GASTRO-OESOPHAGEAL REFLUX

Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's oesophagus

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Background: Barrett's oesophagus is a premalignant condition associated with an increased risk for the development of oesophageal adenocarcinoma (ADCA). Previous studies indicated that oxidative damage contributes to the development of ADCA.
Objective: To test the hypothesis that bile acids and gastric acid, two components of refluxate, can induce oxidative stress and oxidative DNA damage.
Methods: Oxidative stress was evaluated by staining Barrett's oesophagus tissues with different degrees of dysplasia with 8-hydroxy-deoxyguanosine (8-OH-dG) antibody. The levels of 8-OH-dG were also evaluated ex vivo in Barrett's oesophagus tissues incubated for 10 min with control medium and medium acidified to pH 4 and supplemented with 0.5 mM bile acid cocktail. Furthermore, three oesophageal cell lines (Seg-1 cells, Barrett's oesophagus cells and HET-1A cells) were exposed to control media, media containing 0.1 mM bile acid cocktail, media acidified to pH 4, and media at pH 4 supplemented with 0.1 mM bile acid cocktail, and evaluated for induction of reactive oxygen species (ROS).
Results: Immunohistochemical analysis showed that 8-OH-dG is formed mainly in the epithelial cells in dysplastic Barrett's oesophagus. Importantly, incubation of Barrett's oesophagus tissues with the combination of bile acid cocktail and acid leads to increased formation of 8-OH-dG. An increase in ROS in oesophageal cells was detected after exposure to pH 4 and bile acid cocktail.
Conclusions: Oxidative stress and oxidative DNA damage can be induced in oesophageal tissues and cells by short exposures to bile acids and low pH. These alterations may underlie the development of Barrett's oesophagus and tumour progression.

Barrett's oesophagus is a premalignant condition associated with gastro-oesophageal reflux disease (GORD), where normal squamous epithelium is replaced by columnar intestinal-like epithelium containing goblet cells. Importantly, Barrett's oesophagus is closely associated with the development of oesophageal adenocarcinoma.[1–3] Patients with this cancer have a poor prognosis, with a median survival time of <1 year.[4] However, the mechanism of development of Barrett's oesophagus and oesophageal adenocarcinoma is not well understood.

It is speculated that metaplastic columnar epithelium is formed in response to stress induced by the refluxate. Gastric acid and bile acids seem to be two major risk factors for the development of Barrett's oesophagus.[5 6] Clinical studies have identified glycocholic acid, taurocholic acid, glycodeoxycholic acid and glycochenodeoxycholic acid as the predominant bile acids appearing in the oesophagus of patients with GORD.[7–9] More toxic, unconjugated bile acids, such as deoxycholic acid (DCA), are normally formed by the action of colonic bacteria on primary bile acids. Interestingly, DCA is also present in the refluxate of patients with Barrett's oesophagus.[5] Patients taking proton pump inhibitors have a significantly higher incidence of overgrowth of gastric bacteria and, consequently, increased concentrations of unconjugated bile acids, including DCA.[10] Bile acids induce mitochondrial alterations,[11–13] oxidative stress[11 12 14 15] and DNA damage.[14–16] We have also shown that perturbation of mitochondria can lead to apoptosis resistance.[13] In addition, gastric acid and bile acids may alter signalling pathways. Low pH and/or bile acids induce the expression of cyclo-oxygenase 2 and prostaglandin E_2 and activate the extracellular signal-regulated kinase, p38 mitogen-activated protein kinase and nuclear factor-κB pathways, which are associated with increased proliferation and decreased apoptosis of oesophageal cells.[17–21]

Previous studies indicated that oxidative damage contributes to the development of oesophageal adenocarcinoma,[22] and that a higher intake of antioxidants, such as vitamin C, β-carotene and α-tocopherol, is associated with a decreased risk for oesophageal adenocarcinoma.[23 24] Despite the importance of reflux in the pathogenesis of Barrett's oesophagus, there are no studies evaluating the direct effect of low pH and bile acids on the production of reactive oxygen species (ROS) in oesophageal cells or human biopsy specimens.

Since reflux disease is a major risk factor for the development of Barrett's oesophagus, we tested the hypothesis that exposure to low pH, in combination with bile acids, induces oxidative stress. We tested this hypothesis using human oesophageal biopsy specimens obtained from patients with Barrett's oesophagus with different grades of dysplasia and oesophageal cells. The biopsy specimens were also incubated in medium acidified to pH 4 and supplemented with a bile acid cocktail. We show that Barrett's oesophagus tissue, especially dysplastic

Abbreviations: CMXRos, Chloromethyl-X-Rosamine; DCA, deoxycholic acid; GORD, gastro-oesophageal reflux disease; HE, hydroethidine; HGD, high-grade dysplasia; LGD, low-grade dysplasia; 8-OH-dG, 8-hydroxy-deoxyguanosine; MMP, mitochondrial membrane potential; ND, non-dysplastic; PBS, phosphate-buffered saline; ROS, reactive oxygen species

tissue, is under increased oxidative stress and that gastric acid in combination with bile acids induces oxidative DNA damage. Our results indicate a central role for gastric acid and bile acids in the pathogenesis of Barrett's oesophagus.

MATERIALS AND METHODS

Tissue biopsy specimens from patients and ex vivo incubations

Forty-five patients with Barrett's oesophagus with various degrees of dysplasia were included in the present study. Endoscopic biopsy specimens, oesophageal squamous mucosa and duodenum were taken from patients with Barrett's oesophagus who were undergoing surveillance procedures. Patients were men aged 41–83 years. Patients gave written informed consent as approved by the institutional human subjects committee at the University of Arizona, Tucson, Arizona, USA. Biopsy specimens were immediately fixed in 10% buffered formalin. Barrett's oesophagus was defined histologically as the presence of intestinal-like epithelium containing goblet cells after staining with H&E and Alcian blue (pH 2.5) to identify goblet cells. The grade of dysplasia was independently evaluated by two pathologists.

