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Research Paper

Effect of bile salts and bile acids on human gastric mucosal epithelial cells ☆

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Abstract

Objective: To explore the effect of bile salt and bile acid on cultured eternalized human gastric mucosa epithelium GES-1 cells. Methods: Cultured eternalized human gastric mucosa epithelium GES-1 cells were treated with media containing 6 different kinds of bile salts and 3 different kinds of bile acids and their mixture with different concentrations: GCDC(glycochenodeoxycholate), GDC (glycodeoxycholate), GC(glycocholate), TCDC(taurochenodeoxycholate), TDC(taurodeoxycholate), TC (taurocholate), LCA (lithocholicacid), CA(cholic acid), DCA(deoxycholic acid)(50 μ mol/L,250 μ mol/L,500 μ mol/L,1000 μ mol/L), DY(mixture of bile salts) and DS(mixture of bile acids)(250 μ mol/L,500 μ mol/L,1000 μ mol/L,1500 μ mol/L, 2000 μ mol/L), in comparison with the control group(in normal media without bile salts and bile acids). Cell proliferation was assessed by MTT(3-[4,5-Dimethylthiaolyl]-2,5-diphenyl-tetrazolium bromide) assay for 72 hours with different concentrations and the apoptotic cells were assayed by flow cytometry (FCM) with Annex V-FITC conjugated with propidium iodide(PI) staining for 24 hours with different concentrations(1500,2000 μ mol/L). Results: There was no significant difference in morphology and cell proliferation in GC group after 24~72 h. Low concentration(50 μ mol/L) of GCDC, GDC, TCDC, TDC and TC accelerated gastric epithelial cell growth in a dosage-time dependent manner. At middle concentration (250-500 μ mol/L), it showed positive effect after 24~48 h, while negative effect after 72 h. At high concentration(1000 μ mol/L), it accelerated gastric epithelial cell growth after 24h and show consistent inhibition even leading to necrosis after 48~72 h. LCA and CA showed a positive effect on the concentration of 50 μ mol/L after 24~72 h, while 250-1000 μ mol/L showed a trend towards apoptosis after 24~72 h. At 50-500 μ mol/L, DCA showed proliferation after 24 h and apoptosis after 48~72 h, but showed necrosis after 24~72 h at 1000 μ mol/L. DY and DS could facilitate normal gastric mucosa epithelial cell growth at low concentration (250-500 μ mol/L), however at 1000-2000 μ mol/L the trend shifted from apoptosis to necrosis. FCM with Annexin-V conjugated with PI staining revealed that GCDC, GDC, GC, TCDC, TDC, TC, LCA, CA, DCA, DY and DS induced apoptosis of human gastric mucosal epithelial cells. They were all significantly higher than that of the control($P < 0.05$), but there was no significant difference in GC group ($P > 0.05$). The bile salts induced apoptosis in a time-dose-dependent manner. Conclusion: Our results suggested that bile acid and bile salt is the trigger of injury in human gastric mucosal epithelial cells.

Key words: bile salts; bile acid; duodenogastric reflux; gastric mucosal epithelial cells; apoptosis

INTRODUCTION

Recent studies found that bile, pancreatic juice and intestinal juices and duodenum contents refluxing to the stomach over a long time may lead to gastric mucosal epithelial cells injury. However, the injury mechanism of bile salts on human gastric mucosal epithelial cell is not clear at present. Here we study the mechanism of bile damaging the human gastric epithelial cell line (GES-1) through the experiment of bile salts and bile acids and their mixture acting on the human gastric mucosal epithelial cells.

MATERIALS AND METHODS

Materials

RPMI1640 was purchased from GIBCO, fetal bovine

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serum(FBS) from Hangzhou Jiangbin Biotechnology Co. Ltd. GCDC(glycochenodeoxycholate), GDC (glycodeoxycholate), GC(Glycocholate), TCDC (taurochenodeoxycholate) and TDC(taurodeoxycholate), TC(taurocholate),LCA (lithocholicacid), CA (cholic acid), DCA (deoxycholic acid)were from Sigma. Eternalized human gastric mucosa epithelium GES-1 cells line came from Beijing Institute for Cancer Research. Annexin V-FITC apoptosis detection kit I was from BD Biosciences.

