. Then each sample was aspirated into a black 96-well plate and the fluorescence intensity of each well was measured using a fluorescence micro-plate reader with 485 nm excitation wavelength and 525 nm emission wavelength. The intensity of the sample groups was compared with that of the control ones to obtain the relative fluorescence intensity value.

Intracellular Ca²⁺ measurement

The intracellular Ca²⁺ concentration was measured using the fluorescent probes Fluo 4-AM according to the operation manual (DoJindo Chemical Technology Co. Ltd, Shanghai, China). Cells were washed 5 times with HBSS buffer in order to remove Ca²⁺ in medium, and incubated with 2 µM Fluo 4-AM in HBSS buffer at 37°C for 30 min. After that, the samples were washed with HBSS buffer to remove Fluo 4-AM and used for real-time PCR. The sequences of the primers used are listed in Table 1. Real-time PCR analysis was performed on Roche LightCycler® 96 (Roche Diagnostics, Mannheim, Germany) using SYBR green I as the fluorophore (Takara Clontech, Japan). Each sample was run in triplicate. β-actin was used as the internal control. The 2-ΔΔCt method was used for data analysis.

**Table 1. Sequences of Real-Time PCR Primers.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egr-1</td>
<td>5'-TACTCTCCTGTCTCCCCCTTGTT-3'</td>
<td>5'-GAAAGGTTGTGCTATGTTCC-3'</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5'-CGGCTGACGTTGAGTGTTAATAC-3'</td>
<td>5'-GGTCTCGTCAAGATCCTTG-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-TCCCTGTCACTGCTGCCTTG-3'</td>
<td>5'-TGCTTCACATGTCCTCACA-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-CCCTGTAACCCATTTGGTACGA-3'</td>
<td>5'-TTGAAAGAGGACTCGGAGTAG-3'</td>
</tr>
<tr>
<td>CXCL-1</td>
<td>5'-GGCAGCGAAAGGTTGTGCTACATCG-3'</td>
<td>5'-GCCTCTTACGGAACAGCCACACA-3'</td>
</tr>
<tr>
<td>CXCL-2</td>
<td>5'-GATCCGCCCATTGGTAAAGAAAAT-3'</td>
<td>5'-TCTTTGGAAACAGCCACCAATA-3'</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>5'-AGTTGAGGATGCGGGAGTAT-3'</td>
<td>5'-GGATGCAAAAATAGAGCACGAG-3'</td>
</tr>
<tr>
<td>CEBPα</td>
<td>5'-ACAGTTGACGTTGACAGGAG-3'</td>
<td>5'-TATCACTGCAACAGGAGACG-3'</td>
</tr>
<tr>
<td>c-Myc</td>
<td>5'-CCGCTTCCTGACGGTGTCGTC-3'</td>
<td>5'-CGTCGTCGGGTGCGTACGATG-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CCCTGCATGCGGAG-3'</td>
<td>5'-GCAGAGAGCTCTCGCTT-3'</td>
</tr>
</tbody>
</table>
remove residual Fluo 4-AM, and then the samples were observed under a fluorescence microscope.

**Statistical analysis**

Data from at least three experiments were expressed as mean ± SD. One-way analysis of variance (ANOVA) was conducted for comparisons among multiple groups. Differences between any two groups were analyzed using two-tailed Student’s t-tests. p values of <0.05 were considered to be statistically different.

**Results**

**The effect of TCA on HepG2 and THLE-2 cells**

Figure 1 demonstrates that TCA inhibited the HepG2 cell growth and the inhibitory effect became more significant as time went on. Meanwhile, TCA did not show any inhibitory effect on the THLE-2 cells for the first 8-day treatment and started to slowly inhibit the THLE-2 cells growth in the later treatment period.

**Flow cytometry analysis**

Flow cytometry analysis was conducted to further investigate whether the cell inhibition was caused by apoptosis or necrosis. Apoptosis ratios (including the early and late apoptosis ratios) of 50 µM TCA-treatment in HepG2 and THLE-2 cells for 16 consecutive days are shown in Figure 2. Cells without TCA treatments were used as controls. As shown in Fig. 2, the data suggested that the cell inhibition is mainly caused by apoptosis, and the inhibitory effect of TCA on HepG2 cells is more significant than that of THLE-2 cells, which is consistent with the growth curves.

**Real-time PCR analysis**

The mRNA expression changes of related genes were investigated through real-time PCR to explore the underlying mechanism for cell growth inhibition. As demonstrated in Fig. 3A, the expression level of oncogene c-Myc in HepG2 is much higher than that in THLE-2 at the beginning. Interestingly, the c-Myc mRNA content in HepG2 almost doubled in day 4 and then maintained almost the same level later on, while that of THLE-2 maintained a similar low level until day 8 and then highly overexpressed in later days. Also as shown in Fig. 3B, the mRNA expression of potential tumor suppressor CEBPα increased significantly in HepG2 cells right from early stage TCA intervention, while that in THLE-2 only significantly increased in later stages. The tumor necrosis factor α (TNF-α), intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), C-X-C Motif Chemokine Ligand 2 (CXCL-2), and early growth response protein 1 (Egr-1) were significantly elevated in both the THLE-2 and HepG2 cells after exposure to the TCA treatment with a time-dependent manner, with the elevation level of CXCL-2, and Egr-1 in the THLE-2 cells more prominent than that in HepG2 cells, while the elevation level of CEBPα.

