Biochemical and Biophysical Research Communications 429 (2012) 191–196

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hyperoxia-induced ciliary loss and oxidative damage in an *in vitro* bovine model: The protective role of antioxidant vitamins E and C

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ARTICLE INFO

Article history:
Received 23 October 2012
Available online 6 November 2012

Keywords:
Bronchial epithelium
Oxidative stress
Cilia
Antioxidant
Oxygen

ABSTRACT

Although elevated oxygen fraction is used in intensive care units around the world, pathological changes in pulmonary tissue have been shown to occur with prolonged exposure to hyperoxia. In this work a bovine bronchus culture model has been successfully used to evaluate the effects of hyperoxia on ciliated epithelium *in vitro*. Samples were cultured using an air interface method and exposed to normoxia, 21% O_2 or hyperoxia, 95% O_2. Cilial coverage was assessed using scanning electron microscopy (SEM). Tissue damage (lactate dehydrogenase, LDH, in the medium), lipid peroxidation (thiobarbituric acid reactive substances, TBARS), DNA damage (comet assay), protein oxidation (OxyBlot kit) and antioxidant status (total glutathione) were used to assess whether the hyperoxia caused significant oxidative stress. Hyperoxia caused a time-dependent decline ($t_{½}$ = 3.4 d compared to 37.1 d under normoxia) in cilial coverage ($P < 0.0001$). This was associated with a significant increase in the number of cells ($2.80 \pm 0.27 \times 10^6$ compared to $1.97 \pm 0.23 \times 10^6$ ml^{-1} after 6 d), many apparently intact, in the medium ($P < 0.05$); LDH release (1.06 ± 0.29 compared to 0.83 ± 0.36 μmol min^{-1} g^{-1} after 6 d; $P < 0.001$); lipid peroxidation (352 ± 16 versus 247 ± 11 μmol MDA g^{-1} for hyperoxia and normoxia, respectively); % tail DNA (18.7 ± 2.2 versus 11.1 ± 1.5); protein carbonyls ($P < 0.05$); and total glutathione (229 ± 20 μmol g^{-1} versus 189 ± 15 μmol g^{-1}). Vitamins E (10^{-7} M) and C (10^{-6} or 10^{-7} M) alone or in combination (10^{-7} M and 10^{-6} M, respectively) had a significant protective effect on the hyperoxia-induced reduction in percentage cilial coverage ($P < 0.05$). In conclusion, hyperoxia caused damage to cultured bovine bronchial epithelium and denudation of cilia. The antioxidant vitamins E and C significantly protected against hyperoxia-induced cilia loss.

1. Introduction

Elevated oxygen fraction, or hyperoxia, is commonly used in the treatment of critically ill patients [1]. However, excessive exposure to high oxygen concentration has adverse effects. Previous studies using animal tissues both *in vivo* and *in vitro* as well as human cell culture have demonstrated this. These studies have shown that hyperoxia significantly affects cellular physiology by promoting the formation of reactive oxygen species (ROS), increasing expression of pro-inflammatory cytokines and activating cell death pathways by altering oxidative signalling [1,2]. Of particular interest are the effects of hyperoxia on the ciliated cells that cover most of the surface of the respiratory tract. Ciliary loss and dysfunction [3–5], high permeability oedema, hyaline membrane formation and pulmonary fibrosis are common findings [1].

A range of models has been used to investigate the effects of hyperoxia on ciliated epithelium [4,5]. There are some disadvantages with these models. For example, as a consequence of metabolic scaling, the basal metabolic rate for small mammals is much more rapid that for humans [6], and the distribution of cilia in the small mammal trachea may be discontinuous, i.e. cilia occur in bands [7]. The use of human tissue samples is possible, but these samples are more difficult to obtain. For example, bronchial samples can be obtained after surgical intervention (e.g. removal of lung tumours), but these are potentially contaminated with cancer cells [4]. Another possibility is the use of post mortem material, but again this is difficult to obtain and likely to be in poor condition.

