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## Deoxycholic acid at neutral and acid pH, is genotoxic to oesophageal cells through the induction of ROS: the potential role of anti-oxidants in Barrett's oesophagus

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**Bile acids are often refluxed into the lower oesophagus and are candidate carcinogens in the development of oesophageal adenocarcinoma. We show here that the secondary bile acid, deoxycholic acid (DCA), is the only one of the commonly refluxed bile acids tested here, to show genotoxicity, in terms of chromosome damage and mutation induction in the human p53 gene. This genotoxicity was apparent at both neutral and acidic pH, whilst there was a considerable increase in bile-induced toxicity at acidic pH. The higher levels of cell death and low cell survival rates at acidic pH may imply that acid bile exposure is toxic rather than carcinogenic, as dead cells do not seed cancer development. We also show that DCA (at neutral and acid pH) induced the release of reactive oxygen species (ROS) within the cytoplasm of exposed cells. We further demonstrate that the genotoxicity of DCA is ROS mediated, as micronucleus induction was significantly reduced when cells were treated with DCA + the anti-oxidant vitamin C. In conclusion, we show that DCA, is an effective genotoxin at both neutral and acidic pH. As bile acids like DCA can induce DNA damage at neutral pH, suppressing the acidity of the refluxate will not completely remove its carcinogenic potential. The genotoxicity of DCA is however, ROS dependent, hence anti-oxidant supplementation, in addition to acid suppression may block DCA driven carcinogenesis in Barrett's patients.**

### Introduction

Bile acids play an important role in the digestion and absorption of fats in the small intestine. However, it has been suggested for some time that bile acids are also potential human carcinogens (1,2). However, the mechanism (molecular targets etc.) behind bile acid-induced carcinogenicity is not entirely known. Bile acids are thought to play a major role in oesophageal cancer development in particular (3), with bile acid exposure occurring during episodes of duodeno-gastro-oesophageal reflux. Patients who suffer from chronic reflux often develop a pre-malignant condition known as Barrett's oesophagus and subsequently oesophageal adenocarcinoma (4). As the refluxate is known to be a major

cause of oesophageal adenocarcinoma development, one or more of its constituents must be carcinogenic. As patients who take acid suppression therapy still proceed to adenocarcinoma (5,6), one of the non-acid constituents of reflux may well be involved. Hence our interest in bile acids as potential carcinogens in oesophageal adenocarcinoma. It is well known that Barrett's patients with more severe symptoms (stricture, dysplasia etc) undergo reflux containing relatively higher concentrations of bile acids (3). Furthermore, these complicated patients have refluxates with higher levels of the more damaging secondary bile acids like deoxycholic acid (DCA) (7). It is important to remember however, that bile acid reflux into the oesophagus occurs in an acidic background in most cases. Hence, bile acid effects need to be considered in a background of acidity.

In terms of the mechanism of bile acid-induced carcinogenesis, bile acids are known to be extremely toxic agents at high doses, presumably through damaging cell membranes (8), mitochondrial membranes (9,10) or interfering with cellular function (11). This cytotoxicity/membrane disruption may be involved in stimulating proliferation and hence contributing to cancer (12). At lower doses, bile acids are known to stimulate cell signalling effects involving, protein kinase C (13), c-myc (14), COX-2 (15) and NF-kappaB (16). Obviously, such effects might also contribute to bile acid driven carcinogenicity.

In terms of bile acid-induced genotoxicity (DNA damage), there is inconsistency in the literature. Most genotoxicity studies on bile acids have been performed in hepatocytes and colorectal cell lines, studies in oesophageal cells are extremely rare (17). The available studies have shown that bile acids [taken from familial adenomatous polyposis (FAP) patients] can cause DNA damage (18,19), whilst synthetic bile acids can induce chromosome damage (20) and single strand breaks (21–23). Conversely, other reports have suggested that bile acids may not be genotoxic (24–28) and merely act as tumour promoters (reviewed in ref. 29). It has been speculated that bile acids may cause the release of reactive oxygen species (ROS) in hepatocytes (30,31) and hence may be genotoxic via oxidative DNA damage induction (28). This may well be linked to mitochondrial membrane damage (10) and the release of ROS into the cytosol. This hypothesis would fit with reports that bile acids cannot directly damage DNA, but require key cell-based intermediates (32,33). This involvement of ROS would also fit well with observations that tissues from patients with oesophagitis and Barrett's oesophagus show elevated levels of ROS (34) and that Barrett's tissues bear p53 mutation types indicative of ROS exposure (35).