![Figure 1](image1.png)

**Figure 1.** The proliferation curve of THLE-2 and HepG2 cells treated with 50 µM TCA for 16 days. Data are expressed as a mean ± SD (n = 3). *means P<0.05.

![Figure 2](image2.png)

**Figure 2.** Effect of TCA on apoptotic rate in THLE-2 (Figure A-E) and HepG2 (Figure F-J) cells. These cells were treated with 50 µM TCA for 16 consecutive days and cells without TCA treatments (0 day) were used as control sample. Q1, necrotic cells; Q2, late apoptotic cells; Q3, viable cells; Q4, early apoptotic cells.

![Figure 3](image3.png)

**Figure 3.** Real-time PCR analysis of THLE-2 and HepG2 cells after exposure to 50 µM TCA for 16 consecutive days. Data are expressed as a mean ± SD (n = 9). *and #means P<0.05 when THLE-2 and HepG2 treated with 50 µM TCA in comparison to the untreated samples, respectively.
TNF-α, ICAM-1 and VCAM-1 was more significant in HepG2 cells.

**Intracellular ROS analysis**

ROS analysis was conducted to help further investigate the mechanism of cell growth inhibition. As shown in Fig. 4, the relative content of ROS was significantly increased in HepG2 cells, while that in THLE-2 cells did not change much until day 12, which is consistent with the results shown in Figure 1.

**Intracellular Ca^{2+} analysis**

Intracellular Ca^{2+} analysis was conducted to further investigate whether intracellular Ca^{2+} contributed to the cell inhibition. The data in Figure 5 suggested the green fluorescence intensity in HepG2 cells was increased and fluorescence area was also expanded as time went on, which indicated that the Ca^{2+} content in HepG2 cells was significantly increased. However, as shown in Fig 5A-5E, the green fluorescence of THLE-2 cells, which is sporadic and faint, did not show until day 12.

**Discussion**

In our previous study, it has been demonstrated that TCA is highly elevated in the serum of patients with liver cirrhosis. The short term effects of TCA on HepG2 cells have also been investigated (9). Therefore, TCA with a concentration of 50 µM (the concentration in human cholestatic samples) was used to treat HepG2 and THLE-2 cells for 16 consecutive days based on our previous short term study, and its effect on both cell lines were investigated.

The retention of bile acids (10) plays an important role in cholestatic diseases, which could eventually lead to cancer development (11). Studies showed that the concentration of TCA in serum was significantly elevated in cholestatic liver cirrhosis patients (2, 7). However, the effects of elevated TCA involved in this process are unclear (6, 7). One concern is whether TCA contributes to the development of liver cancer from cholestatic disease. In this study, the normal hepatocytes (THLE-2 cell line) and hepatocarcinoma cells (HepG2 cell line) were exposed to long-term intervention of TCA under the concentration of human cholestatic serum sample in vitro, and their growth characteristics and the underlying mechanisms were investigated and explored. The results indicated that when HepG2 and THLE-2 cells were treated with 50 µM TCA (the concentration in human cholestatic samples) for 16 consecutive days, the HepG2 cells’ growth was significantly inhibited while the THLE-2 cells growth was not affected significantly. Meanwhile, cell biology analysis proved that the cell growth inhibition was mainly caused by ROS- and Ca^{2+}-induced apoptosis, and the inhibitory effect of TCA on HepG2 cells was more significant than that on THLE-2 cells, which is consistent with the growth curves.

Real-time PCR analysis was also conducted in an attempt to further investigate the underlying mechanism. As shown in Figure 3, the expression level of oncogene c-Myc in HepG2 is much higher than that in THLE-2 in the start; interestingly, the c-Myc mRNA content in HepG2 almost doubled in day 4 and then maintained the same level later on, while
that of THLE-2 maintained a similar low level until day 8 and then highly overexpressed in later days. Numerous studies have shown that c-Myc is amplified or overexpressed in human tumors (12-14) which our data is consistent with. However, when c-Myc is further overexpressed, the induced response is cell cycle arrest or apoptosis through enhanced ARF/p19 or p53 function pathway which may explain that the HepG2 cell growth inhibition starts from day 4 while the THLE-2 cells growth did not slow down until day 12 (15). Interactions between CEBPα and cell cycle proteins potentially inhibit cell proliferation via different pathways such as inhibition of cdk2 and cdk4 (16). As shown in figure 3B, the mRNA expression of potential tumor suppressor CEBPα increased significantly in HepG2 cells right from early stage of TCA intervention, while that in THLE-2 only significantly increased in later stages which further explained that the HepG2 cells’ growth was significantly inhibited while the THLE-2 cells growth was not changed significantly until later days of intervention.