It would be useful to have a convenient and readily available alternative to these models. Bovine respiratory explants have almost identical epithelial histology compared to the human airway and have already proved to be a good model in mechanistic studies of drug transport [8]. Therefore, in the work described here we have used an *in vitro* bovine bronchial tissue culture model to investigate hyperoxia-induced cilia loss and to evaluate the protective effects of supplemental vitamins E and C. To the best of our

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http://dx.doi.org/10.1016/j.bbrc.2012.10.113

knowledge, this is the first study to use this model for this purpose. Little is known about the effects of hyperoxia on ciliary coverage in large mammalian airways, and no corresponding studies on the protective effects of antioxidant vitamins against hyperoxia-mediated cilia coverage loss have been conducted.

2. Materials and methods

All media and cell culture associated supports were purchased from Invitrogen, UK. Transwell inserts and well plates came from Becton Dickinson, NJ, USA. NADH and NADPH were from Melford (Ipswich, UK) and the OxyBlot kit from Chemicon International (MilliporeS7150). All other reagents were obtained from Sigma–Aldrich (Poole, UK). Gas mixes were supplied by Plymouth Hyperbaric Medical Centre.

2.1. Sample collection and dissection

Bovine lungs were collected from a local abattoir (Newton Abbot, Devon, UK) and transported to the laboratory for immediate processing. Secondary bronchi (~25 mm diameter) were dissected out and cleaned with cell culture grade phosphate-buffered saline (PBS) to remove mucus and blood. Matching pairs of tissue samples (3–4 mm^2), i.e. from the same region of bronchus, were then dissected out.

2.2. Tissue culture and exposure to hyperoxia

Bronchial samples were cultured as described by Rankin et al. [4] with slight modification. Briefly, each pair of bronchus samples was placed ciliated surface uppermost within 12 well-plate cell culture membrane inserts (3.0 μm, high-density membrane). Growth medium consisted of a 1:1 mixture of LHC-9 and RPMI-1640, supplemented with 1% antibiotic/antimycotic solution (10,000 U mL^{-1} penicillin G, 10 mg mL^{-1} streptomycin and 25 μg mL^{-1} amphotericin B). The plates were then placed within custom-made gastight polypropylene boxes (5 L) prepared at Plymouth Hyperbaric Medical Centre. The boxes were flooded with gas mixes (either normoxic, 21% O_2, 5% CO_2 and 74% N_2 or hyperoxic, 95% O_2, 5% CO_2), and then placed in an incubator at 37 °C.

2.3. Co-culture with antioxidant vitamins

Bronchial tissue samples were prepared and cultured as described above and supplemented with medium containing ether vitamin E or vitamin C. Six experimental conditions were used, where samples were exposed to normoxia (21% O_2); hyperoxia (95% O_2); hyperoxia with 10^{-7} M vitamin E; hyperoxia with either 10^{-6} M or 10^{-7} M vitamin C; and hyperoxia with a combination of vitamin E and C at 10^{-7} M and 10^{-6} M, respectively. Stock vitamin solutions were prepared essentially as described by Jyonouchi et al. [9]; briefly, vitamin E was dissolved in an ethanol, methanol and isopropanol mixture (19:1:1, respectively) at 10^{-3} M and then diluted to give the final concentration (10^{-7} M) with culture medium. Vitamin C was dissolved in PBS at 10^{-3} M and diluted with culture medium to give final concentrations 10^{-6} M and 10^{-7} M. After four days culture, samples were prepared for scanning electron microscopy.

2.4. Scanning electron microscopy (SEM) and image analysis

Samples of bronchial tissue were immediately fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer before processing using CO_2 critical point drying and observation in a JEOL 5600 LVSEM operated at 15 kV. For each sample, ten contiguous images were acquired at 2500× magnification and analysis of cilial coverage was carried out using Image J. The method was based on counting cilia-containing squares using the grid option. Analysis of SEM images was carried out blind. The images were converted into 8-bit format, and the cropping tool used to remove any microscope-generated text, e.g. the scale bar. After this, the Plugins option was chosen, and the analyse option was used to apply a grid of 10×6, 2.1 μm^2 squares (settings: grid type, lines; area per point, 0.21 in^2; colour, cyan). The cell counter tool (Plugins, analyse) was used to count squares containing cilia in a manner similar to counting cells using a haemocytometer. Finally the percentage of ciliated squares was calculated.