Additional biopsy specimens were evaluated from 10 patients for induction of oxidative DNA damage after ex vivo incubation at pH 4 and a bile acid cocktail. In this series, we had 10 patients with non-dysplastic (ND) Barrett's oesophagus. Two biopsy specimens from each patient were immediately washed in minimal essential medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Omega Scientific, Tarzana, California, USA), pH 7.4, 2 mM L-glutamine, 5 mM HEPES (N′-2-hydroxyethylpiperazine-N′-ethanesulphonic acid), 1 mM non-essential amino acids (Sigma Chemical Co, St Louis, Missouri, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). The biopsy specimens were then incubated ex vivo for 10 min in this control media or in media acidified to pH 4 and supplemented with 0.5 mM bile acid cocktail at 37℃ and 5% CO_2.[25] After 10 min, the tissues were washed with the medium and then incubated for an additional 170 min in the medium. Previously, we had performed several experiments using different time points and found that after incubation for 3 h, the tissue remains relatively normal histologically (data not shown). However, longer incubation periods induce sloughing and death of epithelial cells. Thus, the 3 h time point was chosen as an optimal condition for this assay. After incubation, biopsy specimens were fixed and embedded in paraffin wax for histological evaluation and immunohistochemical studies. In addition, one biopsy specimen from each patient was immediately fixed in 10% buffered formalin.

Cell line and chemicals

The human oesophageal adenocarcinoma Seg-1 cell line was kindly provided by Dr David Beer (University of Michigan, Ann Arbor, Michigan, USA). Seg-1 cells are used frequently as a model cell line to study Barrett's oesophagus and Barrett's oesophagus-associated signalling pathways after exposure to low pH and bile acids. The Seg-1 cells were cultured in D-MEM media (Gibco, Carlsbad, California, USA) supplemented with 10% (vol/vol) heat-inactivated bovine calf serum (Hyclone Laboratories, Logan, Utah, USA), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). HET-1A cells were provided by Dr Curtis C Harris (National Cancer Institute, Bethesda, Maryland, USA). HET-1A is a human oesophageal epithelial cell line immortalised by transfection of the Simian Virus 40 T antigen early region gene. These cells have retained epithelial morphology, stain positively for cytokeratin 13 and have remained non-tumorigenic in athymic, nude mice for >12 months.[26] The cells were in serum-free BRFF-EPM2 medium (Athena Environmental Sciences, Baltimore, Maryland, USA) supplemented with 50 μg/ml gentamicin and 0.25 μg/ml amphotericin B. Barrett's oesophagus CP-D cells immortalised by hTERT (human catalytic subunit of telomerase reverse transcriptase) transfection were kindly provided by Dr Peter Rabinovitch (Fred Hutchinson Cancer Research Center, University of Washington, Seattle, Washington, USA). The cells were maintained in MCDB 153 medium supplemented with 5% fetal calf serum (Hyclone Laboratories), 20 ng/ml epidermal growth factor (R&D Systems, Minneapolis, Minnesota, USA), 140 μg/ml bovine pituitary extract (Sigma), 10 mM cholera toxin (Sigma), 0.4 μg/ml hydrocortisone (Sigma), 20 μg/ml adenine (Sigma), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium (Sigma), 4 mM L-glutamine, 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 7.5% wt/vol sodium bicarbonate (Gibco) and 0.25 μg/ml amphotericin B (Gibco), as described previously.[27] All cell lines were grown at 37℃ in 5% CO_2 on fibronectin–collagen substrate (BRFF, Ijamsville, Maryland, USA).

A bile acid cocktail was prepared, consisting of an equimolar mixture of sodium salts of glycocholic acid, taurocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid and DCA. This cocktail reflects the mixture of bile acids to which the distal oesophagus is ordinarily exposed during gastrooesophageal reflux, based on the studies of Kauer *et al*,[8] Nehra *et al*[5] and Theisen *et al*.[10] The concentration of each of the five bile salts was 0.02 mM for a total bile acid concentration of 0.1 mM. This cocktail was completely soluble in medium at pH 4 or pH 7.4, and no precipitate was formed.

MitoSOX Red, MitoTracker Red, hydroethidine (HE), YOYO-1 and Hoechst 3222 were obtained from Molecular Probes (Eugene, Oregon, USA). All other chemicals were of the highest purity available and were obtained from Sigma Chemical, unless otherwise noted.

Immunohistochemical staining

A specialised immunohistochemical technique was necessary to detect specific nuclear staining with 8-hydroxy-deoxyguanosine (8-OH-dG). Paraffin wax-embedded sections were deparaffinised, rehydrated, placed in 3% hydrogen peroxide for 30 min and rinsed with deionised water. To open up chromatin, the slides were immersed in 4 N HCl for 20 min, rinsed with water and placed in 0.1 M borax for 5 min, followed by two rinses in water. Slides were immersed in phosphate-buffered saline (PBS) for 5 min and then incubated for 1 h in 2% horse serum made in PBS. 8-OH-dG monoclonal antibody (2 μg/ml, QED Bioscience, San Diego, California, USA) made in 2% bovine serum albumin/PBS was then added and slides were refrigerated overnight. After three rinses with PBS, the secondary biotinylated anti-mouse IgG antibody (1:400, Dako, Carpenteria, California, USA) was applied for 30 min. PBS rinses were followed with applications of Vectastain ABC reagent and diaminobenzidine. The slides were counterstained with haematoxylin. Mouse IgG_{2a} immunocontrols were routinely included with each experiment.

A simple grading system (0–3) routinely used in our laboratory, based on the intensity of staining in the nucleus of epithelial cells, was used to grade the level of expression of 8-OH-dG in Barrett's oesophagus glands (0, 0–5% cells stained; 1, 5–30% cells stained; 2, 30–60% cells stained; and 3, >60% cells stained). Staining was evaluated independently by three experienced investigators. In our incubation studies, to quantify positively and negatively stained nuclei specifically in Barrett's oesophagus glands, we used a digital image analysis system. Images of Barrett's oesophagus glands were visualised using a Nikon Eclipse E400 brightfield microscope and captured by a Sony DXC-390 colour video camera. Barrett's oesophagus glands on each slide (average 8 (2) glands, range 5–13 glands)

were analysed using Media Cybernetics Image Pro V.4.5.1 software (Media Cybernetics, Silver Spring, Maryland, USA). The conditions, brightness, filter and light exposure were initially adjusted to optimise the brightness and contrast of the image. Once established, the settings remained constant for comparison of biopsy specimens.

For fluorescence microscopy, the cells were grown on slides, fixed with 4% formaldehyde, permeabilised with 100% methanol and incubated with primary antibody as described above. Next, Alexa Fluor594 secondary antibody (1:100; Molecular Probes) was applied for 60 min. The slides were then treated for 1 h with 50 μg/ml RNase to digest RNA. Nuclei were counterstained with YOYO-1 (Molecular Probes) and the slides coverslipped using VectaShield HardSet mounting medium (Vector Laboratories, Burlingame, California, USA). Appropriate filters were used to evaluate the expression of 8-OH-dG.