Cell culture

GES-1 cells were cultured by RPMI1640 with 10% FBS. In the following study, different bile salts, bile acids and their mixture were added into the K-SFM (pH 7.1-7.3). The dissociated cells were collected and grown in K-SFM(pH 7.4) in a humidified atmosphere consisting of 50 ml/L CO_2/95% air at 37℃.

Assessment of morphological and cell proliferation

3-[4,5-Dimethylthiaolyl]-2,5-diphenyl-tetrazolium bromide(MTT) assay was conducted to determine the cell proliferation. Cells were treated with 6 different kinds of bile salts, 3 different kinds of bile acids; at concentrations of 50, 250, 500 and 1000 μmol/L, 6 bile salts mixture(DY); at concentrations of 250, 500, 1000, 1500 and 2000 μmol/L(at the ratio of 2:2:1:2:2:) and 3 bile acids mixture(DS) at the concentrations of 2000, 1500, 1000, 500 and 250 μ mol/L at the ratio of 1:8:4[1]. Cells were seeded on a 96-well plate(4-6 × 10^4 cells/well). After 24h of seeding, cells were treated with different bile salts for 3d and untreated cells served as a control. Then we observed morphological change of GES-1 cells under a phase-contrast video microscope. Prior to the determination, 10 μl of the 2.5 g/L stock solution of MTT was added to each well. After 4 h of incubation, the culture media were discarded followed by addition of 100mL of DMSO to each well and vibration for 10 min. The absorbance(A) was measured at 492 nm with a microplate reader. The percentage of viable cells was calculated as follows:(A of experimental group/A of control group)× 100%.

Flow cytometry by Annexin V-FITC conjugated with PI staining

Cells from the medium supernatant and adherent cells treated with bile salts and bile acids(500, 1000 μmol/L) and their mixture(1500, 2000 μmol/L) after 24 h were collected and pelleted at 1200 U/min. Pellets were washed twice with cold PBS and were then resuspended in a binding buffer at a concentration of 1 × 10^6 cells/ml, and two volumes of the 100 μl solution(1 × 10^5 cells) were transferred to two 5 ml culture tubes. Five microliters of Annexin V-FITC and 5 μl of PI were added into each of the 100 μl solution, and the cells were gently vortexed and incubated for 15 min at room temperature in the dark. 400 μl of 1 × binding buffer was added to the samples and were analyzed by FACSCalibur FCM within 1h. A total of 2 × 10^4 cells were detected by FCM in each of the samples.

Statistical analysis

All values were analyzed with SPSS15.0 software and expressed as mean ± SD. Statistical differences between means were calculated by the One-Way ANOVA test. $P < 0.05$ was considered statistically significant.