2.5. Determination of lactate dehydrogenase (LDH) in medium

LDH released into the culture medium was measured in triplicate essentially as described by Bergmeyer and Bernt [10]. The oxidation of NADH was monitored by measuring the absorbance at 340 nm for 15 min in a plate reader (SpectraMax, Molecular Devices, Sunnyvale CA). Enzyme activity was calculated using an extinction coefficient of 6.3 mM^{-1} cm^{-1}.

2.6. Measurement of lipid peroxidation in tissue

The thiobarbituric acid reactive substance (TBARS) assay has been used widely to assess levels of lipid peroxidation [11]. Tissue was homogenised using a Potter homogenizer at a ratio of 1 g of tissue to 4 mL of RIPA buffer (25 mM Tris-chloride, pH 7.6, containing 150 mM NaCl, 0.1% SDS and 1% Triton X-100). After centrifuging, ice-cold 10% TCA (1:1) was added to the supernatant, which was then mixed 1:1 with of 0.67% (w/v) thiobarbituric acid. The mixture was incubated at 80 °C for 30 min and the absorbance was measured at 532 nm using a plate reader. The concentration of malondialdehyde (MDA) was determined by reference to a standard curve produced using 0–50 μM 1,1,3,3-tetramethoxypropane.

2.7. Comet assay for DNA damage detection

Single-cell gel electrophoresis (SCGE) or the 'Comet assay' is a technique used to detect DNA damage in individual cells [12]. To prepare the cells, bronchial tissue that had been exposed to oxygen was incubated in minimum essential medium (MEM) containing 0.1% bacterial protease (*Streptomyces griseus*, Sigma P8811) at 37 °C for 30 min. The bronchus samples were rinsed several times in DMEM containing 10% FBS. After 10 min in the final wash the detached epithelial cells had settled. They were then collected and counted [13]. Slides with cells (10^5/slide) were prepared following a standard protocol [12], and visualised using fluorescence microscopy (Leica DMR). Komet 5.0 image analysis software (Andor Technology plc, Belfast, UK) was used to score Comets from individual cells for % tail DNA. Cell viability was assessed using trypan blue, and found to be ⩾90% throughout.

2.8. Detection of protein carbonyls

As a consequence of protein oxidation, carbonyl groups are formed on the side chains of some amino acid residues [14]. An OxyBlot kit was used to detect protein carbonyl content as a marker of oxidative stress. Samples were prepared following the manufacturer's protocol. Bovine serum albumin (BSA) treated with iron sulphate was used as a positive control. The intensity of chemiluminescence due to oxidised protein was analysed using Image J. The Bradford assay [15] was used to determine the concentration of protein in the homogenized tissue.

2.9. Estimation of total glutathione in tissue

Total glutathione was determined using a method based on that of Owens and Belcher [16]. The absorbance recorded at 412 nm for 5 min using a plate reader. In each run there was a blank (buffer) and a standard (20 μM reduced glutathione).

2.10. Statistical analysis

Statistical analysis was performed using Minitab 16 (Minitab Ltd, Coventry, UK). All results are presented as means ± SEM. Significance was accepted at $P \leqslant 0.05$. Two-way ANOVA was used to analyse LDH data and data for the effect of hyperoxia on ciliary coverage, whereas the antioxidant vitamin treatment data was analysed by one-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) method. Other data were analysed using Student's *t*-test.