In this study we aimed to assess the genotoxicity of several bile acids at neutral and acidic pH, as well as investigating whether ROS induction was responsible for bile acid-induced genotoxicity in oesophageal cells. We have mainly used the micronucleus (MN) assay (36) to monitor overall

**Abbreviations:** DCA, deoxycholic acid; MN, micronucleus; ROS, reactive oxygen species; RSM, restriction site mutation.

genotoxicity. This assay examines the appearance of small DNA containing bodies (micronuclei) in the cytoplasm of cells after they have divided. The micronuclei can either be the result of chromosome fragmentation or due to the loss of whole chromosomes during mitosis. Kinetochore staining (which stains the centromeres of whole chromosomes) of the micronuclei can distinguish between these eventualities. Micronuclei are counted in recently divided cells where cytokinesis has been blocked by the addition of cytochalasin B. The appearance of these 'binucleated cells' (with and without micronuclei present) can also be used as a measure of cell division and hence of the toxicity of the bile acid treatment. In order to assess the potential genotoxicity of bile acids contained in duodeno-gastro-oesophageal reflux, we have used types of bile acids and concentrations of bile acids shown to be present in the oesophagus during reflux episodes (7).

We have specifically investigated the role of ROS induction as a mechanism for bile acid-induced genotoxicity. There has been some evidence in hepatocytes and colorectal cells that bile acids are ROS inducers, we test that possibility here in oesophageal cells. We have predominantly used OE33 cells, derived from an oesophageal adenocarcinoma to test the genotoxicity of bile acids, but have also confirmed some results with KYSE-30 cells (derived from a squamous cell carcinoma). Specifically, we have used two complementary methods to assess first, if bile acids can induce ROS in oesophageal cells and, second, to see if anti-oxidants can prevent this ROS release. Finally, to conclusively prove if ROS induction is responsible for bile acid driven genotoxicity, we have used the MN assay to determine if vitamin C supplementation can block the genotoxicity of a genotoxic bile acid.

## Materials and methods

### Cell culture

OE33 cells (derived from an oesophageal adenocarcinoma) were obtained from the European Collection of Cell Cultures (Salisbury, UK) and cultured in 25 or 80 cm<sup>2</sup> flasks with RPMI 1640 media (Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum [FCS; Gibco BRL (Invitrogen) Paisley, UK], 100 U/ml penicillin, 100 µg/ml Streptomycin and 1 mmol/l Glutamine (Gibco BRL). KYSE-30 cells (derived from a squamous cell carcinoma) also purchased from the ECCC, were incubated in 50% RPMI, 50% HAMS (Life Technologies), 1% Glutamine and 2% FCS. The cells were grown in 5% CO<sub>2</sub>, 95% air at 37°C and were subcultured through trypsin mediated detachment every 2–3 days.

### Measurement of DCA-induced ROS

Both OE33 and KYSE-30 cells were grown on sterile microscope slides for 2–3 days prior to ROS measurement. The slides were exposed to bile acid (DCA) at 100 µM in fresh pre-warmed culture medium within sterile petri dishes. After a 1 h incubation with DCA (pH 7.4 and 6), the ROS levels in the cells was assessed using the Image-iT Live Green ROS detection kit (Molecular Probes-Invitrogen, Paisley, Scotland). The reduced fluorescein dyes are sensitive to a range of different ROS types (OH radical, peroxy nitrates, NO etc). This kit uses Carboxy-H<sub>2</sub>DCFDA, a reliable fluorescent detection reagent for ROS generation (37), this reagent is non-fluorescent in its native state but becomes fluorescent upon exposure to ROS. The conversion of H<sub>2</sub>DCFDA to fluorescent carboxy-DCF in the cell involves initial de-acetylation by intracellular esterases and then oxidation by intracellular ROS. The ROS generated within the cells (untreated and DCA treated at pH 7.4 and pH 6) were visualised using a Zeiss LSM 510 META confocal microscope. The microscope software allowed the fluorescent intensity of the various treatments to be measured. Five separate fields of view at random were quantified per slide to obtain average ROS levels in each group. In addition quantified ROS levels were also obtained from untreated, DCA treated and DCA+vitamin C co-treatments in duplicate sets

of slides, established independently. Thus 10 separate ROS levels were obtained for each treatment group. The *t*-test was used to assess the significance of increases/decreases in ROS levels.

ROS induction in bile treated cells was also assessed by quantifying the levels of carbonyl groups in the cellular proteins. Carbonyl groups are formed during protein oxidation by ROS, these irreversible protein adducts are readily detectable biomarkers of intracellular ROS. As KYSE-30 cells and OE33 cells behaved similarly with H<sub>2</sub>DCFDA, only OE33 cells were used in the carbonyl study. Briefly, OE33 cells were cultured in 25 cm<sup>2</sup> flasks and were either untreated or treated with acid (pH 6), neutral pH DCA (100 µM) or acidic DCA (pH 6, 100 µM) for 1 h at 37°C. Cellular proteins were then extracted from the cells using the following method. The cells were scraped off the tissue culture flask in 500 µl of lysis buffer (250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 20 mM DTT and 0.01% bromophenol blue). The proteins were then extracted by boiling the solution at 100°C for 5 min. The protein extract was centrifuged at 13 000 r.p.m. for 5 min to pellet the debris, the protein containing supernatant was then quantified using a 2D Quant kit (Amersham, Bucks, UK), adjusted to a concentration of 2 µg/µl and aliquoted at –80°C. Protein aliquots (10 µg) were then assessed for carbonyl groups using the OxyBlot Protein oxidation kit (Chemicon, Hamps, UK). After derivitization of the carbonyl groups to 2,4-dinitrophenylhydrazine with dinitrophenylhydrazine for 15 min, the proteins were spotted onto nitrocellulose membranes (TransBlot, Bio-Rad, Hemel Hempstead) in 1 µl additions and allowed to dry. A positive control (DNP containing proteins supplied by manufacturer) was always included. A set of control reactions was also initially set up without the derivitization to ensure specificity of the antibody (data not shown). The derivitized proteins were subsequently detected by dot blotting using an anti-DNP antibody, as detailed by the manufacturers. The oxidised proteins were visualised by incubation with ECL reagent for 1 min (Amersham). The membranes were then exposed to hyper-film (Amersham) for 5–10 min to visualise the spots and each spot on the membrane was also quantified using a chemiluminescent image analyser (Chemi-Doc system, Bio-Rad). Blots were carried out in triplicate and average spot intensities were obtained for the different treatment groups. The *t*-test was used to assess significant increases in oxidised proteins in the different groups.

