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Role of ascorbate in lung cellular toxicity mediated by light-exposed parenteral nutrition solution

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Abstract

Neonatal lung injury has been induced experimentally by infusion of multivitamin-containing light-exposed parenteral nutrition (PN) solutions. The objective was to explore the role of ascorbate in toxic effects of light-exposed PN on primary cultured foetal rat lung epithelial cells. Hydroperoxides were measured in 3% amino acid solutions at baseline, immediately after addition of either multivitamins or ascorbate alone (400 μg/mL) and again after a 24-h period of exposure to (or protection from) ambient light. Cellular toxicity was assessed by [C^{14}]adenine release. Multivitamins or ascorbate alone increased hydroperoxides in PN, which was attenuated by light protection. Light-exposed PN containing multivitamins was more toxic to cells than baseline or light-protected PN. Exposure to ascorbate at concentrations both lower (< 5 μg/mL) and higher (> 1000 μg/mL) than normally contained in PN-induced oxidant-mediated cell death, as indicated by protective effects of hydroperoxide and hydroxyl radical scavengers. This study concludes that ascorbate generates toxic amounts of peroxide in PN solutions. The types and physiological importance of hydroperoxides induced by pro-oxidant effects of ascorbate require further evaluation *in vivo*.

Keywords: *Oxidative stress, hydroxyl radical, hydroperoxides*

Abbreviations: *ASCOOH, Ascorbylperoxide; BPD, Bronchopulmonary Dysplasia; DPPD, N,N'-diphenyl-para-phenylenediamine; FOX, Ferrous oxidation of Xylenol Orange;* H_2O_2*, Hydrogen Peroxide; OOH, Hydroperoxide; PN, Parenteral Nutrition.*

Introduction

Premature infants are at increased risk of oxidant stress [1,2] and morbidities in which oxidant stress is believed to play a causative role, including bronchopulmonary dysplasia (BPD) and retinopathy [3,4]. Light-exposure of parenteral nutrition (PN) solutions has been reported to increase hydroperoxide (OOH) generation [5,6], which may contribute to oxidative stress-related morbidities in pre-term infants [7,8]. Since OOHs, such as hydrogen peroxide (H_2O_2), are known to mediate oxidant-induced lung epithelial cell injury *in vitro* [9], it is reasonable to assume that increased OOH load may directly mediate effects of light-exposed PN on the lungs of experimental animals [10]. Although evidence in human infants remains lacking, intravenous infusion of light-exposed (compared to light-protected) PN solution containing multivitamins was recently shown to increase oxidative stress, to impair alveolarization and to increase apoptosis in the lungs of neonatal guinea pigs [10,11]. Previous work by others has shown that the addition of multivitamin solution is necessary for OOH generation in PN [6], with a particular role identified for riboflavin [11]. Ascorbate, which is a major component

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of intravenous multivitamin solutions, is known to possess both pro- and anti-oxidant effects, depending on local concentrations and other factors [12,13], but its direct contributory role toward OOH generation and toxic effects of light-exposed PN solutions has to date received limited attention [6,14]. In particular, an interaction between ferrous iron and ascorbic acid has been shown to generate hydroxyl radicals and to produce oxidative injury [15,16]. Exposure of multivitamins to light was previously shown to induce a rapid loss of ascorbate and to cause generation of a specific ascorbate byproduct, ascorbylperoxide (ASCOOH) [17,18], which may cause DNA breakage [19]. Although the physiological importance of ascorbate-derived peroxides is currently unknown, recent work in pre-term infants has suggested that urinary ascorbylperoxide concentrations during the first week of life predicted the development and correlated with severity of BPD [20]. Our objective in the present study was to explore the contribution of ascorbate toward generation of peroxides and toxicity to premature rat foetal lung cells *in vitro*.

Materials and methods

Materials

Primene® amino acid solution was from Baxter (Toronto, Ontario, Canada). Multivitamins (Multi-12/K1® Pediatric) and Ascorbate were from Sandoz (Boucherville, Québec, Canada). [8-^{14}C]Adenine was from ICN Biomedicals (Costa Mesa, CA). Cell culture media, collagenase, trypsin and DNase were from GIBCO BRL (Burlington, Ontario, Canada). All other chemicals and reagents were purchased from Sigma (Oakville, Ontario, Canada).

Institutional review

All procedures involving animals were conducted according to criteria established by the Canadian Council on Animal Care. Approval for the studies was obtained from the Animal Care Committee of the Hospital for Sick Children Research Institute.