RESULTS

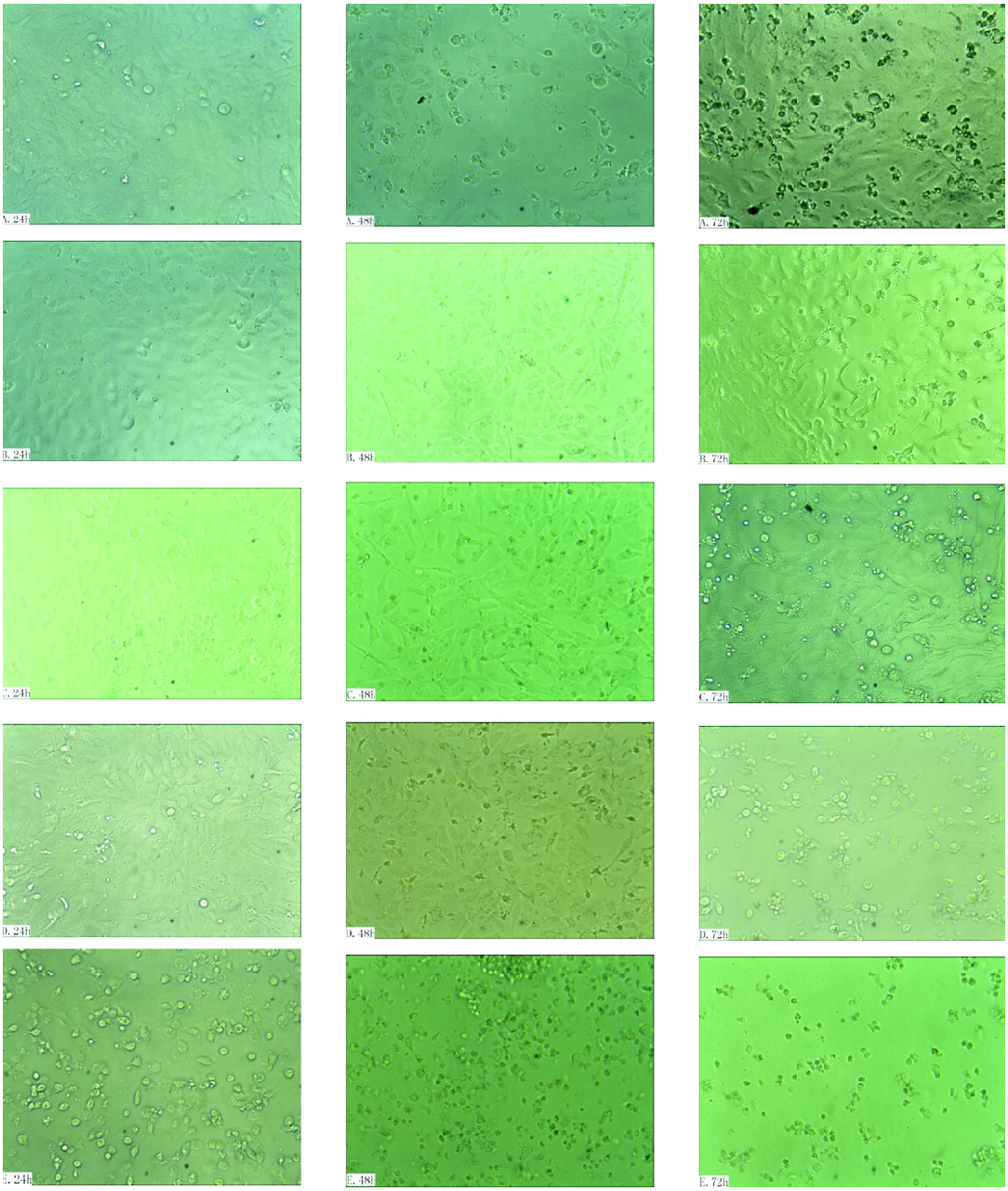
Morphological changes of GES-1

We observed morphological change of GES-1 cells under a phase-contrast video microscopy. Cells of the control group grew well(nucleus big and regular, nucleolus clear). The density of cell gradually rose from 24 h to 72 h. Cells after 24~72 h treatment with GC presented no significantly differences from the control in density and morphology.

24 h treatment with GCDC, GDC, TCDC, TDC, TC (50 μmol/L) stimulated the growth of GES-1 cells, the density was gradually higher than that of the control, while morphology had no change. At 250-500 μmol/L, after 24 h the cells were crowded and wrapped, but regular. After 48 h the density was lower, nucleus smaller, nucleolus was not clear and lost typical shape. After 72 h parts of cells appeared crimple, became round, cytoplasm crinkle and vacuole came into being. At 1000 μmol/L after 24h had appeared apoptosis cells. Apoptosis cells obviously increased after 48 h. Parts of cells broke away from near cells after 72 h.

At 50 μmol/L, LCA and CA stimulated the growth of GES-1 cells, the density was gradually increased after 24~72 h. At 250-1000 μmol/L, after 24~72 h Apoptosis cells rose. At 50-500 μmol/L of DCA after 24 h the density increased, on the contrary, after 48~72 h decreased and led to apoptosis. At 1000 μmol/L, after 24~72 h crimpled and round cells, cytoplasm with crinkles and vacuoles rose.