Determination of oxidative stress

Oxidative stress in oesophageal cells was assessed by staining with the mitochondrial superoxide indicator, MitoSOX Red. The cells were exposed to control medium or medium adjusted to pH 4 and/or supplemented with a 0.1 mM bile acid cocktail. After treatment, the cells were washed twice with normal medium. For fluorescent microscopic studies, the cells were incubated with 5 μM MitoSOX Red for 15 min and then for 5 min with 10 μM Hoechst 33342—a cell-permeant dye, which stains the nuclei. To confirm the data, the Seg-1 cells were also incubated with HE, a non-fluorescent dye that is oxidised by ROS, primarily by superoxide. The Seg-1 cells were treated as described above and then incubated with 2 μM HE in serum-free medium for 30 min, and then for 5 min with 10 μM Hoechst 33342. Images of live cells were taken using a Nikon TE300 fluorescence microscope with a digital camera equipped with appropriate filters for individual dyes. Digital images were captured using Image-Pro Plus software (Media Cybernetics).

For flow cytometry studies, the Seg-1 cells were trypsinised and stained with 5 μM MitoSOX Red at 37℃ for 15 min.[28] Experiments were performed in triplicate and a minimum of 10 000 events were collected. Fluorescence intensity of MitoSOX Red was analysed using a BD FACScan flow cytometer (BD Biosciences, San Jose, California, USA) with excitation at 488 nm and emission at 620 nm. Data are expressed as a graph indicating the percentage of cells staining positively with MitoSOX Red after various treatments compared with treatment with control medium.

Determination of mitochondrial membrane potential

Seg-1 cells were evaluated for changes in mitochondrial membrane potential (MMP) after treatment with pH 4 and/or bile acid cocktail. For these studies, the lipophilic cationic dye, MitoTracker Red Chloromethyl-X-Rosamine (CMXRos), which is concentrated in intact mitochondria based on the MMP, was used, along with flow cytometry and confocal microscopy.[29] For confocal microscopy, the cells were washed after treatment with normal medium and incubated for 30 min with 200 nM CMXRos at 37℃. The cells were then fixed, stained with 4′-6-diamidino-2-phenylindole to identify nuclei and evaluated by fluorescent microscopy using appropriate lasers (Nikon PCM 2000; Nikon, Melville, New York, USA). The cells were also analysed by flow cytometry as described previously.[28] Cells treated with 0.5 mM sodium deoxycholate for 3 h were used as positive controls for decreased MMP.

Quantification of apoptosis using brightfield microscopy

For apoptotic studies, Seg-1 cells were treated for 10 min with control medium or medium adjusted to pH 4 and/or supplemented with a 0.1 mM bile acid cocktail. Cells were then washed and incubated for 24 h in control medium, trypsinised, cytospun onto the slides using Cytospin 2 (Shandon, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA), fixed with 100% methanol for 2 min, air-dried and stained with Giemsa. Two hundred cells were evaluated for apoptosis using brightfield microscopy (100× oil immersion) as described previously.[30] The criteria used to identify apoptotic cells included chromatin condensation, formation of apoptotic bodies and cellular shrinkage.[31]

Statistical analysis

The relationships of intensity of 8-OH-dG staining with grade of dysplasia were analysed using contingency table methods (table 1). Significance testing was based on Cochran–Mantel–Haenszel statistics for ordered contingency tables. These statistics account for the ordered levels of staining (0–3) and can also account for ordering of categories based on grade of dysplasia.

Statistical significance of flow cytometry data was determined by analysis of variance at the 95% confidence level. We compared the mean fold increase in fluorescence intensities after various treatments compared with control. All other data are expressed as the mean (SEM). Differences between groups were compared using the two-tailed Student's t test.

RESULTS

Expression of 8-OH-dG in Barrett's oesophagus

Using immunohistochemical staining with a monoclonal antibody against 8-OH-dG, we examined whether biopsy specimens from patients with Barrett's oesophagus with different grades of dysplasia are associated with increased oxidative cellular stress. 8-OH-dG antibody can recognise oxidised nucleotides as markers of oxidative damage to DNA. Fifteen patients were evaluated in each of three groups (ND, low-grade dysplasia (LGD) and high-grade dysplasia (HGD)). We also evaluated oesophageal squamous epithelium ($n = 5$) and duodenal mucosa ($n = 5$) as control tissues for expression of these markers.

Increased nuclear staining of 8-OH-dG was demonstrated in Barrett's oesophagus glands compared with squamous and duodenal epithelia (fig 1). The strongest and most abundant staining of 8-OH-dG was observed in the epithelial cells of dysplastic glands classified as LGD and HGD. A less intense 8-OH-dG signal was found in ND glands of patients in whom Barrett's oesophagus had never been diagnosed as dysplastic. The isotype-matched immunocontrols did not show any positive staining. Figure 1A shows representative images.

As fig 1B and table 1 demonstrates, there was a significant trend (increased 8-OH-dG immunostaining with dysplasia) across the three categories (ND, LGD, HGD; $p = 0.046$).

Incubation of biopsy specimens ex vivo in medium acidified to pH 4 and bile acid cocktail results in increased formation of 8-OH-dG, a marker of oxidative DNA damage

To detect whether ROS are formed in biopsy specimens after exposure to low pH and bile acid cocktail, we incubated paired biopsy specimens from 10 patients for 10 min, in control medium or in medium acidified to pH 4, and supplemented with a 0.5 mM bile acid cocktail. The biopsy specimens were stained with 8-OH-dG antibody to determine DNA oxidative damage. The percentage of positively stained nuclei in Barrett's oesophagus glands was determined using Media Cybernetics Image Pro V.4.5.1 software image analysis. In this ex vivo experiment, 8 of 10 biopsy specimens showed a significant increase in percentage of nuclei that stained positively for 8-OH-dG after exposure to pH 4 and bile acid cocktail compared with the biopsy specimens exposed only to control medium ($p<0.05$, fig 2). By contrast, when samples were

Table 1 Statistical evaluation of intensity of 8-hydroxy-deoxyguanosine immunostaining in human biopsy specimens of Barrett's oesophagus with different degrees of dysplasia based on Cochran–Mantel–Haenszel statistics

Patients with Barrett's oesophagus	Immunostaining intensity for 8-OH-dG					
	0	1	2	3	Total	Mean score
ND	5 (33%)	4 (27%)	4 (27%)	2 (13%)	15	1.20
LGD	0 (0%)	2 (13%)	6 (40%)	7 (47%)	15	2.33
HGD	2 (13%)	0 (0%)	10 (67%)	3 (20%)	15	1.93
Comparison of the 3 categories (ND, LGD, HGD): p = 0.046						

HGD, high-grade dysplasia; LGD, low-grade dysplasia; 8-OH-dG, 8-hydroxy-deoxyguanosine; ND, non-dysplastic.

treated with pH 4 only or bile acid cocktail only, the effects were small and the data inconsistent.