PN solutions

Twenty 500-mL bags of each of the following solutions were used: (1) Primene® 3% (wt/vol) containing 10% (wt/vol) dextrose and electrolytes only, (2) Primene® 3% with dextrose, electrolytes and multivitamins (including 400 μg/ml ascorbate (see Table I) and (3) Primene® 3% with dextrose, electrolytes and 400 μg/ml ascorbate. These concentrations are identical to those received by the patients in our newborn intensive care unit (NICU). Following removal of an aliquot for baseline measurement (baseline sample), solutions were divided into two sub-groups (10 bags each) which were either exposed to or protected from ambient fluorescent light. Photo-exposure was performed by placing the PN bags 50 cm from an UV-filtered fluorescent lamp (F40T12/CW/IS, 40W, T12 instant start fluorescent lamp, Cool White phosphor, 4200K colour temperature, 62 CRI, Cat. No. 226068, Sylvania fluorescent, Canada) with a Mean Spherical Candle Power of 251 ft-candles, which is the same light source used in the NICU. Photo-protection was achieved by wrapping the PN bags in aluminum foil. Both interventions were performed for 24 h, after which a second sample was taken for assay.

Measurement of hydroperoxides

Hydroperoxide levels in PN solutions and *tert*-butyl OOH standards were measured in duplicate (in PN or conditioned media samples stored at −80°C in the dark immediately upon collection) by the ferrous oxidation of xylenol orange (FOX) assay, as previously described in detail [21,22]. At low pH, Fe^{2+} is oxidized to Fe^{3+} in the presence of OOHs. Fe^{3+} reacts with xylenol orange to produce a chromophore with maximal adsorbance at 560 nm [23]. Hydroperoxide content was expressed in μmol/L equivalents of *tert*-butyl OOH. The lower limit of detection by this assay was 5 μmol/L, using an Ultrospec 3100 Pro Spectrophotometer (Biochrom, Cambridge, UK).

Cell culture preparation

Cellular toxicity of PN solutions was tested on primary cultured lung epithelial cells obtained from d-19 (term = 22 d) gestation foetal Sprague-Dawley rats (Charles River, St. Constant, Québec, Canada), as previously described in detail [24]. We used foetal rat lung epithelial cells for these experiments due to our extensive experience with this cell type [9,24,25]. Briefly, lungs were isolated and separated from major vessels and airways, then minced and gently vortexed to remove erythrocytes. Minced tissue was subjected to proteolytic digestion with trypsin and DNase and the resultant cell suspension eluted through 100-μm-mesh nylon cloth. Eluted cells were subjected to a collagenase digestion and fibroblasts removed by differential adherence, resulting in epithelial cells of −95% purity. Cells were seeded in DMEM with 10% (vol/vol) foetal bovine serum at a sufficient cell density to allow near-confluence 24–48 h after seeding. Cells were maintained in a humidified gas mixture of 3% O_2, 5% CO_2 and 92% N_2. This gas mixture provides a PO_2 of ~ 20 mm Hg, equivalent to that observed *in utero*, thus avoiding any potential artifact induced by an acute change in environmental PO_2. Sub-confluent cells were serum-starved for 24 h and incubated with 0.2 μCi/ml

Table I. Constituents of Primene® 3% (italics) and Primene® 3% with electrolyte, multivitamins and trace elements.

Constituent	Amount per 100 ml	Constituent	Amount per 100 ml
Glucose	*10 gm*	Manganese	91 μM
Protein	*3 gm*	Vitamin A	1150 IU
Sodium	*2 mmol*	Vitamin D	200 IU
Potassium	*1.3 mmol*	Vitamin E	3.5 IU
Chloride	*1.2 mmol*	Vitamin C	40 mg
Calcium	*0.9 mmol*	Vitamin K	0.1 mg
Phosphate	*0.9 mmol*	Folate	0.07 mg
Acetate	*0.8 mmol*	Biotin	10 μg
Magnesium	*0.2 mmol*	B1 (Thiamine)	0.6 mg
Iodine	5.9 μM	B2 (Riboflavin)	0.7 mg
Selenium	5 μg	B3 (Niacin)	8.5 mg
Zinc	760 μM	B6 (Pyridoxine)	0.5 mg
Chromium	19 μM	B12 (Cyanocobalamin)	0.5 μg
Copper	160 μM	Pantothenic acid	2.5 mg

[8-^{14}C] adenine for 2 h to achieve intracellular incorporation of [8-^{14}C] adenine after which cells were treated as described below.