At 250-500 μmol/L, DY and DS stimulated the growth of GES-1 cells and the density gradually increased after 24~72 h. At 1000-1500 μmol/L, the density was higher than that at 250-500 μmol/L after 24h. Morphological criteria of cell apoptosis, such as membrane blebbing, cell shrinkage, and nuclear condensation, were assessed after 48~72 h. At the concentrations of 2000 μmol/L there were lots of necrosis cells. Characterization of DCA-induced morphological changes of eternalized human gastric mucosa epithelium GES-1 cells was shown in(Fig. 1).



A: Morphological changes of control group after 24~72 h; B: Morphological changes of DCA(50 μmol/L) group after 24~72 h; C: Morphological changes of DCA(250 μmol/L) group after 24~72 h; D: Morphological changes of DCA(500 μmol/L) group after 24~72 h. E: Morphological changes of DCA(1000 μmol/L) group after 24~72 h.

Fig. 1 Characterization of DCA-induced morphological changes of eternalized human gastric mucosa epithelium GES-1 cells

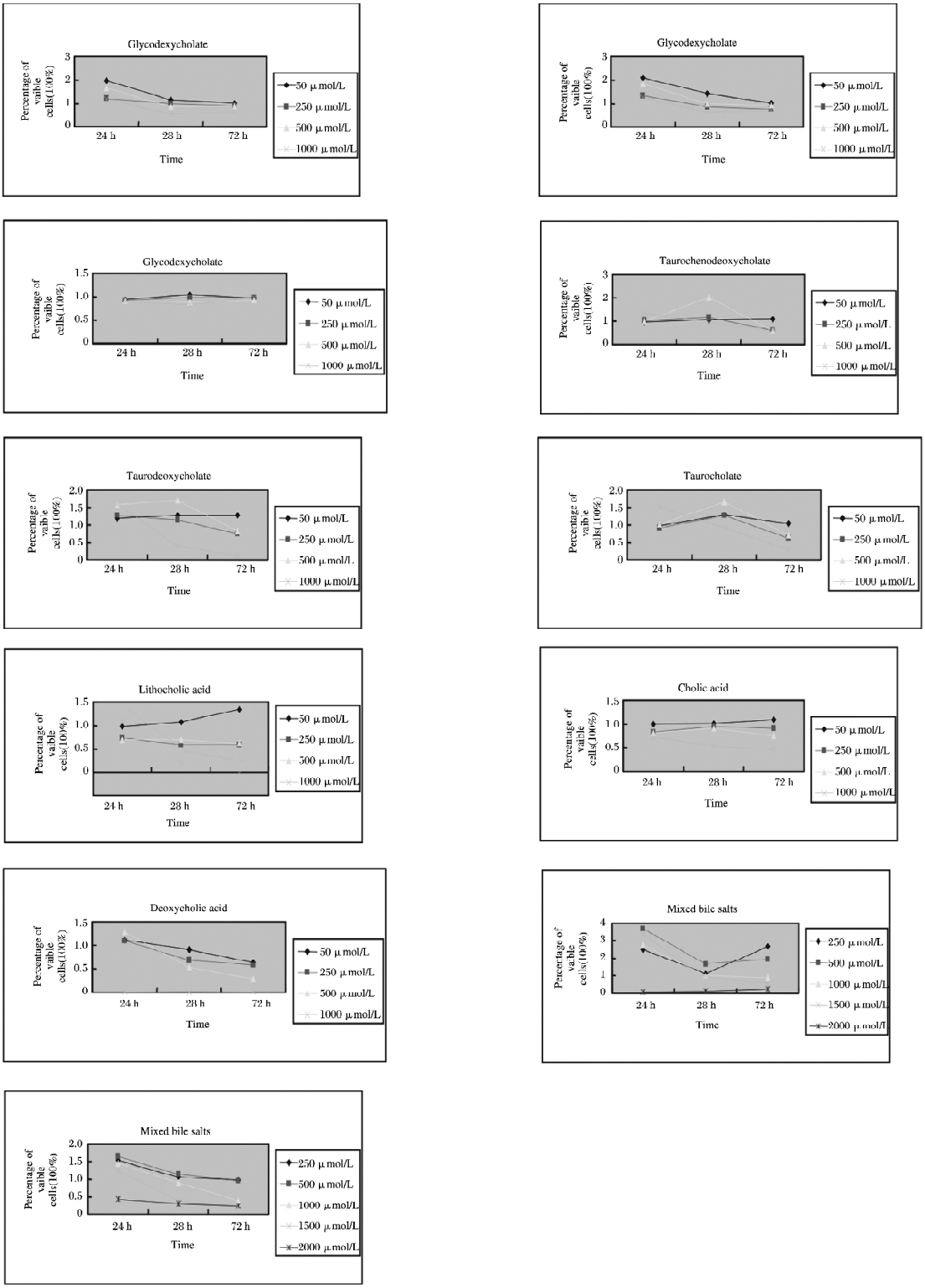
Inhibiting growth

There were no significant differences in the percentage of viable cells between GC and that of the control after 24~72 h treatment. The other five bile salts and their mixture effected the proliferation of GES-1 cells in a dose- and time-dependent manner. After 24 h treatment, the percentage of viable cells