LABORATORY STUDY

Effect of bile acids on fibroblast proliferation and viability

K. RAMAESH, F.A. BILLSON, M.C. MADIGAN

Abstract

Purpose **To examine the *in vitro* effects of bile acids and salts on the viability, growth and morphology of human Tenon's capsule fibroblasts.**

Methods **Human Tenon's capsule fibroblasts were grown from explants in culture and used between passages 3 and 5. Fibroblasts ($n = 3$) were treated in triplicate with deoxycholic acid (DA), sodium deoxycholate (NaD) and cholic acid (CA) at concentrations between 50 μM and 1 mM. Cell viability and growth were assessed with trypan blue staining and haemocytometer counting, and a colorimetric (MTT) assay. Morphology was assessed with light microscopy and cresyl violet staining.**

Results **A dose-dependent decrease in viability was observed following bile acid treatment (24 and 48 h) where the effects of DA > NaD > CA. The LD_{50} values at 48 h for DA, NaD and CA were 300 μM, 400 μM and 720 μM respectively. Cell growth following bile acid treatment was reduced compared with controls. Fibroblasts treated with bile acids displayed a loss of normal spindle-shaped morphology and multiple processes, some with varicosities, extended from many cells. These changes were dose-dependent, and at higher concentrations cells became detached and were non-viable (trypan-blue-positive). Extensive cell death was observed in cultures treated with DA 400 μM, NaD 500 μM and CA 800 μM.**

Conclusions **Lower doses of bile acids and salts reduced *in vitro* fibroblast growth associated with morphological changes; higher doses induced fibroblast cytotoxicity. These observations suggest that bile acids and salts, in particular DA, may be useful in regulating wound fibrosis following trabeculectomy surgery.**

Key words Bile acids and salts, Human Tenon's capsule, Fibroblasts, Glaucoma, Trabeculectomy, Wound healing

Glaucoma filtration surgery or trabeculectomy reduces intraocular pressure (IOP) by forming a new drainage channel for aqueous humour outflow.[1,2] The successful lowering of IOP following trabeculectomy depends on the healing response after surgery; trabeculectomy failure is most likely to occur when subconjunctival scarring, secondary to fibroblast proliferation, occludes the trabeculectomy and encystation of the filtering bleb occurs.[1,2] The failure rate is significantly higher in the paediatric age group where the wound healing response is more vigorous.[1,3,4] Agents that can inhibit fibroblast proliferation and scar formation, including 5-fluorouracil (5-FU) and mitomycin C, are commonly used to reduce the incidence of trabeculectomy failure.[2] However, these agents may require frequent injections and have been associated with ocular side-effects including epithelial toxicity, wound leaks, and an increased risk of endophthalmitis.[1,2]

Clinical observations and *in vitro* studies indicate that bile acids and salts may regulate wound healing and can inhibit human fibroblast proliferation.[5,6] Recent studies have also shown that bile acids and salts can inhibit proliferation and induce differentiation of HL-60 promyelocytic leukaemia cells,[7] and may induce apoptosis of colonic goblet cells[8] and Epstein–Barr virus (EBV)-transformed lymphocytes.[9] Bile salts have been trialled on rabbit corneas as possible penetration-enhancing agents for various drugs, without apparent ocular irritation or increases in corneal hydration.[10] Systemically administered bile acids (ursodeoxycholic and chenodeoxycholic acid) have also been used over prolonged periods, with minimal side-effects, for patients with primary biliary cirrhosis and choleostasis,[11,12] further suggesting these compounds have low toxicity.

To further assess the potential of bile acids and salts to inhibit fibroblast growth, we investigated the effects of three bile acids and salts (deoxycholic acid, sodium deoxycholate and cholic acid) on the viability, growth and morphology of cultured human Tenon's capsule fibroblasts.