3. Results

3.1. Hyperoxia-induced ciliary loss in cultured bovine bronchial tissue

On isolation of the bronchial tissue, cilial coverage was essentially 100% in all cases. A significant loss of cilial coverage over time was found on exposure of tissue to 95% oxygen. Cilial loss became apparent within 48 h of exposure and was progressive throughout the period studied. A highly significant difference ($P < 0.0001$) in ciliary coverage in bovine bronchial tissue within concentrations (95% O_2 compared to 21% O_2) between days and within days between concentrations (Fig. 1A). The interaction between time and concentration was also highly significant ($P < 0.0001$), i.e. there was little loss of cilial coverage in tissue exposed to 21% O_2 over 6 days. Histopathological changes induced by exposure to 95% O_2 were observed over time including the appearance of blebs on the surface and sloughed cells, in addition to loss of cilial coverage (Fig. 1B). Likewise the number of cells found in the medium (presumed to have been sloughed from the epithelium either in groups or individually) increased with time (Fig. 1C).

3.2. Effect of hyperoxia on lactate dehydrogenase release from bovine bronchial tissue

Each day, during the exposure of bovine bronchial tissue, samples of culture medium were collected and assayed for lactate dehydrogenase (LDH) activity. In all cases the LDH activity in the medium decreased overtime (2-way ANOVA, $P < 0.001$ between days). However, the cumulative LDH release was much greater for tissue exposed to hyperoxia compared to tissue exposed to normoxia (2-way ANOVA, $P < 0.01$ between treatments; Fig. 2A). After exposure for one day, LDH activities were 0.04 ± 0.03 μmol min^{-1} - ml^{-1} and 0.02 ± 0.02 μmol min^{-1} ml^{-1} in samples exposed to 95% and 21% O_2, respectively; while at the end of the sixth day of exposure, they were 0.14 ± 0.01 μmol min^{-1} ml^{-1} and 0.05 ± 0.01 μmol min^{-1} ml^{-1}, respectively.