Treatment of cells and measurement of cellular toxicity

Cells were either exposed to DMEM only (control) or DMEM containing various concentrations of PN (pre-diluted with DMEM to concentrations of between 1:50–1:10 000) or ascorbate (0.001–5000 μg/ml) for 24 h. Cellular toxicity was assessed by measurement of the release of pre-incorporated [8-^{14}C] adenine into culture medium at 24 h [26]. Results are presented as a cytotoxicity index where the percentage of [8-^{14}C] adenine release has been standardized to the mean percentage release by control cells that was assigned a value of 1 and all other values expressed as a multiple or fraction of the control value. Percentage [8-^{14}C] adenine release by control cells was 20–30% and data were discarded if baseline release exceeded 30%. All treatments were conducted in 3% O_2, to avoid confounding additive effects of oxidant stress secondary to increased PO_2. In order to determine whether ascorbate-induced cytotoxicity was mediated by OOH, cellular toxicity was also assessed after co-addition of non-toxic doses of N,N'-diphenyl-*para*-phenylenediamine (DPPD), a OOH scavenger [27], or Desferrioxamine (Desferal), an Fe^{2+}-chelator [28,29], to the culture medium. Concentration-toxicity curves were performed (data not shown) to establish the non-toxic range for DPPD (0.5 μmol/L) and Desferal (0.5 μmol/L) prior to their use. The effect of DPPD and Desferal were tested on toxicity secondary to low (0.05 μg/mL) and high (5000 μg/mL) doses of ascorbate.

Measurement of hydroperoxides in conditioned medium

Total hydroperoxides were measured in conditioned phenol red-free medium following 24-h exposure of cells to low-dose (50 ng/mL) ascorbate with or without DPPD (0.5 μmol/L).

Statistical analyses

Unless otherwise stated, all values are expressed as means ± SEM from four wells or 10 samples/group. Where error bars are not visible, they fall within the plot point. For studies involving cell culture, at least one replicate from a different litter was performed to ensure reproducibility of results. Statistical significance ($p < 0.05$) was determined by one-way ANOVA followed by pairwise multiple comparisons using the Tukey test.

Results

Hydroperoxide generation in PN solutions was increased by light exposure

Hydroperoxides were undetectable in light-exposed PN solution in the absence of multivitamins or ascorbate. As previously described [5,6], light exposure of PN containing multivitamins led to significantly ($p < 0.001$) increased OOH levels, when compared to baseline (Figure 1A). Light-protected PN with multivitamins had significantly decreased OOH levels ($p < 0.001$) when compared to light-exposed PN (Figure 1A), confirming that light exposure contributed to increased OOH generation.

Hydroperoxide levels in PN solutions were increased by ascorbate

Light exposure of PN containing ascorbate also led to significantly ($p < 0.001$) increased OOH levels, when compared to baseline (Figure 1B), although significantly less than that observed in light-exposed PN containing multivitamins (61.8 ± 2.6 vs 111 ± 8.4; $p < 0.001$). Hydroperoxide levels in PN solutions containing multivitamins or ascorbate alone were similar at baseline ($p > 0.05$; Figures 1A and B).

Cytotoxicity of PN solutions and ascorbate

We have previously determined that concentrations of *tert*-butyl-OOH ≥ 2 μM were toxic to primary cultured