exposed GCDC, GDC, TCDC, TDC, TC at 50 μmol/L was greater than 100%, exhibiting a significant increase in proliferation after 24 h treatment. At 250-500 μmol/L, our results suggested a two-way effect. They showed positive effect after 24~48 h, while negative effect after 72 h. At 1000 μmol/L, they increased after 24h treatment but decreased after 48~72 h.

LCA and CA proliferated at concentration of 50 μmol/L after 24~72 h, while apoptosis was present after 24~72h at 250-1000 μmol/L. At 50-500 μmol/L, the percentage of viable cells on DCA rose after 24 h and displayed apoptosis after 48~72 h. However, it showed necrosis after 24~72 h at 1000 μmol/L.

At 250-500 μmol/L, proliferation increased after 24~72 h treatment with DY and DS. Cell growth increased

after 24 h treatment while was suppressed after 48~72 h at 1500 μmol/L. At 2000 μmol/L, the percentage of viable cells closed to zero, which presented the trend to necrosis. The percentage of viable cells after 24~72 h treatment with all groups was shown in Fig. 2.

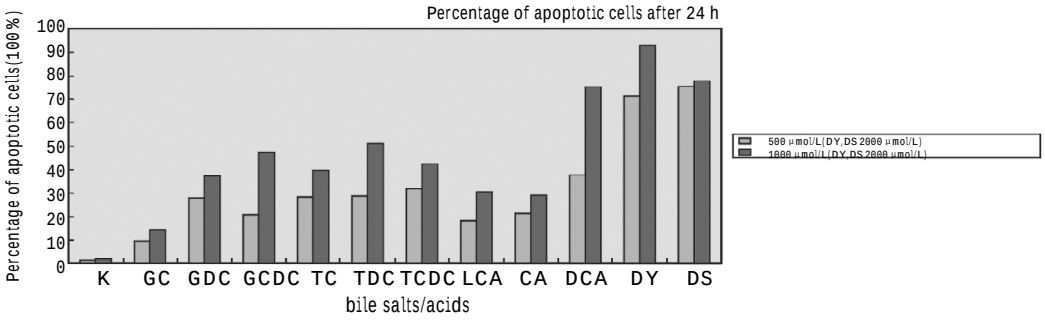


bile salts and bile acids at the concentration ◆(50 μmol/L), ■(250 μmol/L), ▲(500 μmol/L), △(1 000 μmol/L), and *(1 500 μmol/L), mixture of bile salts and mixture of bile acids at the concentration ◆(250 μmol/L), ■(500 μmol/L), ▲(1000 μmol/L), △(1500 μmol/L), and * (2000 μmol/L).