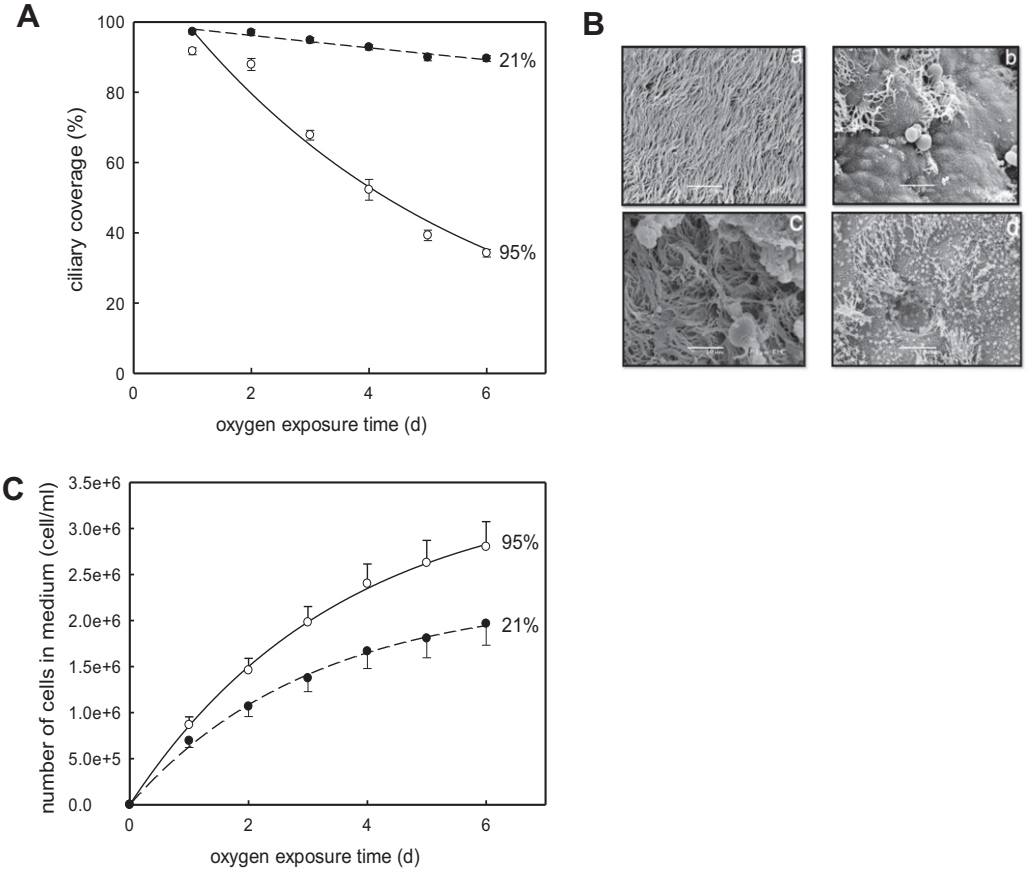

Fig. 1. (A) Ciliary coverage of cultured bovine bronchus tissue exposed to normoxia (●, 21% O_2) and hyperoxia (○, 95% O_2) for up to 6 days. Data are means ± SEM, $n = 9$; (B) scanning electron microscopy images of cultured bovine bronchial tissue exposed to either normoxia (21% O_2) (a), or hyperoxia (95% O_2) for 4 days, where cellular blebbing (b), sloughing cells (c) and cavities left by sloughing cells (d) have been noted; (C) cumulative cell number in the medium from bovine bronchial tissue samples that had been exposed to either normoxia (●, 21% O_2) or hyperoxia (○, 95% O_2). Data are means ± SEM, from triplicate samples from a single animal.