K. Ramaesh
F.A. Billson
M.C. Madigan
Department of Clinical Ophthalmology
Save Sight Institute
University of Sydney
NSW 2006
Australia

M.C. Madigan ✉
Department of Clinical Ophthalmology
Sydney Hospital
GPO BOX 4337
Sydney
NSW 2001
Australia
Tel: +61 2 9382 7315
Fax: +61 2 9382 7318
e-mail:
michele@eye.usyd.edu.au

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Materials and methods

Fibroblast cultures

Pieces of Tenon's capsule were obtained with consent at the time of cataract surgery, from 4 patients aged 17, 39, 65 and 70 years. The tissue was transported in Hank's buffered saline solution (HBSS; Trace Biosciences). The biopsy specimens were minced into small pieces and explants incubated at 37 °C in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum, 50 mg/ml penicillin and streptomycin and 2 mM L-glutamine (Trace Biosciences). Explants were cultured for 3 weeks and the medium changed twice weekly until fibroblasts reached confluence. Following removal of the explants, adherent fibroblasts were rinsed twice in HBSS and trypsinised with 0.05% trypsin/0.02% EDTA in HBSS (Trace Biosciences) and passaged into 25 cm^2 flasks. When confluent, fibroblasts were again trypsinised and then passaged into 75 cm^2 flasks. Fibroblasts between passage 3 and 5 were used for all experiments.

Immunocytochemistry

A mouse anti-human fibroblast antibody (Dako Aust.) that stains for prolyl 4-hydroxylase, an enzyme found in fibroblasts, was used to assess the homogeneity of the fibroblast cultures. Fibroblasts grown on coverslips for 48 h were immunostained as follows. Coverslips were rinsed in Dulbecco's phosphate-buffered saline (DPBS; pH 7.2), fixed in acetone at −20 °C, rinsed again, then incubated in primary antibody (diluted 1:50). After further rinsing, cells were incubated in a biotinylated secondary antibody (diluted 1:50) (Amersham Aust.), and bound antibody detected with an avidin–biotin peroxidase labelling technique (Vectastain, Vector Labs, USA) using 3,3'-diaminobenzidine tetrahydrochloride.

Bile acids

Stock solutions of deoxycholic acid (DA), sodium deoxycholate (NaD) and cholic acid (CA) (Sigma Aust.) were prepared as follows. CA (20 mM) and DA (50 mM) were dissolved in absolute ethanol; NaD was prepared as a 50 mM stock solution in HBSS. The CA and DA stock solutions were stored at 4 °C and discarded after 2 weeks; fresh stock solutions of NaD were made for each experiment. To examine for pH effects of bile acids and salts prior to viability experiments, the pH of the culture medium plus a range of concentrations of bile salts and acids (from 50 μM to 1 mM) was assessed and found to remain between pH 7.2 and 7.4. Previous *in vitro* studies indicate that doses of bile acids from 25 μM to 3.1 mM can induce growth inhibition and cytotoxicity in a variety of cell types including fibroblasts.[6,7,9] A range of concentrations of bile acids from 50 μM to 1 mM was therefore used in the present study.

Effect on fibroblast viability

To assess the effect of varying concentrations of bile acids on cell viability, fibroblasts from three explants (17, 65 and 70 years) were seeded in triplicate into 24-well plates at a concentration of 10^4 cells per well. After 24 h the medium was replaced and bile acids added to give final concentrations of 50, 100, 200, 300, 400, 500 μM for DA and NaD. For CA, treatments of 600 μM, 800 μM and 1 mM were also studied. For NaD, parallel untreated controls were examined. For DA and CA, where ethanol was used as the diluent, triplicate wells were treated with a volume of ethanol equal to that used for the highest concentration of DA or CA. For example, 20 μl of 50 mM stock DA was added to cells in 2 ml medium to give a final concentration of 500 μM DA; controls were treated with 20 μl ethanol. After 24 h fibroblasts were examined and photographed, then trypsinised as above. Cell viability was assessed with trypan blue staining and haemocytometer counting.