### MN assay

For the MN assay, 1–2 × 10<sup>5</sup> OE33 cells per ml were seeded into 25 cm<sup>2</sup> flasks, 24 h prior to treatment. Only OE33 cells were used as the KYSE-30 cells were less amenable to MN assessment (elongated cells, more difficult to score). The OE33 cells were then treated with the bile acids most commonly found in refluxate (7) i.e. Glycocholic acid (GCA), Taurocholic acid (TCA), Glycodeoxycholic acid (GDCA), Taurodeoxycholic acid (TDCA), Cholic acid (CA) and DCA (SigmaAldrich, Poole, UK) at varying doses (50 µM–1 mM). Mitomycin C (10 µM) was also used as a positive control as it is a well established chromosome damager. Concurrent with the exposure to the bile acids, Cytochalasin B (4 µg/ml) was added and the cells were harvested by trypsin mediated detachment 24 h later (i.e. 48 h after cultures were seeded). In order to obtain the acidic conditions for the bile treatments at pH 5.5, 1 M HCL was added to the RPMI medium and aliquots of the media were tested for pH using a pH meter, until the desired pH was obtained. We deliberately used mild acid conditions (pH 5.5 and 6) as DCA exposures at lower pH's show excessive toxicity. In the case of the vitamin C supplementation, equimolar amounts of vitamin C (200 µM, Sigma) were added 2 h prior to the DCA, and the media changed prior to the addition of DCA to prevent chemical interaction in the media.

Slides were prepared from 100–200 µl of the cell pellets using a Cytospin at 1200 r.p.m. for 8 min. The slides were checked using a light microscope and air dried before being fixed with 90% methanol at –20°C for 10 min (up to 10 slides prepared for each dose). The slides were subsequently stained with 10% Giemsa (BDH, Leicestershire, UK) for 8 min in phosphate buffer at pH 6.8 after being immersed in xylene and allowed to dry. After drying and mounting of the cover slips, the slides were visualised with an Olympus BH2 microscope at ×1000 magnification. For kinetochore labelling, the slides were prepared as above, but a previously developed (38) immunofluorescent labelling step was employed. In short, an anti-kinetochore antibody (Quadrach, Epsom, UK) was used to identify which micronuclei were kinetochore positive (K<sup>+</sup>) or kinetochore negative (K<sup>–</sup>). One hundred micronuclei were scored for kinetochore status. Kinetochore positive cells were assumed to contain whole chromosomes, whereas kinetochore negative cells were assumed to contain chromosome fragments. Any increase in K<sup>+</sup> frequency in the treated cells was taken to suggest induction of aneuploidy.

All slides were scored blind and where possible, 1000 binucleated cells were counted per dose and assigned as MN positive or negative. Standard criteria for selecting micronuclei were used (39). To assess statistical significance between untreated cells and cells treated with varying doses of

**Table I.** PCR primers, restriction enzymes used in RSM

P53 Exon	Restriction enzymes	Digestion temperature (°C)	Forward primer	Reverse primer	PCR size (bp)	Anneal temperature (°C)	Cycle number
Exon 5 Codon 175	HaeII GCGC	37	gtgcagctgtgggtgattc	ctgagcagcgctcatggt	124	60	31
Exon 6 Codon 213	TaqI TCGA	65	gtcccaggcctctgattcctc	taaccctctctcccagagaccaccag	188	65	34
Exon 7 Codon 248, Codon 249	MspI CCGG HaeIII GGCC	37 37	tggctctgactgta ccac	agtggtgcagggtgg caag	137	60	31
Exon 8 Codon 282	MspI CCGG	37	cctcttgettcttttctatcc	cttggctctcccaccgcttcttg	262	60	31

bile acids, a two tailed Fishers exact test was employed. Duplicate cell cultures were established for the MN study. The slides obtained from the duplicate cultures were scored together and the total number of micronuclei in each exposure group (as a % of the binucleate frequency) was noted.