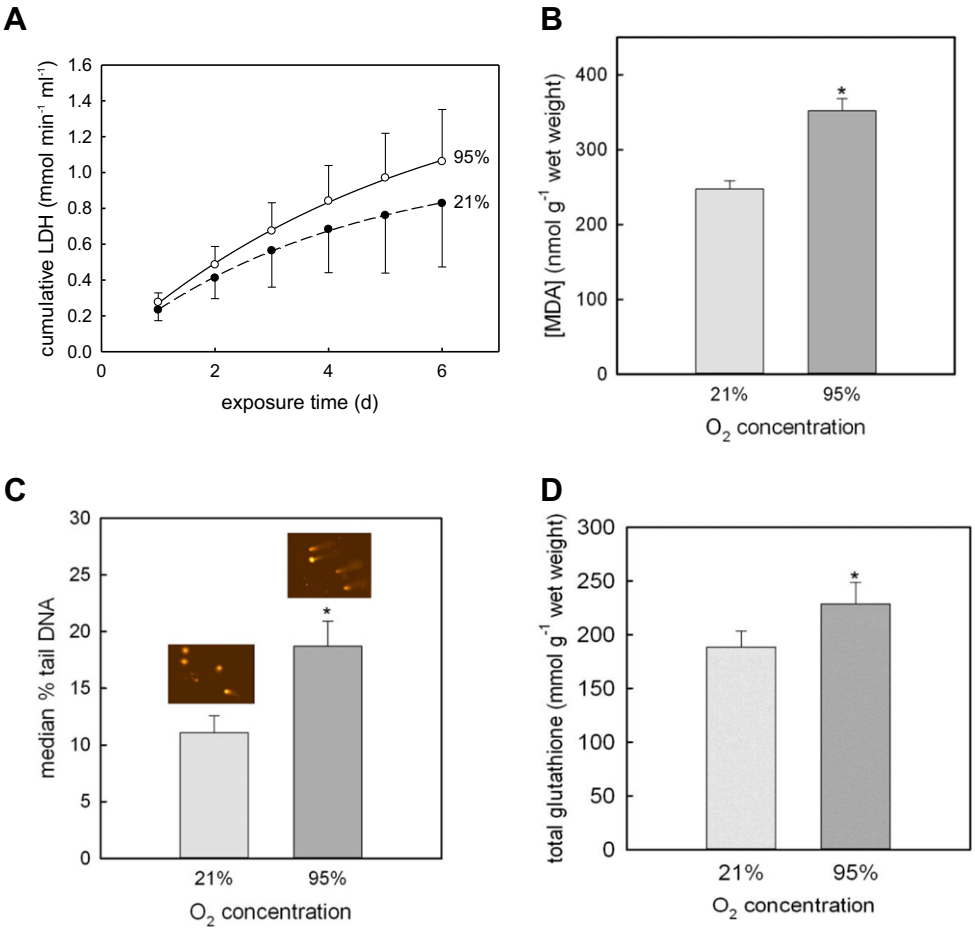

Fig. 2. (A) Cumulative LDH activity in the medium from bovine bronchial tissue samples that had been exposed to either normoxia (●, 21% O_2) or hyperoxia (○, 95% O_2), $n = 9$. (B) Lipid peroxidation in extracts of homogenised bovine bronchus tissue cultured for four days under normoxic and hyperoxic conditions, $n = 9$. (C) DNA damage (% tail DNA) in cells isolated from bronchus tissue exposed to hyperoxia and normoxia. Cells were released from the tissue by incubation with protease, and then the level of DNA damage was assessed using the comet assay (see Section 2). Samples from three different animals were used and 500 nuclei from each animal were scored. Inset comet images. (D) Total glutathione in extracts of homogenised bovine bronchus tissue. Data are means ± SEM, $n = 9$. *Significant differences relative to control.

3.3. Detection of lipid peroxidation, DNA damage and total glutathione

After four days of culture, homogenised tissue was used for the measurement of TBARS. There was a significant increase (*t*-test, $P < 0.05$) in bronchial tissue exposed to hyperoxia (351.9 ± 16.3 μmol g^{-1} wet weight tissue) compared to tissue cultured under normoxia (247.4 ± 10.8 μmol g^{-1} wet weight tissue) (Fig. 2B). The Comet assay was used to quantify hyperoxia-induced DNA oxidative damage after four days of culture. For each treatment 500 nuclei/animal were analysed from five slides, i.e. 100 nuclei/slide. A significant increase ($P = 0.048$) in DNA strand breaks was observed under hyperoxia (% tail DNA = 18.7 ± 2.2) compared to normoxia (11.1 ± 1.5) (Fig. 2C). There was a significant increase ($P < 0.05$) in total glutathione in samples exposed to hyperoxia (228.9 ± 19.9 μmol g^{-1} wet weight tissue) compared to samples exposed to normoxia (188.6 ± 14.6 μmol g^{-1} wet weight tissue) (Fig. 2D).

3.4. Determination of protein oxidation

Oxidised protein bands were observed in the range 43–68 kDa (Fig. 3A). There was a significantly higher level of protein carbonyls in samples exposed to hyperoxia ($P < 0.05$) compared to normoxia (Fig. 3B).

3.5. Effects of antioxidant vitamins on the decline in cilial coverage induced by hyperoxia

The antioxidant vitamins (vitamins C and E individually or in combination) provided some protection against hyperoxia-induced loss of cilial coverage ($P < 0.05$) (Fig. 4). However, this protection was relatively modest, and there was still a highly significant difference ($P < 0.0001$) in the % cilial coverage of bronchial tissue exposed to hyperoxia in the presence of the vitamins compared to the normoxia control.

4. Discussion

As airway epithelium is the first target for oxidative injury, we have tested a bovine bronchus tissue culture model that uses abattoir material to study the effects of high oxygen concentrations on epithelial cells and ciliary coverage. This appears to be the first use of this type of model for this purpose. A simple method for quantifying ciliary coverage has been developed, based on blind scoring of images. The computer-aided method based on thresholding of images that we had previously used [4] was found not to be applicable to the bovine model; this is probably because bovine cilia are longer than human cilia, and tend to 'flop' in SEM preparations. Moreover, the current method, although simple, provided more detailed information on loss of ciliary coverage compared to methods