Fig. 2 Effect of GC(A), GCDC(B), GDC(C), TC(D), TCDC(E), TDC(F), and mixture of bile salts(G), mixture of bile acids(H) on the growth of cultured eternalized human gastric mucosa epithelium GES-1 cells

Apoptosis induced by the bile salts and bile acids

FCM with Annexin-V conjugated with PI staining showed that GC did not induce apoptosis($P > 0.05$). The assays revealed that GCDC, GDC, GC, TCDC, TDC, TC, LCA, CA, DCA, DY and DS induced apoptosis of human gastric mucosal epithelial cells. They were all significantly higher than that of the control(1.88 ± 0.05)%($P < 0.05$). The bile salts and bile acids induced apoptosis in a time-dose-dependent manner. The percentage of apoptosis in cells treated with different bile salts and bile acids for 24 h were shown in Fig. 3.



k:Control; DY:mixture of bile salts; DS:mixture of bile acids.

Fig. 3 Percentages of apoptotic cells after 24 h

DISCUSSION

It is well known that bile reflux cause injury of gastric mucosa and lead to inflammation, ulcer, and even tumor[2-4]. Bile acid is an important constituent in bile. The research in vitro has indicated that the bile acid can make cultured gastric mucosa epithelium produce cytotoxicity and have a close correlation with its concentration and the working time[5]. It is reported that some bile salts directly induce hepatic cell colorectal cancer cell necrosis at high concentration(500-2000 μmol/L), while middle concentration(200-500 μmol/L)[6,7] cause cell apoptosis, low concentration(10-100 μmol/L) stimulate proliferation and migration on intestinal cell lines[8-10] and differentiation on hapatocyte[11], and even induce marrow stem cell differentiating to mononuclear leukocyte[12]. It has been reported that GCDC,GDC[13], TCDC[14], TDC[15], TC[16], LCA, CA[17,18], and DCA[19] all can cause gastric mucosa epithelial cell damage, while GC[20] presents beneficial effects on gut mucosal damage. Maria[21] used the cell line known as AGS cells to determine whether necrosis or apoptosis was the predominant mechanism responsible for gastric mucosal cellular death. Cells were exposed to various concentrations of deoxycholate(DC; 50-500 μmol/L) for periods ranging from 30 min to 24h. No evidence of cytotoxity (by LDH assay) was discernible when cells were exposed to DC(50-300 μmol/L) for periods as long as 8 h; instead, clear evidence of apoptosis was noted that was time and dose dependent. When cells were exposed for 24 h to these DC concentrations, cytotoxicity was also present, indicating necrosis as well. Their results indicated that physiological concentrations of DC(50-300 μmol/L) primarily induce cellular death through an apoptotic process. Only after prolonged exposure to DC or acidification of the bathing solution does necrosis also occur. Low concentrations of DC, such as 100 and 150 μmol/L, showed mild degrees of apoptosis by the TUNEL assay, whereas concentrations of 200 μmol/L or greater demonstrated more pronounced effects. At concentrations of DC approaching 300 μmol/L or greater, 50% of cells were observed to be apoptotic by this assay. The results for DNA laddering were similar. Cells exposed to 100 μmol/L DC demonstrated no consistent evidence of laddering until 4h of exposure. In contrast, cells exposed to 300 μmol/L DC had clear evidence of DNA laddering as early as 1h following exposure to this damaging agent.

Piepoli[15] used a well-known amphibian model of gastric mucosa, and studied the effects of taurodeoxycholic acid(TDCA) on electrical transepithelial parameters, acid secretion and histology. Mucosal exposure to TDCA caused a reduction in transepithelial potential difference(V(t)) and transepithelial resistance (R(t)) and a decrease in acid secretion. Moreover, TDCA primarily affected the neck cells. Yohei[14] evaluated whether the elevated serum level of bile acids was a trigger of gastric mucosal damage in rats. Taurocholic acid(TCA), taurodeoxycholic acid(TDCA) and taurochenodeoxycholic acid(TCDCA) were used as bile acid samples and saline was administered to the rat stomach via a tube in a rat model 3 h after ligation of the common bile duct. Moreover, gastric mucosal blood flow and gastric content of epidermal growth factor (EGF) were evaluated in rats with severe mucosal damage. As a result, significant mucosal damage was detected in rats administered TDCA or TCDCA com-

pared to controls. These data indicated that gastric mucosal injury was readily induced by an elevated serum level of TDCA, TCDCA and their unconjugated bile salts and this effect was directly due to cytotoxicity of the bile acid.