#### Cell viability assay

OE33 cells ( $10^5$  cells/ml) were cultured in 6 well culture plates at pH 7.4 and pH 6 and exposed to DCA at various doses (0–400  $\mu$ M) in triplicate for 24 h. After 24 h of DCA exposure, the cells were assessed for survival and viability by using the MTS cell proliferation assay (Promega, Southampton, UK). After incubation with the MTS solution (containing the tetrazolium compound), the formation of the formazan product was assessed by spectrophotometry at 490 nm. The levels of this formazan product are proportional to the levels of de-hydrogenase enzyme in the cells and hence, to the number of living cells. The average absorbance readings were then plotted against dose, as a measure of DCA-induced toxicity.

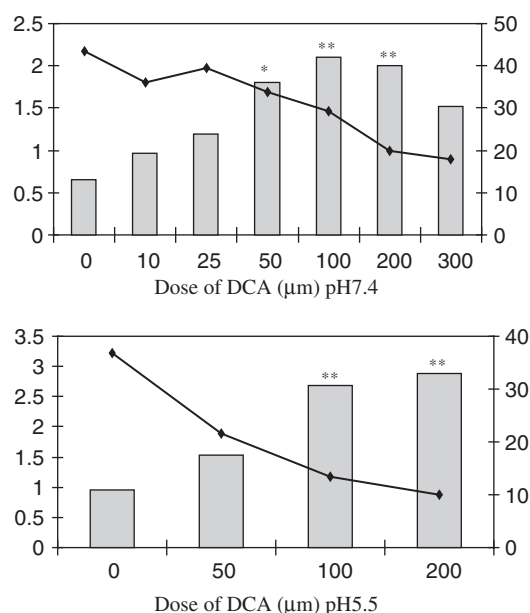
#### Restriction site mutation (RSM) analysis of DCA-induced mutations

We sought p53 mutations in KYSE-30 and OE33 cells treated with DCA. Both these cell lines have been reported to carry p53 mutations, although the reported mutations (splice site of intron 6 for KYSE-30 cells and codon 135 for OE33 cells) are not at the same codons analysed here. Hence there is no interference of existing mutations in this study. In order to assess if DCA was a mutagen, both OE33 cells and KYSE-30 cells were exposed to 100 and 200  $\mu$ M DCA (at pH 7.4 and 6) for 24 h, followed by a 48 h recovery period in fresh media, for the mutations to become fixed. DNA was extracted from the washed and pelleted cells using a high salt kit (Stratagene, Cambridge, UK). The DNA was checked for quantity and quality by spectrophotometry at 260/280 nm. The RSM method was used to analyse six restriction enzyme sites of the human p53 gene for KYSE-30 cells (Table I contains details of the restriction enzyme sites, gene locations and the PCR primers used to amplify the mutated PCR products). In the case of OE33 cells, only codon 247/248 and 249/250 were analysed as it was here that most of the KYSE-30 mutations were found. RSM analysis was performed as previously described (35,40). As with the MN assay, the acidified bile was obtained by acidifying the media using HCL. As this acidified bile was highly toxic, various doses (50–100  $\mu$ M) and various exposure times (4–24 h) were used. Mutated RSM products were sequenced using a CEQ2000 automated DNA sequencer (Beckman Coulter, High Wycombe, UK). Both strands of the PCR products were sequenced and mutations were only accepted if present on both strands.

## Results

### The secondary bile acid DCA, at neutral pH induces micronuclei in oesophageal cells

We exposed OE33 cells to a range of concentrations (up to 1 mM) of six different bile acids and determined the corresponding MN frequencies. The conjugated bile acids TCA, GCA, TDCA, GDCA and the primary bile acid CA, did not induce any significant increases in MN at these doses, only high levels of toxicity at the higher doses, as measured by the drop in binucleate frequency. The secondary bile acid DCA, was the only bile acid tested that showed a significant increase in the number of micronuclei ( $P = 0.02$ , 0.006 and 0.009 for 50, 100 and 200  $\mu$ M, respectively) (Figure 1). In comparative terms the 3-fold increase in DCA-induced micronuclei at 50  $\mu$ M, is slightly less than that of mitomycin C, a common positive control in MN experiments, which induces 3-fold increases in micronuclei at 10  $\mu$ M (mitomycin

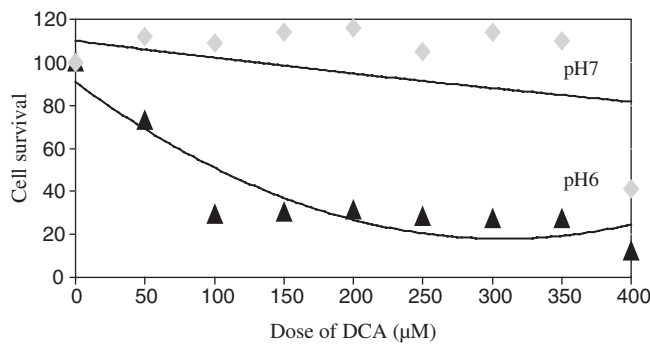


**Fig. 1.** The MN frequency (% micronucleated binucleated cells) is plotted against dose of DCA (columns, left hand scale). The binucleate frequency (% binucleated cells) is also plotted as a measure of toxicity (line graph, right hand scale). Upper graph shows neutral pH bile, lower graph shows acidic pH (5.5) bile. \* $P < 0.05$ , \*\* $P < 0.01$ .