Oxidative stress in oesophageal cells exposed to pH 4 and bile acid cocktail

Oesophageal cells exposed to control medium, medium acidified to pH 4, medium supplemented with 0.1 mM bile acid cocktail, and medium acidified to pH 4 and supplemented with 0.1 mM bile acid cocktail (combination of bile acid cocktail and acid) were evaluated for oxidative stress using the fluorescent dyes MitoSOX Red and HE. Whereas MitoSOX Red detects mitochondrially derived ROS, particularly superoxide, in live cells, HE detects all superoxide formed in cells. Because both these dyes are not oxidised by other ROS or

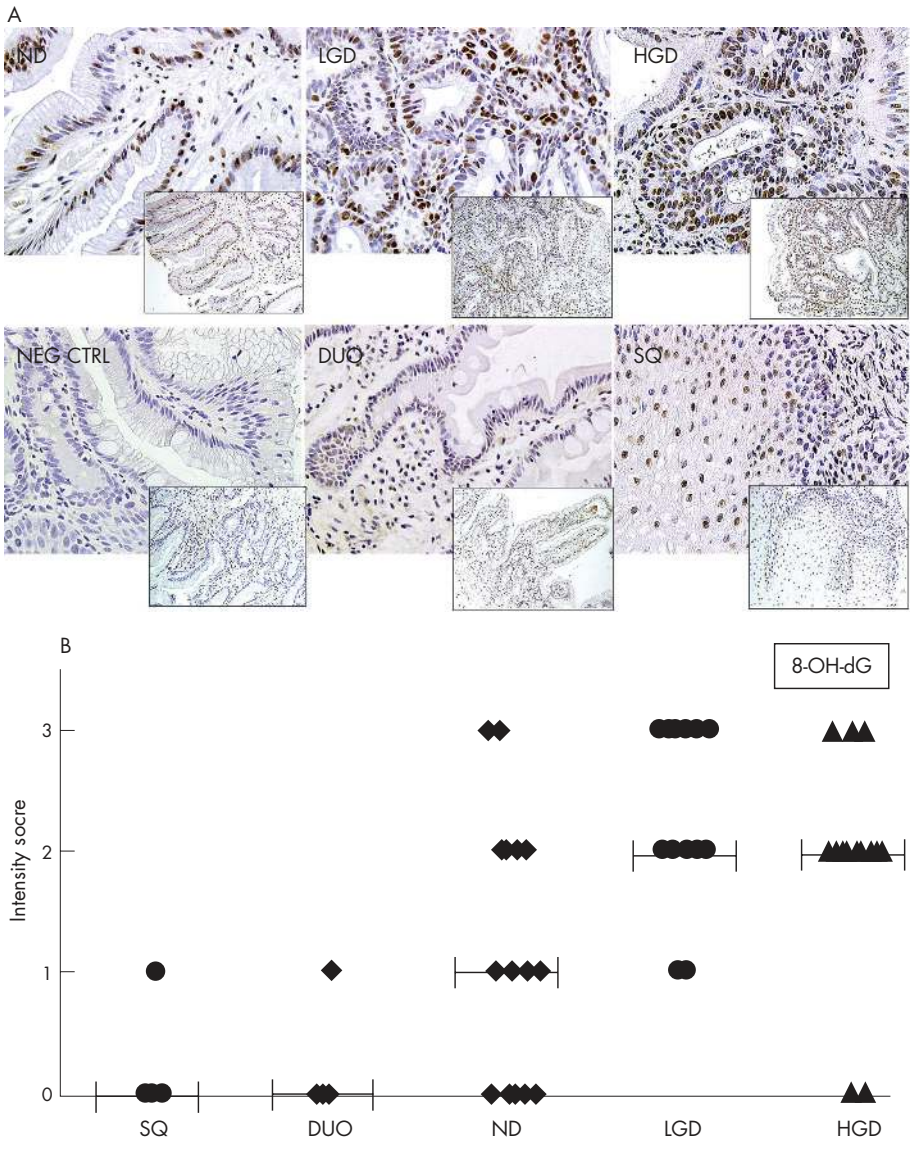

Figure 1 Immunohistochemical staining pattern for 8-hydroxy-deoxyguanosine (8-OH-dG) in different tissues. Representative images are shown in panel (A): ND, non-dysplastic Barrett's oesophagus; LGD, Barrett's oesophagus with low-grade dysplasia (LGD); HGD, Barrett's oesophagus with high-grade dysplasia (HGD); NEG CTRL, IgG_{2a} immunocontrols (negative control); DUO, duodenum; SQ, squamous epithelium; original magnification 400×. The small insets show tissue samples at 200× magnification. Panel (B) shows expression patterns of 8-OH-dG in individual patient biopsy specimens from patients with ND Barrett's oesophagus (n = 15), Barrett's oesophagus with LGD (n = 15), Barrett's oesophagus with HGD (n = 15), SQ (n = 5) and DUO (n = 5). The scoring used a simple grading system (0–3). Median values are indicated by horizontal bars.