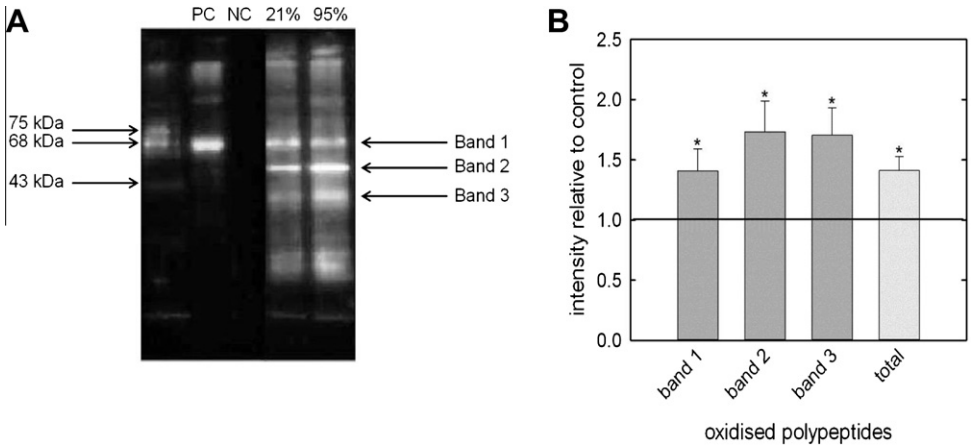

Fig. 3. (A) Immunoblotting assay of protein carbonyl groups as a marker of oxidative damage to polypeptides in extracts from bovine bronchial samples exposed to normoxia (21% O_2) or hyperoxia (95% O_2), where PC = positive control and NC = negative control, and concentration of O_2 is as indicated. (B) Fluorescence intensity relative to the control, quantified using Image J. Bands 1–3 refer to the prominent bands on the blot (labelled in A), whereas total refers to the total fluorescence intensity from all bands. Data in B are means ± SEM, $n = 4$. *Significant differences relative to control blots.

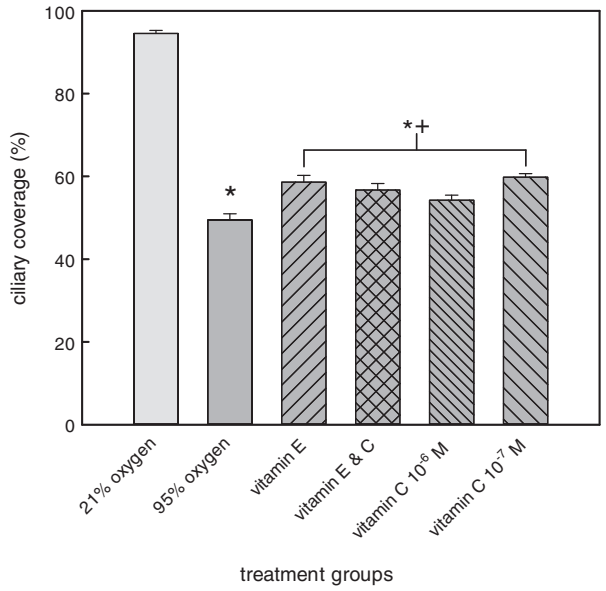

Fig. 4. Ciliary coverage in bovine bronchus cultured tissue exposed to normoxia (21% oxygen) and hyperoxia (95% oxygen) for 4 days and supplemented with vitamin E or C or combination of both. *Significantly different relative to control, +significantly different relative to 95% oxygen. Data expressed as means ± SEM, $n = 9$.

previously used where images were scored to broad categories of coverage (e.g. <50%, 50–75% and >75% [3]).