C data not shown). Importantly, the increase in micronuclei was observed at physiological doses (50–100  $\mu$ M) and at low levels of toxicity as measured by the binucleate frequency. Hence, these micronuclei were not primarily induced by toxicity-related mechanisms. Furthermore, kinetochore staining was performed on these DCA-induced micronuclei to assess if fragments or whole chromosomes were present in the micronuclei. The kinetochore data showed that the majority (60–70%) of DCA-induced micronuclei were  $K^-$  (hence mostly chromosome fragmentation or ‘clastogenicity’). The proportion of  $K^+$  MN did not increase with DCA treatment, hence no aneugenic effect was observed.

### Acidic DCA (pH 5.5) induced equivalent numbers of micronuclei, but more toxicity, than neutral DCA

The conjugated bile acids (TCA, GCA, TDCA and GDCA) and the primary bile acid (CA) did not induce significant levels of micronuclei at acid pH, merely cytotoxicity (results not shown). However, the treatment of OE33 cells with DCA at acidic pH (5.5) resulted not only in increased toxicity, but also resulted in a significant increase in MN frequency ( $P = 0.006$  and 0.002 for 100 and 200  $\mu$ M DCA, respectively). These micronuclei were  $K^-$ , hence a similar clastogenic effect to that of neutral pH DCA. There was no significant difference in DCA’s genotoxicity at acidic pH compared to



**Fig. 2.** MTS data for cell survival after exposure to acidic and neutral pH DCA. Neutral pH DCA (diamonds) shows little reduction in survival up to 400  $\mu\text{M}$ . Conversely, acidic DCA (triangles) show substantial loss of survival at doses  $>50$   $\mu\text{M}$ .

neutral pH ( $P = 0.47$  and  $0.31$  at 100 and 200  $\mu\text{M}$  DCA, respectively). Although acid DCA induced slightly more MN than neutral pH DCA, the appearance of these MN against a background of marked toxicity (low binucleate frequencies) should be borne in mind. Hence it is possible that some of these MN were the by-product of the high levels of background toxicity. Comparing the two untreated controls in Figure 1, we can see a small increase in MN frequency at acid pH compared to neutral pH (without DCA), from 0.65 to 0.96%. This increase in MN frequency as a result of acid exposure alone (pH 5.5) was not significant ( $P = 0.6$ ).

#### Acidic DCA induces enhanced cytotoxicity in OE33 cells

As the binucleate frequency is only a measure of early toxicity (i.e. a block to cell division), we wished to assess the relative effect of acidic and neutral DCA on OE33 cell survival using the MTS assay that measures mitochondrial function and is a more general indicator of toxic effects. In order to further examine the effect of acidity on DCA-induced cytotoxicity, we treated OE33 cells for 24 h with DCA (100–400  $\mu\text{M}$ ) at pH 7.4 and 6. Subsequent to this treatment, we examined the cells by microscopy and evaluated their viability using the MTS assay. Figure 2 exemplifies this toxicity and shows the striking enhancement of DCA-induced toxicity at acidic pH compared with neutral pH.

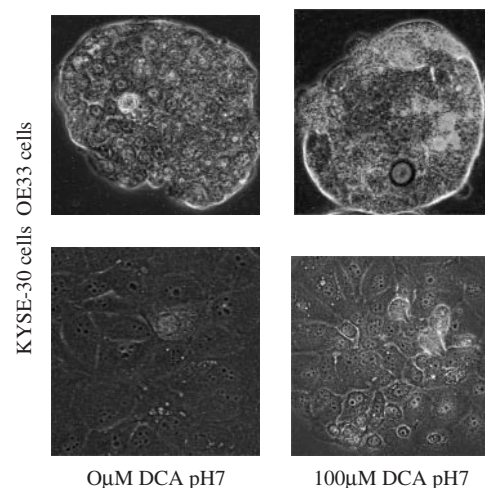
#### DCA-induced mutations in KYSE-30, but not OE33 cells

The RSM method was then used to interrogate the DNA taken from DCA treated KYSE-30 and OE33 cells for the presence of p53 mutations. Only DCA was assessed for mutagenicity, as it was the only bile acid tested for MN induction that exhibited a genotoxic effect. Twelve resistant PCR products were recovered from KYSE-30 treated cells, no mutations were observed in OE33 cells (Table II). In order to identify the mutation types induced by exposure to DCA, the codon 247/248 mutant PCR products were sequenced. The mutations recovered were exclusively GC to AT mutations at the last base of codon 247 (the first base of the MspI recognition sequence). This mutation does not alter the amino acid sequence (Asn to Asn).