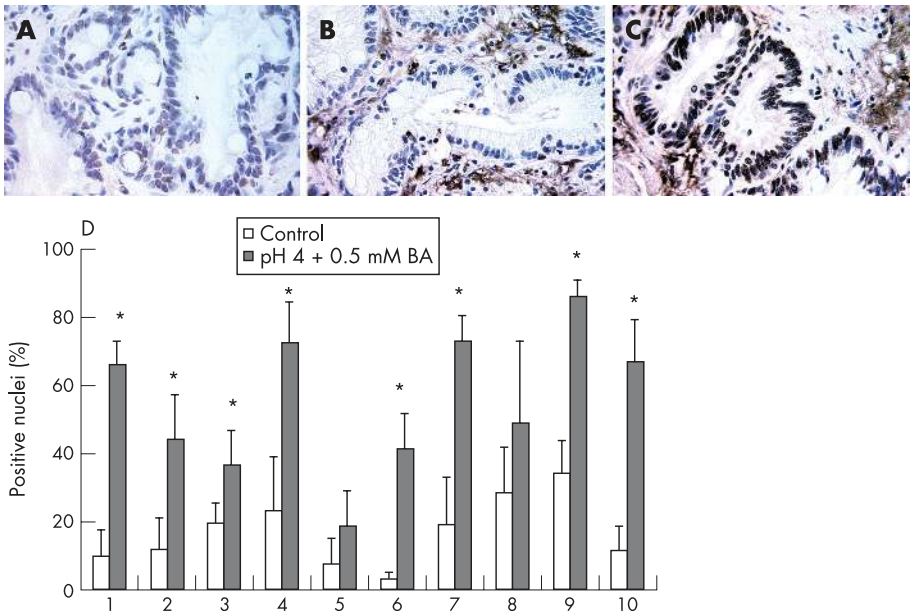

Figure 2 Immunohistochemical staining pattern and evaluation of 8-hydroxy-deoxyguanosine (8-OH-dG) in Barrett's oesophagus tissues exposed to pH 4 and bile acid (BA) cocktail for 10 min. The biopsy specimens of Barrett's oesophagus were either immediately fixed (A) or incubated in control medium (B) or medium acidified to pH 4 and supplemented with 0.5 mM BA cocktail (C). Note the intense nuclear staining of 8-OH-dG in epithelial cells after incubation with pH 4 and BA cocktail (C). (D) Percentage of nuclei positive for 8-OH-dG in Barrett's oesophagus glands from individual biopsy specimens incubated for 10 min in control medium (open bars) or in medium acidified to pH 4 and supplemented with a 0.5 mM BA cocktail (dark bars). Asterisks indicate a significant difference ($p<0.05$).

reactive nitrogen species (http://probes.invitrogen.com/media/pis/mp36008.pdf?id = mp36008), the total level of ROS/reactive nitrogen species may be underestimated.

Our data using fluorescence microscopy and flow cytometry indicated that Seg-1 cells exposed for 10 min to pH 4 and 0.1 mM bile acid cocktail experience mitochondrial oxidative stress (fig 3). A strong signal of MitoSOX Red was detected in the cells treated for 10 min with the combination of bile acid cocktail and acid using fluorescence microscopy (fig 3A). By contrast, a low signal was found in untreated control cells or cells treated with pH 4 alone or with the bile acid cocktail alone. When cells were subjected to flow cytometry, we found a significant increase in fluorescent intensity after treatment with the combination of bile acid cocktail and acid ($p<0.05$, fig 3C). No significant increase in fluorescent intensity was detected when the cells were treated with pH 4 alone or 0.1 mM bile acid cocktail alone (fig 3 B,C). Furthermore, we investigated whether longer exposure to the bile acid cocktail alone or pH 4 alone induces oxidative stress. Despite longer exposure (20 and 30 min) to bile acid cocktail or pH 4, only the combination of bile acid cocktail and acid resulted in mitochondrial oxidative stress as indicated by MitoSOX staining (data not shown). Similar results were observed when we used HE. The cells treated for 10 min with the combination of bile acid cocktail and acid using fluorescence microscopy experienced oxidative stress, as indicated by a strong HE signal (data not shown), whereas untreated control cells or cells treated with pH 4 alone or with the bile acid cocktail alone exhibited low HE staining.

In addition, we evaluated oxidative DNA damage after exposure for 10 min to pH 4 and/or 0.1 mM bile acid cocktail in Seg-1 cells using immunohistochemical analysis with 8-OH-dG monoclonal antibody. No signal of 8-OH-dG was detected in the cells treated with bile acid cocktail alone or pH 4 alone. However, a strong signal of 8-OH-dG was primarily seen in the cytoplasm of Seg-1 cells treated with the combination of bile acid cocktail and acid, suggesting that this treatment induces oxidative DNA damage mostly in mitochondrial DNA (fig 4).

We also evaluated whether the bile acids and/or low pH could induce apoptosis in Seg-1 cells. After 24 h of treatment, small, but significant, differences in the percentage of apoptotic cells were noted after treatment with pH 4 alone and the combination of acid and bile acid cocktail ($p<0.05$, fig 5). In the control cells, we found 1.5% (0.5%) of apoptotic cells; in the cells treated with pH 4 alone, we found 3.8% (1.2%) of apoptotic cells; in the cells treated with 0.1 mM bile acid cocktail, we found 2.3% (0.8%) of apoptotic cells; and in the cells treated with the combination of acid and bile acid cocktail, we found 6.2% (1.0%) of apoptotic cells. As a positive control, we included the cells treated for 3 h with 0.5 mM DCA; this treatment induced apoptosis in 27.4% (2.5%) of the cells.

To test whether these findings were unique to the Seg-1 cell line or representative of findings in other oesophageal lines, we used immortalised Barrett's cell lines and normal squamous oesophageal HET-1A cells. However, since these cells are more sensitive to stresses than Seg-1 cells, shorter exposures were used in the assays for Barrett's oesophagus CP-D cells and HET-1A cells to avoid cell detachment and loss of viability. Figure 6 shows the images of Barrett's oesophageal CP-D cells and HET-1A cells treated for 5 and 1.5 min, respectively, with 0.1 mM bile acid cocktail and/or pH 4 and stained with MitoSOX. The findings in Barrett's oesophagus cells are consistent with those of Seg-1 cells (fig 6A). In HET-1A cells, the MitoSOX Red signal was detected after treatment with the combination of bile acid cocktail and acid, and also with acid alone (fig 6B).

MMP after treatment with pH 4 and/or bile acid cocktail

We observed no significant decrease in CMXRos staining in the cells treated for 10 min with the combination of bile acid cocktail and acid compared with control cells (fig 7). As a positive control, the cells were treated with the sodium salt of DCA for 3 h, which resulted in a marked decrease in staining, with CMXRos as an indicator of MMP ($p<0.01$, fig 7A,B).

DISCUSSION

The major aim of this study was to determine whether short exposure to low pH and/or bile acids induces oxidative stress. Immunohistochemical studies revealed that Barrett's oesophagus epithelial cells, especially dysplastic cells, frequently form 8-OH-dG, a marker of oxidative DNA damage (fig 1). Furthermore, Barrett's oesophagus tissues, stressed ex vivo with a combination of bile acid cocktail and acid, show evidence of increased oxidative DNA damage (fig 2). Our studies using oesophageal cell lines indicate that exposure to a combination of bile acid cocktail and acid also induces oxidative stress (figs 3–5).