Significant progressive decline in ciliary coverage with time during exposure to hyperoxia (95% O_2) compared to the normoxia (21% O_2) was found (Fig. 1A), with the observation of some histopathological changes (Fig. 1B). The regulation of the balance of proliferation and cell death in the airway epithelium has been found to be changed via oxidant stress resulting from a hyperoxia-induced increase in ROS production [8]. Features consistent with both apoptotic and necrotic cell death, such as organelle swelling, apical blebbing, ciliary border damage and DNA damage consequent upon hyperoxia have been demonstrated [17,18]. Moreover, the presence of the large number of mitochondria in the ciliated cells especially near the basal bodies of the cilia might make cilia and ciliated cells particularly prone to oxidative stress. Li et al. [19] demonstrated that ρ° respiration-deficient HeLa cells have more resistance to the damaging effects of exposure to 80% O_2 compared to the wild type cells.

Our results indicate that hyperoxia induces bronchial epithelial tissue damage over time: (i) the cumulative LDH level in the medium was significantly higher from the third day of exposure to 95% O_2 (Fig. 2A); (ii) there was significant elevation in the lipid peroxidation in cultured bovine tissue exposed to 95% O_2 (Fig. 2B); (iii) a significant increase in DNA damage in epithelial cells was found after exposure of bovine tissue to hyperoxia (Fig. 2C); and (iv) protein carbonyl levels increased significantly under hyperoxic conditions (Fig. 3B). In addition, total glutathione levels were significantly higher after treatment of bovine tissue with 95% O_2 compared to 21% O_2 (Fig. 3C). Our findings are in accordance with the study by Alttas and Al-Said [20] who found elevated ROS levels were associated with elevated extracellular LDH levels and reduced antioxidant capacity in rats exposed to hyperoxia. A time-dependent increase in the cholesterol/fatty acid ratio in the cell membrane, as a result of oxidative stress, was found in human U87 (glioblastoma) cells cultured under hyperoxia. This affected the properties of the membrane leading to the formation of membrane blebs [21]. The ubiquity of lipids in cells and the ease of their peroxidation can lead to loss of cell function through changes in membrane fluidity and weakening of cellular compartmentation. These changes can lead to altered membrane receptor mobility, and could lead to leakage of intracellular enzymes [22]. Hence, the increase in lipid peroxidation seen here could account for the elevated LDH levels in the medium seen after exposure to 95% O_2.

There are two general mechanisms by which oxidative stress can lead to DNA damage (a) formation of $OH^{\bullet}$ from the reaction of H_2O_2 with metal (Fe and Cu) ions that are bound to the DNA [23] and (b) activation of nucleases that lead to DNA strand breaks [24]. Both types of damage have been detected in lung cells as a consequence of exposure to hyperoxia [25,26]. The propagation of oxidative damage in cells can also extend to protein modification. A series of papers by Davies [27,28] has described the major oxidative modifications of proteins, and the cell damage that follows. Our results with glutathione are consistent with the study by Pietarinen-Runtti et al. [29] in which the glutathione significantly increased after 24–48 h of exposure of BEAS-2B bronchial epithelial cells to hyperoxia.

Given the evidence of the involvement of oxidative stress in the hyperoxia-induced cilial loss, the bovine model was used to quantitatively evaluate whether supplementation with the antioxidant vitamins, C and E could protect against this. As far as we are aware,

this is the first study that shows protection against oxygen-induced loss of pulmonary cilial abundance by vitamins C and E (Fig. 4). Vitamins E and C have been shown to play a valuable role in protecting against cell death due to oxidative injury [8] and vitamin E deficiency makes the lung more susceptible to oxidative damage and inflammation [30]. In addition, vitamin E has been reported to significantly minimize the carbonyl content increase associated with hyperoxia in retinal pigmented epithelial cells [31].

In conclusion, the bovine culture model we used in this study has proved to be a promising model for the investigation of mechanisms by which hyperoxia leads to ciliary loss and roles of antioxidants in protecting against this.

Acknowledgments

Hanady Al-Shmgani thanks Prof. Waleed Al-Murrani for his statistical advice, and Peter Russell for his organization of sample collection. We are also grateful to Peter Bond and Glen Harper of Plymouth Electron Microscopy Unit, and Dr Gary Smerdon at Plymouth Hyperbaric Medical Centre. This study was supported by the Ministry of Higher Education, Iraq.

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