When the KYSE-30 cells and OE33 cells were exposed to acidic bile (50–200  $\mu\text{M}$  for 4–24 h), no mutations were recovered. There were substantial amounts of cytotoxicity noted in these cells, as determined by the numbers of dead cells and the reduced DNA yield. Due to this cytotoxicity,

**Table II.** Mutational analysis of DCA treated KYSE/OE33 cells

Cell	Dose of DCA ( $\mu\text{M}$ )	pH	Number of mutated bands and codon
KYSE	0	7	0
KYSE	100	7	3 at 247/248
KYSE	200	7	9 (3 at 247/248, 2 at 249/250, 4 at 175)
KYSE	0	6	0
KYSE	100	6	0
KYSE	200	6	0
OE33	0	7	0
OE33	100	7	0
OE33	200	7	0
OE33	0	6	0
OE33	100	6	0
OE33	200	6	0

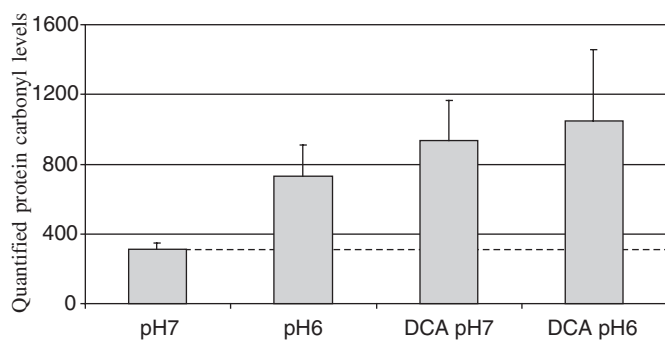


**Fig. 3.** Confocal images of ROS levels in both OE33 and KYSE-30 cells +/- neutral pH DCA. The green fluorescence is produced in the cells by the action of ROS and hence the amount of fluorescence is proportional to the level of ROS. These images show that in both cell lines, neutral pH DCA caused ROS release in the cells. Acidic DCA (pH 6) also caused ROS induction, but acid alone did not (data not shown).

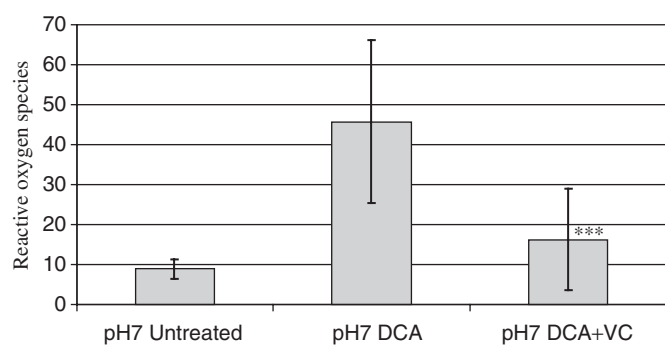
shorter exposures (4 h) and lower doses of DCA (50  $\mu\text{M}$ ) were employed, but it may be possible that these exposure conditions were not sufficient to induce detectable mutations.

#### DCA generates ROS in both OE33 and KYSE-30 cells

It has been suggested that bile acids may interfere with membrane function, and that by specifically interfering with mitochondrial membranes, bile acids could stimulate the release of mitochondrial ROS. We considered that bile-induced ROS could well explain the genotoxicity of bile acids and hence explored the effect of DCA, on ROS generation within OE33 and KYSE-30 cells. In order to assess if DCA-induced ROS could be the source of the DNA damage noted in this study, we measured cellular ROS levels after exposure of cells to DCA for 1 h using two methods. First, confocal microscopy was employed in conjunction with a ROS-sensitive fluorescent dye ( $\text{H}_2\text{DCFDA}$ ). Using this approach, we were able to show that neutral pH DCA and acidic DCA (pH 6), but not acid alone, could induce ROS within both KYSE-30 and OE33 cells (pH 7 data shown in Figure 3). The fact that acid alone did not cause ROS



**Fig. 4.** Protein oxidation levels as measured by dot blots of OE33 proteins using an antibody against derivatized protein carbonyl groups. This graph shows an increase in protein carbonylation (and hence cellular ROS) when cells are exposed to acid, DCA or acidic DCA. Acidic DCA causes most protein oxidation.