In separate experiments, fibroblasts were grown on coverslips, treated with various doses of bile acids as described above, fixed in 2% paraformaldehyde/DPBS (pH 7.2), and stained with cresyl violet. The coverslips were mounted on slides in glycerol and photographed.

Effect on fibroblast growth

To test the effects of DA, NaD and CA on cell growth, fibroblasts from three explants (39, 65 and 70 years) were seeded in triplicate at 2×10^4 cells per well into 24-well plates. After 24 h, the medium was replaced and cells treated with DA and NaD to give final concentrations of 200 μM, 300 μM and 400 μM. For CA, concentrations of 200 μM, 400 μM and 600 μM were also tested. At 24 and 48 h, cultures were examined and photographed. The total number of viable cells was then counted using trypan blue staining and a haemocytometer. Parallel untreated and alcohol-treated (for DA and CA) controls as described above were also examined. Cell counts from the three specimens, assessed in triplicate wells, were averaged. Differences in cell counts between bile-acid-treated specimens and control specimens (untreated controls and ethanol-treated controls) were assessed for each bile acid and the appropriate control at 24 and 48 h using a paired *t*-test, where $p<0.05$ was considered significant.

The effects of DA, NaD and CA on fibroblast growth were also assessed using a colorimetric assay (MTT Kit 1; Boehringer Mannheim Aust.). Fibroblasts from three explants (39, 65 and 70 years) were seeded in triplicate at 5×10^3 cells per well in 96-well plates, and treated for 24 and 48 h with bile acids and salts as described above. Tetrazolium dye (3-[4,5-dimethylthiazol]-2-yl-2,5-diphenyl tetrazolium bromide or MTT) was added, and cells incubated for 4 h at 37 °C (MTT is converted by viable cells to an insoluble, purple formazan salt). Formazan salts were solubilised overnight at 37 °C and the absorbance quantified with an ELISA plate reader (Bio-Rad; 570 nm with 655 nm reference).

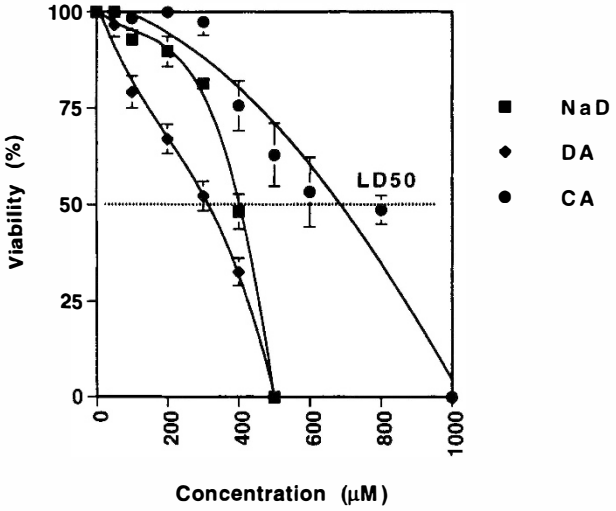

Fig. 1. *Viability of fibroblasts treated for 24 h with a range of concentrations of deoxycholic acid (DA), sodium deoxycholate (NaD) or cholic acid (CA), where the LD_{50} is approximately 300 μM DA, 400 μM NaD and 720 μM CA, respectively. Error bars show the standard deviation (SD).*