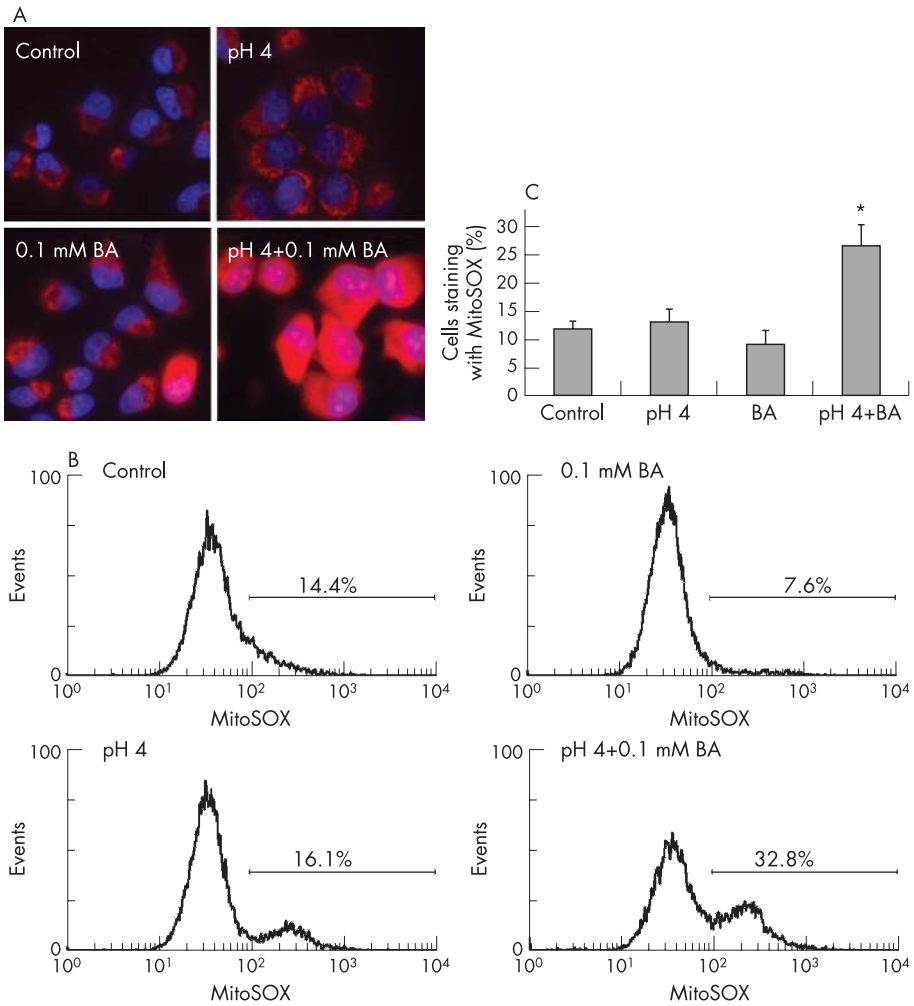

Figure 3 Detection of mitochondrial superoxide in Seg-1 cells after treatment with pH 4 and/or bile acid (BA) cocktail by fluorescence microscopy and flow cytometry. Seg-1 cells were treated for 10 min with control medium (pH 7.4), medium containing 0.1 mM BA cocktail, medium acidified to pH 4 and medium acidified to pH 4 and containing BA cocktail. The cells were then washed and stained with 5 μM MitoSOX Red to measure mitochondrial superoxide (red signal). For fluorescence microscopy, cells were also stained with 10 μM Hoechst 3222 to visualise the nuclei (blue signal, A). The data from flow cytometry are shown in panels (B) and (C). The graphs show the percentage of cells staining positively with MitoSOX Red measured in triplicate by flow cytometry (B). Histograms indicate the percentage of cells that converted MitoSOX to the fluorescent dye (C). Error bars indicate SEM. *$p<0.05$.

During a reflux episode, the normal squamous epithelium in the distal oesophagus is usually exposed to gastric acid and bile acids. An analysis of the oesophageal aspirates of patients with GORD showed that bile acids, primarily glycine conjugates, are present in 86% of patients.[8] The refluxate of patients with GORD consists of glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid and a mixture of taurine-conjugated bile acids.[8] DCA is also present in the refluxate of patients with Barrett's oesophagus taking proton pump inhibitors.[10] However, clinical studies using oesophageal aspirates have shown inconsistent results regarding the concentrations of individual bile acids.[5 32] Therefore, we used equimolar concentrations of bile acids that are ordinarily present in the refluxate. The bile acid cocktail used in this study included sodium salts of glycocholic, taurocholic, glycodeoxycholic, glycochenodeoxycholic and DCA. This cocktail reflects the mixture of bile acids to which the distal oesophagus is exposed during gastroduodenal reflux.[5 8 10] Bile acids conjugated with glycine are toxic at pH 4 since their pKa is ~4. At this pH, glycine-conjugated bile acids are partly non-ionised and may damage oesophageal cells. By contrast, at lower pH they precipitate, and at higher pH they are ionised and thus unable to penetrate through the cell membrane. Total bile acid concentrations in the refluxate of patients with Barrett's oesophagus is ordinarily in the range 0.03–0.82 mM,[5] although bile acid concentrations as high as 7.6 mM have been reported in the refluxate of some patients with Barrett's oesophagus.[7] Thus, the cocktail used in our studies had a concentration in the lower physiological range (0.1 mM for treatment of cultured cells) and in the higher physiological range (0.5 mM for treatment of human biopsy specimens).

Oxidative stress has been implicated in the development of Barrett's oesophagus.[33] Decreased antioxidant capacity and increased oxidative stress have been shown in the GORD–Barrett's oesophagus–ADCA sequence.[34] One postulated mechanism is that an inflammatory response in the oesophagus, mainly involving neutrophils, is a major source of ROS.[33] Animal studies using a surgical model of Barrett's oesophagus and oesophageal adenocarcinoma that induces duodenogastro-oesophageal reflux also indicate the importance of oxidative stress in the pathogenesis of Barrett's oesophagus.[35 36] However, studies evaluating the direct effects of refluxate components (ie, low pH and bile acids) on the production of ROS in human oesophageal cells and human biopsy specimens have been lacking. We speculated that bile acids in combination with low pH might induce excessive oxidative stress, leading to DNA damage, DNA mutations and ultimately cancer.