TNF-α triggers apoptosis, necrosis, or survival signals through binding two different cell surface receptors, TNFR1 and TNFR2. TNFR1-mediated apoptosis activates caspase-8 and induces cell death through downstream signaling (11, 17). It is a well-studied apoptotic pathway in a variety of cell types (18). Recent research found that TNF-α had a statistically significant inhibitory effect on cellular proliferation of HepG2 cells (19). Therefore, since TNF-α was up-regulated both in TCA treated HepG2 and THLE-2 cells with that in HepG2 much more significant, TNF-α played a similar role as CEBPα did.

CXCL-2 plays a pivotal role in cancer progression and carcinogenesis. It has been discovered that CXCL-2 expression was stably down-regulated in 94% of hepatocellular carcinoma (HCC) specimens compared with paired adjacent normal liver tissues and some HCC cell lines (20), and our results (figure 3G) are consistent with the this finding. Moreover, CXCL-2 overexpression profoundly attenuated HCC cell proliferation and induced apoptosis in vitro (20), which explains our results (figure 3G) that the CXCL-2 expression in THLE-2 decreased in the early stage (no cell growth inhibition) and then increased in later stages (cell growth started to decline).

Meanwhile, it is reported that TNF-α, as a pro-inflammatory cytokine, could induce the expression of ICAM-1 and VCAM-1 (21), whose expressions were also up-regulated both in TCA treated HepG2 and THLE-2 cells with that in HepG2 much more significant (figure 3D and 3E). ICAM-1 and VCAM-1, as cell adhesion molecules, are associated with the inflammation induced by T cell infiltrate (21, 22) and cancer metastasis (23, 24). Scientific research found that the expression of ICAM-1 and VCAM-1 are significantly higher in HCC than that in adjacent normal liver tissues, and significantly higher in HCC with high invasive potential than that in HCC with low invasive potential. Therefore, the up-regulation of ICAM-1 and VCAM-1 may imply an intense T cell infiltrate and risk of cancer metastasis at the late term of TCA-exposure in vivo.

Egr-1 has significant tumor suppressing properties and could be induced by various stimuli leading to either growth arrest or cell death in tumor cells (25-27). The up-regulation of Egr-1 in HepG2 cells has a positive influence on the cell growth inhibition. For THLE-2 cells, the fold change of Egr-1 was not so significant in the early days of TCA intervention, which explained that the cell growth was not significantly arrested.

Study has found that p53-induced genes that encode mitochondrial proteins had the potential to directly stimulate ROS production, and ROS production led to human colon cancer cell apoptosis (13). Other research found that silica nanoparticles induce autophagy and autophagic cell death in HepG2 cells triggered by ROS (28). Moreover, zinc oxide nanoparticles selectively induce apoptosis in human cancer cells through ROS (29). These studies suggested that the elevated mitochondria-derived ROS level induces cancer cell apoptosis. ROS analysis conducted in the present study demonstrated that the ROS level was significantly up-regulated in HepG2 cells, and the cell growth inhibition was likely to be partly mediated by ROS via p53 pathway. Some studies demonstrated that ROS production is Ca$^{2+}$ dependent (30) and suggested that mitochondrial Ca$^{2+}$ up-rise could lead to ROS production (31). In this study, data proved that Ca$^{2+}$ content in HepG2 cells was significantly elevated, and it is positively related to ROS production. The resulting HepG2 cell growth inhibition was consistent with the findings in other studies (32).

In conclusion, experimental results proved that long-term intervention of TCA under the concentration in human cholestatic serum samples was capable of inhibiting cell growth and inducing apoptosis in HepG2 cells whereas it had relatively little or no impact on that of THLE-2 cells until later stages of 16-day treatment. Little growth inhibitory effects of TCA on THLE-2 cells may contribute to their unregulated growth, which leads to abnormality or even tumorous change. HepG2 growth inhibition could contribute to the research on the treatment methods of patients already with hepatocarcinoma.

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Interest conflict
We declare there is no interest conflict in our work.

Author’s contribution
Xiujuan Zhang is responsible for providing the experi-
mental materials and part of the equipment used, and also helped write and revise the manuscript. De Nan cultured the cells, did real-time PCR analysis, analysed the data and wrote part of the first version of the manuscript. Chunting Zha did ROS and Ca\(^{2+}\) analysis, and part of the first version of the manuscript. Gaohong He provided part of the equipment used and helped revise the manuscript. Wenjun Zhang helped revise the manuscript. Zhijuan Duan helped with the main idea.

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