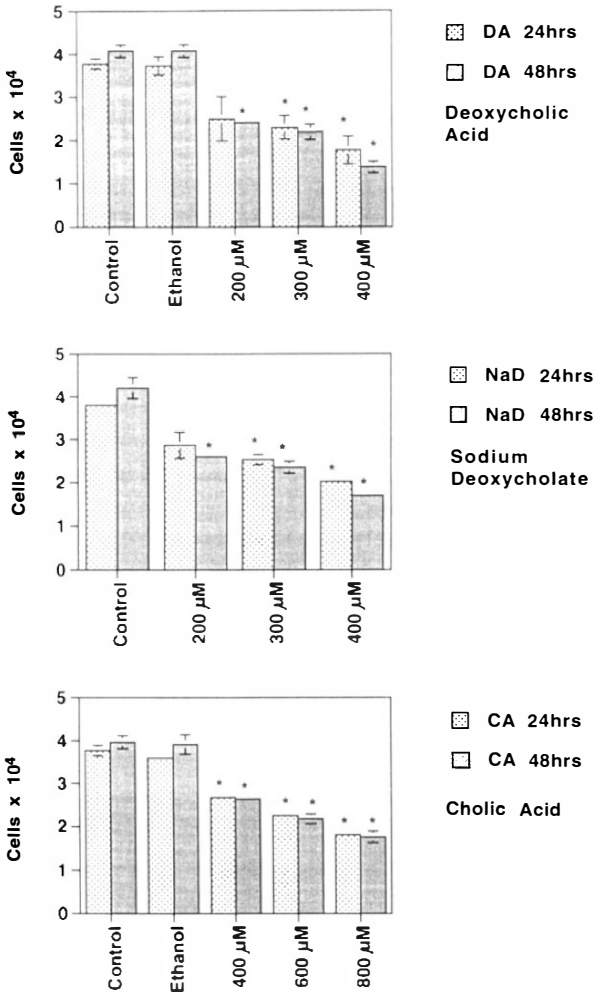

Fig. 2. *Cell counts for a range of concentrations of DA, NaD and CA, after 24 h and 48 h of treatment. The decrease in cell number following treatment with bile acids and salts is time- and dose-dependent, where the effect of DA>NaD>CA. Error bars show the SD; *p<0.05, paired t-test.*

Results

Dose response

In untreated control and alcohol-treated control cultures, cell viability was greater than 95% for all experiments. Following treatment with bile acids and salts for 24 h, viability decreased in a dose-dependent fashion with an LD_{50} of approximately 300 μM DA, 400 μM NaD and 720 μM CA respectively (Fig. 1).

Cell growth

In untreated and alcohol-treated controls, cell numbers increased from approximately 3.8×10^4 cells at 24 h to approximately 4.1×10^4 cells at 48 h (Fig. 2). Cell numbers in cultures treated with a range of concentrations of DA, NaD and CA for 24 and 48 h were