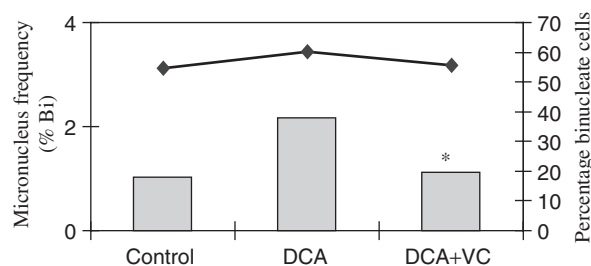


**Fig. 5.** The effect of anti-oxidant supplementation on ROS levels. Quantified ROS levels (fluorescent intensities using the confocal microscope) in cells treated with neutral pH DCA +/- vitamin C (equimolar). Vitamin C significantly reduces the ROS load in DCA treated cells.

induction could be due to the intrinsic chemistry of the fluorescent dye which is optimal at neutral pH. Secondly, we analysed the induction of protein carbonyl groups as a measure of ROS induction in OE33 cells. As shown in Figure 4, acid alone, DCA alone and acidic DCA all increase the levels of ROS as determined by increases in protein carbonyl groups ( $P = 0.002$ ,  $0.0007$  and  $0.0046$ , respectively). Due to the fact that acidic DCA and neutral DCA induced largely similar levels of ROS, it is unlikely that ROS levels contribute to the enhanced cytotoxicity of acidic DCA noted in this study.

#### *Anti-oxidants protect oesophageal cells from DCA-induced DNA damage*

In order to assess if low levels of anti-oxidants could soak up the bile-induced ROS and prevent bile-induced DNA damage, we exposed OE33 cells to neutral pH DCA ( $100 \mu\text{M}$ ) for 1 h. In addition, prior to the DCA exposure, the cells were bathed for 24 h in equimolar amounts of ascorbic acid ( $100 \mu\text{M}$  vitamin C) and then washed to remove the vitamin C. We subsequently measured the induction of ROS using the confocal microscope and a ROS-sensitive fluorescent dye ( $\text{H}_2\text{DCFDA}$ ). Here only neutral pH DCA was assessed to remove the confounding issue of enhanced toxicity when cells are exposed to acidic DCA. The levels of bile-induced ROS detected in cells pre-exposed to vitamin C were shown to be significantly lower ( $P = 0.0008$ ) than in cells exposed to bile alone (Figure 5). Hence, pre-treatment with vitamin C



**Fig. 6.** The effect of vitamin C on DCA-induced micronuclei. Line graph shows binucleate frequency (right hand scale) and the column graph (left hand scale) show MN frequency. OE33 cells were exposed to DCA +/- vitamin C ( $200 \mu\text{M}$  equimolar).

reduced overall levels of DCA-induced ROS. This vitamin C supplementation was in fact, effective whether given 24 h prior to DCA treatment, or given concurrently with DCA (data not shown).

To further investigate if vitamin C could reduce DCA-induced DNA damage and hence strengthen the link between DCA, ROS and DNA damage, we measured MN induction by DCA and by DCA + vitamin C ( $200 \mu\text{M}$ ). Figure 6 shows the result of this study. Pre-incubation (2 h) with equimolar amounts of vitamin C significantly reduced the number of MN induced by neutral pH DCA ( $P = 0.03$ ). Co-incubation of vitamin C and DCA also resulted in reduced MN induction (data not shown). This MN data, confirms that DCA induces DNA damage through an ROS mediated mechanism.

## Discussion

The data presented here shows that physiological doses ( $50$ – $100 \mu\text{M}$ ) of the secondary bile acid DCA, but not the conjugated bile acids GCA, TCA, TDCA, GDCA, nor the primary bile acid CA, are genotoxic to oesophageal cells. In terms of MN induction, this genotoxicity was equally apparent at both neutral and acidic pH, whilst the p53 mutation data showed that only neutral DCA was mutagenic. This implies that DCA's genotoxicity, may favour a neutral pH environment. This supports earlier work examining DCA-induced DNA damage measured by the comet assay (23), but contradicts other earlier work showing that DNA adduct induction by bile favours an acidic pH (18). Our data may have some important implications for future intervention strategies for Barrett's patients (see below). Acid alone caused a non-significant increase in MN frequency, despite earlier reports that acid exposure significantly increases MN frequency (41).

We show here that DCA was a mutagen in KYSE-30 cells but not in OE33 cells. This difference in cell specificity may reflect our observations that KYSE-30 cells were less sensitive to DCA driven toxicity, are generally tougher, and hence may have better survived the DCA exposure. It is also possible that the KYSE-30 cells are more sensitive to mutagenicity than OE33 cells due to some unknown cellular defect (in DNA repair for example). OE33 cells were subject to DCA-induced toxicity (particularly when incubated for 3 days post exposure) and hence mutated cells may have been eliminated from the culture and hence from the DNA extraction process. We show here mutagenicity of DCA, but only at neutral pH. When the KYSE-30 cells were exposed to acidified DCA rather than neutral pH DCA, we

saw an abolition of the mutagenicity seen at neutral pH. This may be a consequence of the enhanced toxicity of acidified DCA, which may have killed the cells that would otherwise harbour DCA-induced mutations. Jolly *et al.* (23) also showed that DCA could induce DNA damage as detected by the comet assay, at neutral pH but not acidic pH. The p53 mutations induced here were synonymous (not changing the amino acid) whereas in oesophageal adenocarcinoma, the p53 mutations seen are non-synonymous [changing the amino acid, usually involving codon 248 (35)]. The reason for this discrepancy between our bile-induced mutations *in vitro* and the mutations seen *in vivo* is probably due to selection. There is very little selection acting in our studies due to the short exposure time (3 days). However *in vivo* there are years (if not decades) of selection acting to expand clones with advantageous p53 mutations (like codon 248 non-synonymous mutations). It is possible that the common codon 248 non-synonymous mutation is induced in our study, but at levels below the detection limit of RSM. Given adequate time, this mutation may expand *in vitro* as it does *in vivo*.