GES-1 cell lines(eternalized human gastric mucosa epithelium cell lines) are accessed from transfected SV240 virus in primary cultured human fetus gastric mucosa epithelial cells. However, it reserves a normal cell skeleton system and does not cause tumors in nude mouse with a lower colony formation rate in soft agar. It has been reported that GES-1 cell has a greater value in investigating the mechanism of gastric mucosa pathologic **in vitro**[22].

Bile acids constitute 67% of the normal bile, and are conjugated with glycine or taurine. The normal bile acids consist of 40% cholic acids, 40% chenodeoxycholic acid, and 20% deoxycholicand acids, and a minor fraction of lithocholic acid and ursodeoxych acids. Here we studied, by culturing GES-1 cell lines, the effect in different bile constituents, including 6 kinds of bile salts(GC,GDC, GCDC,TC,TDC,TCDC), 3 kinds of bile acids(LCA,CA, DCA), mixture of 6 kinds of bile salts(DY) and mixture of 3 kinds bile acids(DS). Bile salts are present in the normal human stomach, with mean concentrations varying from 50-500 μmol/L and ranges up to 9 mmol/L, while in patients who have undergone gastric surgery, concentrations may rise to 25 mmol/L[23]. Someone adapt sustained draw gastric juice for 90 minutes to make a diagnosis as DGR by TBA $>$ 100 μmol/h or $>$ l000 μmol/L[24]. So we chose the concentration from 50 to 1000 μmol/L in the different bile salts and from 250 to 2000 μmol/L in the mixture to study the effect of bile salts and bile acids.

The results presented that there was no significant difference in morphology and cell proliferation in GC group after 24~72 h. At low concentration(50 μmol/L), GDC, GCDC, TC, TDC and TCDC can accelerate gastric epithelial cell growth, in a dosage-time depen-dent manner. At middle concentration(250-500 μmol/L), our results suggested two-way effect. There was shown a positive effect after 24~48 h, while a negative effect after 72h. At high 1000 μmol/L it accelerated gastric epithelial cell growth after 24 h, and showed consistent inhibition even leading to necrosis after 24~72 h. LCA and CA group showed a positive effect at 50 μmol/L after 24~72 h. It presented to apoptosis after 24~72 h at 250-1000 μmol/L. At 50-500 μmol/L, DCA trended towards proliferation after 24h and to apoptosis after 48~72 h. At 1000 μmol/L, it showed necrosis after 24~72 h. DY and DS group can facilitate normal gastric mucosa epithelial cell growth at low concentrations (250-500 μmol/L). At 1000-2000 μmol/L cell presented the trend from apoptosis to necrosis.

Cell apoptosis has a close relation with cell necrosis. Cells often change from apoptosis to necrosis when the dose of apoptosis revulsant increased. While culture time extends with the revulsant dose unchanged, it also can lead apoptosis to secondary necrosis[25]. In our study, we cultured the GES-1 cell lines in vitro to research the effect of bile salts and bile acids on human gastric mucosa epithelial cell with flow cytometry by annexin V-FITC conjugated with PI staining. Our results suggested that apoptosis ratio after 24 h was higher than the control group, and increased with time-dose-dependent except for GC group. Thus, bile salts and bile acids incubated with GES-1 cell can induce gastric mucosa epithelial cell apoptosis, and apoptosis ratio increased obviously with the concentration and incubating time adding of bile salts and bile acids.

In conclusion, bile salts and bile acids are the key factors of gastric mucosa epithelial cell injury. We studied the effect of bile salts and bile acids on normal gastric mucosa epithelial cell and observe the mechanism of injury by cell culture. However, there is a great difference between **in vivo** and *in vitro*, we can only provide a theoretical basis for describing the injury mechanism of bile in the duodenogastric reflux in vitro, which perhaps may find a new way to protect the gastric mucosal cell.

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