We have shown in the present study that the combination of acidic pH and bile acids induces oxidative stress in biopsy specimens of Barrett's oesophagus and three different oesophageal cell lines. An increased nuclear staining of 8-OH-dG, a marker of oxidative DNA damage, was detected in epithelial cells of biopsy specimens, compared with that seen in control tissues—that is, oesophageal squamous epithelium and duodenum,

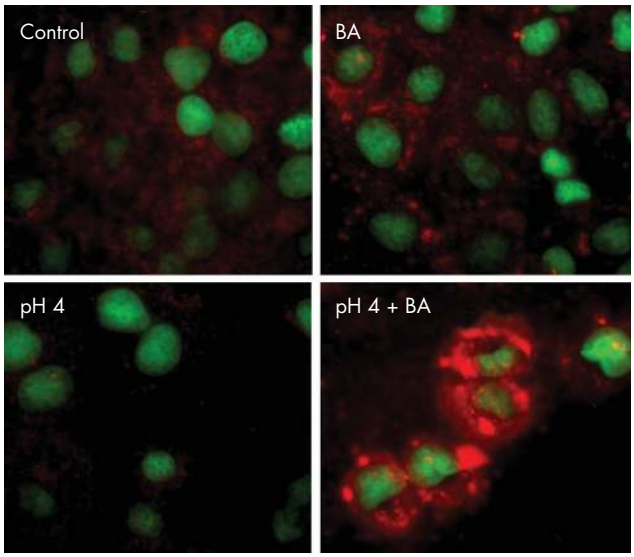

Figure 4 Immunohistochemical staining pattern for 8-hydroxy-deoxyguanosine (8-OH-dG) in Seg-1 cells after treatment with pH 4 and/or bile acid (BA) cocktail by fluorescence microscopy. Seg-1 cells were treated for 10 min with control medium (pH 7.4), medium containing a 0.1 mM BA cocktail, medium acidified to pH 4, and medium acidified to pH 4 and containing BA cocktail. The cells were then incubated for 170 min in normal medium, fixed and immunostained with 8-OH-dG monoclonal antibody to measure oxidative stress (red signal) and with YOYO-1 to visualise the nuclei (green signal).

indicating that Barrett's oesophagus tissue is exposed to ROS. ND Barrett's oesophagus tissue expressed low levels of 8-OH-dG, whereas dysplastic tissue had significantly greater 8-OH-dG staining levels. Furthermore, our ex vivo experiments indicate that biopsy specimens exposed only for 10 min to the combination of 0.5 mM bile acid cocktail and acid show increased oxidative DNA damage compared with biopsy specimens exposed to control medium only. Increased production of mitochondrial ROS was also detected in three oesophageal cell lines, normal squamous epithelial HET-1A cells, Barrett's oesophagus-derived cells and adenocarcinoma Seg-1 cells.

There is substantial evidence that the combination of acid and bile acids (ie, mixed refluxate) is more injurious to the oesophageal epithelium than either acid alone or bile acids alone.[5 9 37–39] This synergism may be caused by the activation of complementary pathways that impinge on mitochondria, inducing the generation of intramitochondrial ROS. Acidic pH conditions activate plasma membrane-associated acidic sphingomyelinases,[40] resulting in the generation of ceramide that can

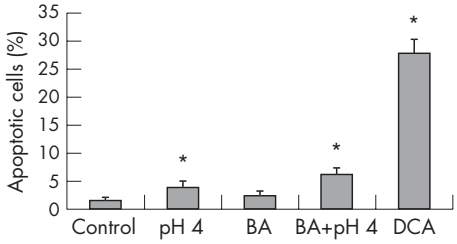

Figure 5 Apoptosis in Seg-1 cells after treatment with pH 4 and/or bile acid (BA) cocktail. Seg-1 cells were treated for 10 min with control medium (pH 7.4), medium containing a 0.1 mM BA cocktail, medium acidified to pH 4, and medium acidified to pH 4 and containing BA cocktail. After treatment, the cells were washed and incubated for 24 h in normal medium. As a positive control, Seg-1 cells treated with 0.5 mM deoxycholic acid (DCA) for 3 h were included. The percentage of apoptotic cells was determined by morphological criteria after staining with Giemsa. *$p<0.05$.

target mitochondria. Bile acids perturb the plasma membrane, thereby activating phospholipase A_2, resulting in the release of arachidonic acid that can also target mitochondria.[41] Indeed, previous studies have shown that mutations in mitochondrial DNA frequently occur in dysplastic Barrett's oesophagus and oesophageal adenocarcinoma, probably as a result of oxidative damage.[42] Another mechanism by which acid and bile acids can synergise to induce oxidative stress is by the activation of NADPH oxidase, the former through endosomal acidification[43] and the latter probably through membrane perturbation.[44] In addition, acidic pH might amplify bile acid-generated ROS through iron-mediated Fenton reactions.[45] Another potential mechanism of synergism between acid and bile acids is intracellular acidification induced by acid[46–48] and the amplification of ROS-generating enzymes activities by bile acids. The mechanisms proposed above by which acid and bile acids might synergise to induce ROS and consequent DNA damage need to be tested in oesophageal epithelial cells in future experiments.

An important part of cellular defence against ROS is the induction of specific genes in response to oxidative stress to permit the cells to adapt to this stress. Oxidative stress is associated with activation of signal transduction pathways, including the nuclear factor-κB, Janus kinase-signal transducers and activators of transcription and mitogen-activated protein kinase pathways that can upregulate expression of antiapoptotic, prosurvival and angiogenic proteins.[49 50] Activation of these pathways and expression of antiapoptotic proteins was shown by us and by others to be increased in Barrett's oesophagus.[18 19 51] In agreement with these reports, low levels of pro-apoptotic markers were found in Barrett's oesophagus.[52] We speculate that oxidative stress induced by low pH and bile acids may result in the activation of pro-survival and anti-apoptotic pathways in