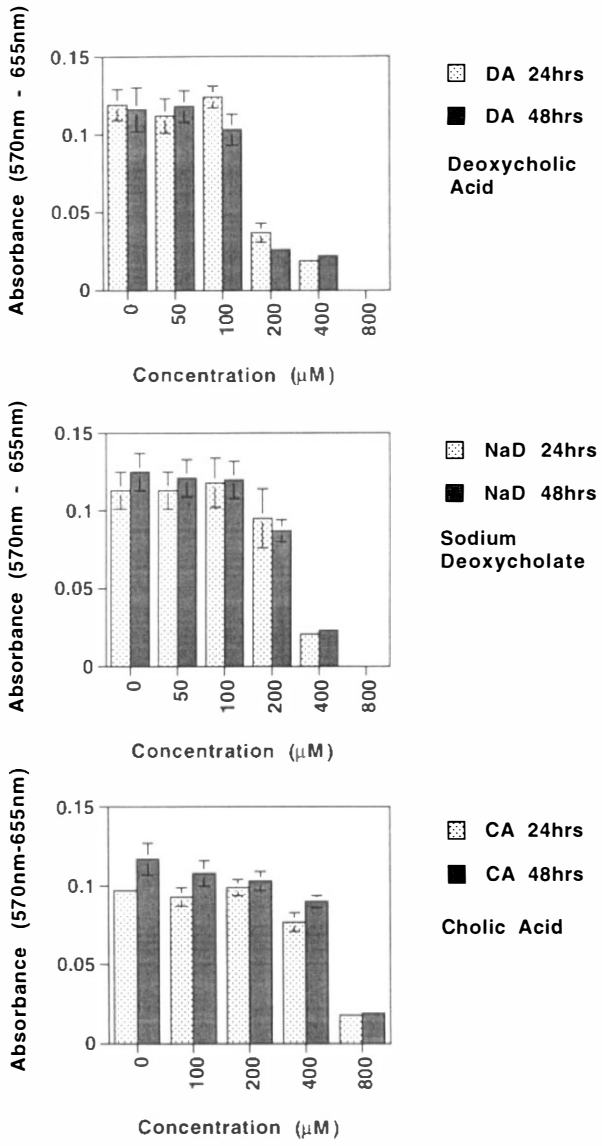

Fig. 3. *MTT assays after 24 and 48 h of treatment. A time- and dose-dependent decrease in absorbance (viable cells) of treated cultures is seen, particularly for higher doses. In untreated and ethanol-treated (not shown) controls, an increased absorbance is seen at 48 h compared with 24 h. Error bars show the SD.*

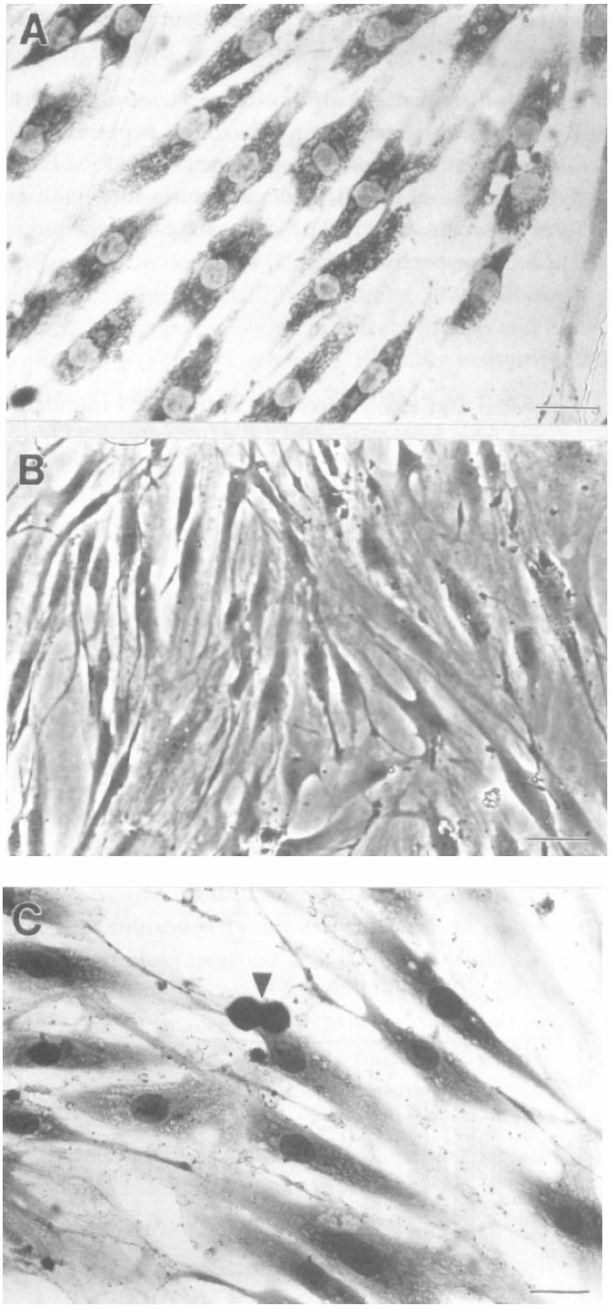

Fig. 4. *Photomicrographs of control Tenon's capsule fibroblasts grown from explants. (A) Immunocytochemistry of fibroblast cultures using an anti-fibroblast antibody; cytoplasmic staining is seen in all cells indicating homogeneous fibroblast cultures. (B) With phase-contrast microscopy, control fibroblasts display a spindle-shaped confluent morphology. Ethanol-treated controls had a similar appearance (not shown). (C) Cresyl violet staining further illustrates the spindle-shaped morphology of control fibroblasts; a mitotic cell is also visible (arrowhead). Scale bars represent: (A), (C), 25 μM; (B), 130 μM.*

significantly decreased compared with controls ($p < 0.05$, paired *t*-test) (Fig. 2). However, for similar concentrations, DA-treated cultures had fewer cells than NaD- or CA-treated cultures (Fig. 2). MTT assays (where absorbance is proportional to viable cell numbers) also indicated that, for similar concentrations, DA induced greater decreases in fibroblast viability than either NaD or CA (Fig. 3).