From these results, it is clear that neutral pH DCA is equally genotoxic (MN data) or more genotoxic (p53 mutation data) than acidic DCA. Another interesting feature of our data is that relatively low levels of acidity (pH 6 or 5.5) increased the toxicity of the bile acids substantially. This emphasises the role of refluxed acidic bile in eroding the lower oesophagus in patients with GORD. It may be argued that as acidic bile is overtly toxic, there is less opportunity for exposed cells to survive and seed cancer development. Whereas, neutral bile (DCA) is less toxic, but overtly genotoxic, hence these damaged cells may well survive and initiate the carcinogenic process more efficiently. Obviously, acid exacerbates the toxicity of bile acids and this may well promote proliferation and ultimately oesophageal carcinogenesis via a different mechanism. However, oxidative DNA damage has been noted in acid suppressed refluxing patients, implicating neutral pH bile acids in ROS induction *in vivo* (42). On the other hand, our data does show that acid alone can induce ROS and hence could contribute to carcinogenesis, indeed it has been reported elsewhere that acid can damage DNA (23,41). However, solely tackling the acidity of the refluxate in Barrett's patients, will not prevent bile acid (DCA) driven DNA damage induction.

In order to investigate if ROS induction by bile acids (DCA) was responsible for the genotoxicity observed here, we took two approaches. Firstly, we assessed whether DCA in particular, could induce ROS in oesophageal cells using two complementary methods. Once we were satisfied that DCA could indeed induce ROS, we determined if these were responsible for the genotoxicity of DCA. Therefore, we treated OE33 cells with DCA (200  $\mu$ M) following a 2 h incubation with the well-known anti-oxidant (vitamin C 200  $\mu$ M) and assessed the impact on DCA driven chromosome damage. We demonstrated that vitamin C could indeed significantly reduce DCA-induced chromosome damage at neutral pH, as well as blocking the release of ROS in the first place. We did not assess this effect at acidic pH due to the confounding influence of increased toxicity, but we see no reason to expect that acidic DCA induces DNA damage through a different mechanism, as it also induced ROS. The source of the DCA-induced ROS is not entirely known, though we suspect that mitochondrial membrane damage and

ROS leakage is most likely. It is known that DCA cannot damage naked DNA directly (32,33) and requires a cellular intermediate. Furthermore, DCA and other bile acids are known to damage mitochondria in hepatocytes and colorectal cells (9,10,43). Consistent with this hypothesis, a recent study has shown mitochondrial swelling in Barrett's tissues exposed *ex vivo* to DCA (44). The central role of cellular ROS in the genotoxicity of bile acids may explain some of the conflicting reports in the literature as to whether bile acids are genotoxic or not. Many of these studies assessed genotoxicity in bacterial cells (e.g. the Ames test), as these possess no mitochondria, it is not surprising that they failed to show genotoxicity.

The confirmation of the role of ROS in bile acid driven genotoxicity has some interesting implications for chemoprevention in Barrett's patients. There is already epidemiological data showing that cancer progression in general (45) and specifically in Barrett's patients is inversely proportional to the levels of ingestion of anti-oxidants like vitamin C (46,47), supporting the notion that anti-oxidants may be chemo-protective. Indeed Terry *et al.* (47) have shown a 40–50% reduced risk of oesophageal adenocarcinoma in Barrett's patients with high levels of anti-oxidant intake. Our data shows a protective effect using 200  $\mu$ M *in vitro*, this is estimated to be the physiological level in gastric juice *in vivo* (48). We (and others) have also shown that bile acids like DCA can activate the NF-kappaB signalling pathway and that this process can be blocked with anti-oxidants like PDTTC (16) as well as other anti-oxidants (like vitamin C, curcumin etc, unpublished findings, our group). Hence DCA driven ROS may be the common upstream event in DCA-induced signalling abnormalities and in the induction of DNA damage. Therefore, anti-oxidants may play a dual-protective role by blocking bile driven genotoxicity and bile driven signalling abnormalities. Clinical trials using anti-oxidants to assess DNA damage levels and histological progression in Barrett's patients, are therefore warranted.

In conclusion, the bile acid DCA is a clastogen, a mutagen and is toxic to oesophageal cells. DCA induces this genotoxicity through an ROS mediated mechanism, hence supporting the view that anti-oxidants may be potent chemo-protective agents. Future chemo-preventative trials should also assess the role of anti-oxidant supplementation. As DCA's genotoxicity appears to be largely independent of pH, altering the pH of the reflux may not abolish DCA driven carcinogenesis. However, coupling acid suppression therapy to adequate anti-oxidant supplementation may be effective at reducing bile driven genotoxicity.

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