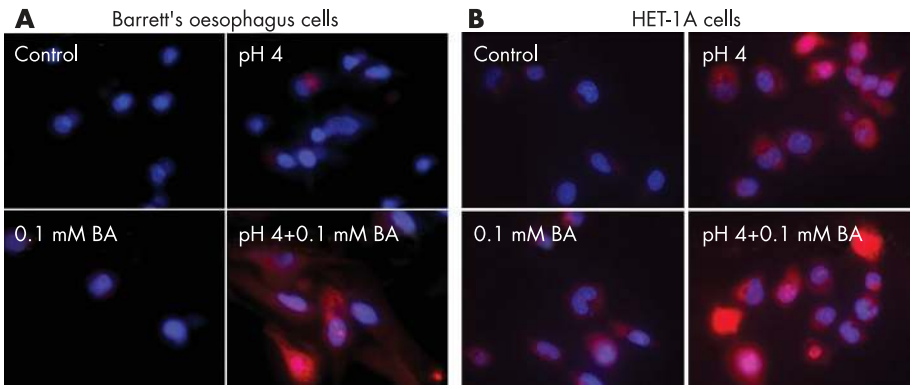

Figure 6 Detection of mitochondrial superoxide in Barrett's oesophagus cells (CP-D cells) and HET-1A cells after treatment with pH 4 and/or bile acid (BA) cocktail by fluorescence microscopy. Cells were treated for 5 or 1.5 min, respectively, with control medium (pH 7.4), medium containing a 0.1 mM BA cocktail, medium acidified to pH 4, and medium acidified to pH 4 and containing BA cocktail. The cells were then washed and stained with 5 μM MitoSOX Red to measure mitochondrial superoxide (red signal). Cells were also stained with 10 μM Hoechst 3222 to visualise the nuclei (blue signal). (A) Barrett's oesophagus CP-D cells; (B) HET-1A cells. DCA, deoxycholic acid.

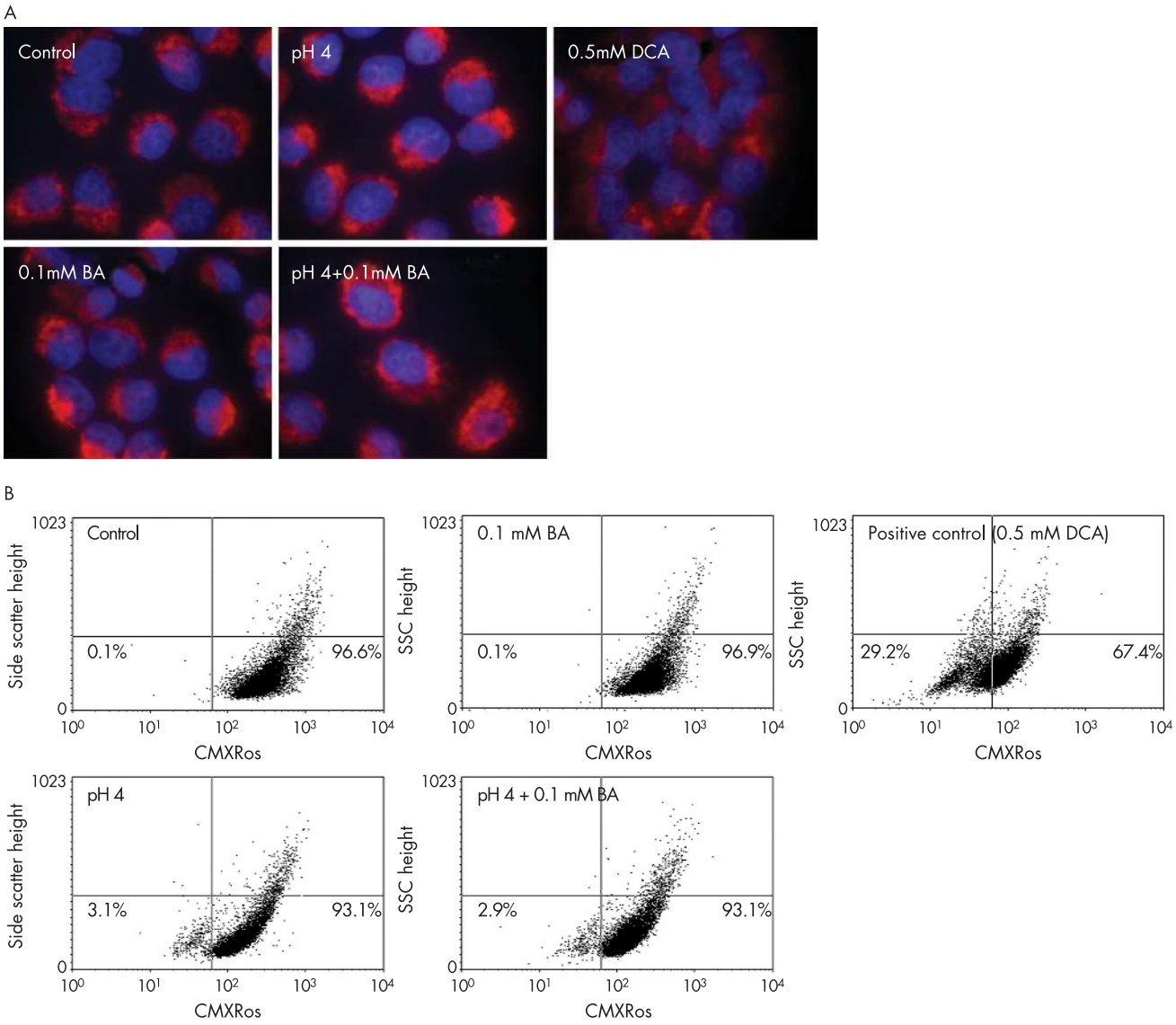

Figure 7 Detection of mitochondrial membrane potential (MMP) using MitoTracker Red (Chloromethyl-X-Rosamine (CMXRos)) in Seg-1 cells after treatment with pH 4 and/or bile acids (BA) by confocal microscopy and flow cytometry. Cells were treated for 10 min with control medium (pH 7.4), medium acidified to pH 4, medium containing a 0.1 mM BA cocktail, or medium acidified to pH 4 and containing BA cocktail. The cells were then stained with CMXRos to measure MMP by fluorescence microscopy (A) or flow cytometry (B). A red signal represents CMXRos and a blue signal represents 4′-6-diamidino-2-phenylindole used to detect nuclei. As a positive control, 0.5 mM deoxycholic acid (DCA) treatment for 3 h was used.

Barrett's oesophagus. DNA damage in apoptosis-resistant cells is a dangerous situation and can lead to mutations caused by replication errors, followed by clonal expansion of mutated cells and neoplastic progression. Indeed, common chromosomal alterations in Barrett's oesophagus lesions and adjacent cancer tissue suggest a process of clonal expansion.[53 54]

In summary, our study is the first to show that bile acids in combination with low pH induce oxidative DNA damage and mitochondrial oxidative stress in oesophageal cells. We speculate that chronic exposure to bile acids and low pH may result in increased genomic instability, abnormal cell signalling and resistance to apoptosis. These alterations probably have a role in the progression to cancer.

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