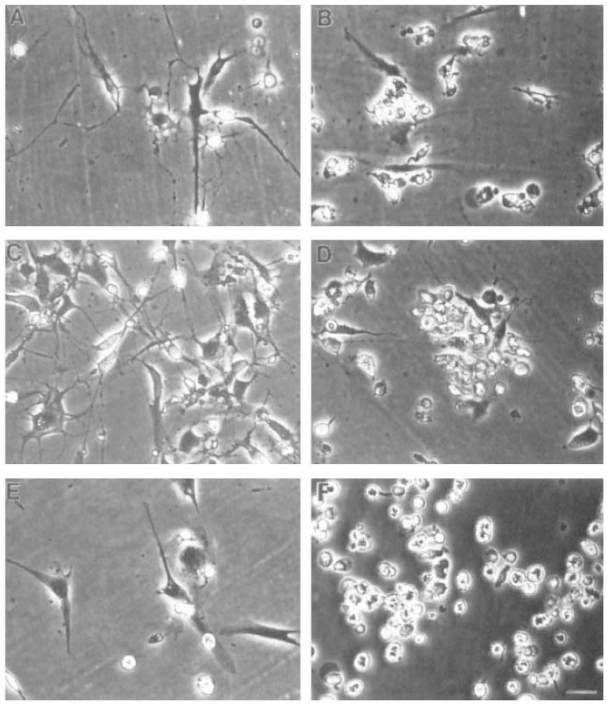

Fig. 5. *Phase-contrast photomicrographs of fibroblasts after 48 h of treatment with bile acids and salts. Fibroblasts treated with DA > 100 μM show loss of spindle-shaped morphology, and multiple processes, some beaded, extend from many cells (A). These changes are more obvious at higher concentrations and for DA ⩾300 μM numerous rounded detached (non-viable) cells are seen (B). Similar morphological changes were observed in NaD-treated (C, D) and CA-treated fibroblast cultures (E, F). Extensive cell death was observed in cultures treated with DA 400 μM, NaD 500 μM or CA 800 μM. Scale bar represents 110 μM.*

Morphology

Immunocytochemistry of cultures from each explant, using an anti-fibroblast antibody, revealed homogeneous fibroblasts with cytoplasmic staining of all cells (Fig. 4A). Untreated or alcohol-treated fibroblasts grew as attached flat spindle-shaped cells in culture (Fig. 4B, C).

Concentrations of 100 μM and 200 μM NaDA, DA and CA did not produce marked changes in cell morphology or cell detachment. However, fibroblasts treated with 300 μM and 400 μM DA and NaD showed evidence of cell detachment, especially with DA, and dead cells were also visible; some viable cells were still attached 48 h after treatment (Fig. 5A–D). The attached cells displayed distinct changes, with loss of the spindle-shaped morphology normally seen in control cultures, and extension of multiple long thin processes from the cell body (Fig. 5A–D). Varicosities were sometimes visible along these processes (Fig. 5A, C). Similar changes were noted for 600 μM and 800 μM CA (Fig. 5E, F). With phase-contrast microscopy, fibroblasts incubated for 24 h with ⩾500 μM NaD or DA, and ⩾1 mM CA, were detached and non-viable, as indicated by trypan blue staining.

In separate experiments, fibroblasts were grown on coverslips, treated as above with bile acids and salts, and stained with cresyl violet. Control and alcohol-treated

fibroblasts displayed normal spindle-shaped morphology; cell nuclei were clearly stained with cresyl violet and mitotic cells were sometimes visible (Fig. 4C). The loss of spindle-shaped morphology associated with higher doses of bile salts and acids was also clearly visible in cresyl-violet-stained cultures (not shown).

Discussion

The outcome of glaucoma filtration surgery has been considerably improved with the use of agents such as 5-FU (an antimetabolite) and mitomycin C (an antiproliferative), which can inhibit fibroblast proliferation and scarring of the trabeculectomy site.[1,2] Patients followed for up to 5 years after trabeculectomy with 5-FU treatment are reported to have about a 50% failure rate compared with 74% in patients where 5-FU was not used.[13] Several studies indicate, however, that adverse effects may be associated with 5-FU, including the necessity for frequent injections, ocular epithelial toxicity, wound leaks and endophthalmitis.[14–16] Trabeculectomy failure rates have also been reduced with the use of mitomycin C, although serious complications associated with its use, including chronic repeated bleb leaks, scleral necrosis and avascular bleb formation, have also been observed in some cases.[17–19] *In vitro* studies indicate that mitomycin C can be cytotoxic to vascular endothelial cells.[20] A variety of other agents that may potentially modulate fibroblast function and proliferation continue to be investigated *in vitro*,[2] including bleomycin, daunorubicin and mithramycin,[21] interferon-α 2b,[22] cytarabine,[15] taxol and colchicine.[2]

In the present study, bile acids and salts induced a time- and dose-dependent reduction in fibroblast viability. Both cell counts and MTT assays indicated that bile acids and salts (DA, NaD and CA) inhibited growth of human Tenon's capsule fibroblasts compared with controls; fibroblast morphology was also affected by bile acids and salts, with an obvious reduction in cell density and extension of processes from fibroblast cell bodies. Both DA and NaD were more potent than CA (DA was slightly more potent than NaD). Previous *in vitro* studies have similarly observed that DA and its conjugates are more toxic than other bile acids to a variety of cells including cultured human fibroblasts.[7,8] The cytotoxicity of bile acids has been related to several factors including bile acid modulation of protein kinase C activity,[23] enhancement of intracellular calcium levels[24,25] and generation of free radicals associated with cell membrane damage and activation of phospholipase C.[26] Cell membrane damage appears to be a function of bile acid detergency, where increased interaction between bile acids and cell membranes increases toxicity.[27] More specifically, the increased potency of DA (and conjugates) may be due to increased detergency of these compounds compared with other bile acids such as CA; bile acid interactions with cellular membranes (and thus toxicity) appear to be lowered by the number and orientation of hydroxyl groups, and the formation of β-epimers.[27]

At the higher doses used, we observed fibroblast cytotoxicity, with marked morphological changes and significant reductions in viability. While bile acids and salts have been suggested to induce cytotoxicity via necrosis,[8] recent *in vitro* studies indicate that bile salts can induce apoptotic involution of cells.[8,9,28,29] For example, sodium deoxycholate treatment of EBV-transformed lymphocytes can induce either apoptotic cell death or necrosis depending on concentration,[9] and may also induce apoptotic involution of cultured colon goblet cells[8] and colorectal tumour cells.[29] The potential of bile acids and salts to induce apoptotic involution of human Tenon's capsule fibroblasts is currently being investigated.

Clinical observations in patients with obstructive jaundice and following bile duct surgery, have implicated bile acids and salts in regulating wound repair.[5] Human bile, bile acid mixtures and individual bile salts, in particular deoxycholate salts, have also been reported to induce time- and dose-dependent cytotoxic effects in cultured human fibroblasts.[6] Together with the present study, these observations suggest that bile acids and salts may be useful for inhibiting fibrosis associated with failure of trabeculectomy surgery. Further studies are required to investigate the cytotoxicity of bile acids and salts to other ocular cells, and the potential of bile acids and salts, particularly DA, to modulate fibroblast function.

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