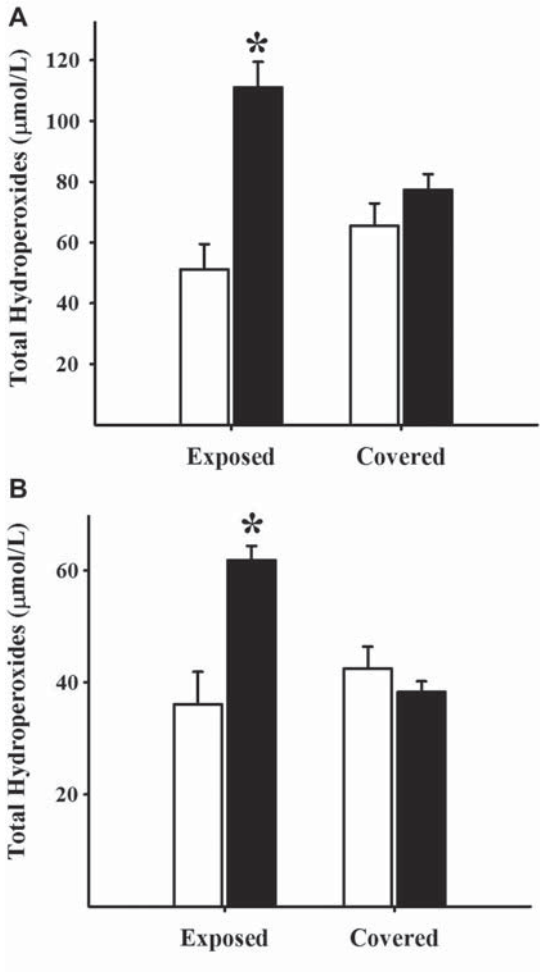

Figure 1. Total hydroperoxides measured in Primene® 3% PN containing (A) multivitamins or (B) ascorbate alone at baseline (open bars) and 24 h (closed bars) following light exposure (exposed) or light protection (covered) for 24 h. $^{*}p < 0.01$, by ANOVA, compared to all other groups.

rat foetal lung epithelial cells (data not shown). We therefore reasoned that if OOH in PN solution is a major cause of cell toxicity, a dilution of PN by 1:50 should show differences between light-exposed and baseline or light-protected solutions (< 2 μM in light protected or in baseline and > 2 μM in light-exposed). As hypothesized, we found that light-exposed PN containing multivitamins diluted 1:50 with DMEM caused significantly increased cell death ($p < 0.01$), when compared with baseline or light-protected PN (Figure 2). However, contrary to expectation, further dilution of PN increased (rather than decreased) cellular toxicity (Figure 2). This phenomenon was also observed in baseline or light-protected PN but at greater dilutions than was observed with light-exposed PN (Figure 2). We speculated that the responsible factor may be ascorbate [17,30]. In accord, a concentration-toxicity curve for ascorbate demonstrated cellular toxicity at concentrations lower (as well as higher) than that normally added to our PN solutions (400 μg/ml, indicated by dashed line; Figure 3A.

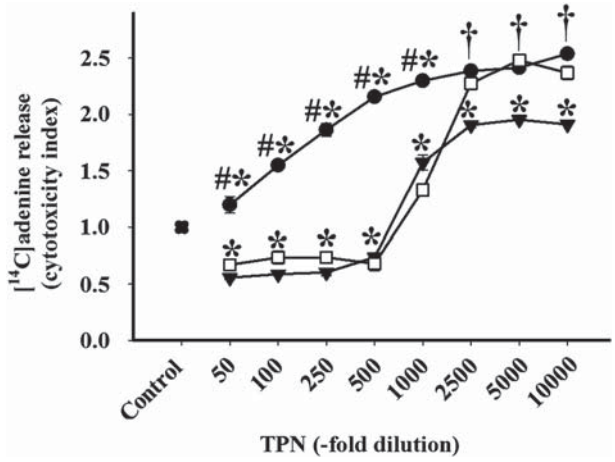

Figure 2. Cytotoxicity of various dilutions of light exposed Primene® 3% PN (closed circles) containing multivitamins and trace elements at 24 h compared to baseline (open square) or compared to light-protected (closed triangle) samples. $^{*}p < 0.01$, by ANOVA, compared to control (medium only) group. $^{\#}p < 0.001$, by ANOVA, compared to light-protected group and baseline group at the same dilution. $^{\dagger}p < 0.001$, by ANOVA, compared to light-protected group at the same dilution.

Cytotoxicity secondary to ascorbate was abolished by hydroperoxide and hydroxyl radical scavengers

As shown in Figure 3B, DPPD (0.5 μmol/L) almost completely abolished cytotoxicity secondary to both low- and high-dose ascorbate. Similar effects were also observed with Desferal (0.5 μmol/L; Figure 3B), an Fe^{2+}-chelating agent that is known to limit hydroxyl radical production. Together, these data suggest that ascorbate-induced cytotoxicity was mediated by hydroxyl radical-induced OOH generation.

Hydroperoxide content in cell culture medium was increased by ascorbate

Total hydroperoxides were significantly increased by the presence of ascorbate (41.6 ± 2.1 vs 27.4 ± 2.8 μmol/L in DMEM-only controls; $p < 0.001$), which was normalized by DPPD (27.3 ± 1.4 μmol/L; $p < 0.001$ compared to ascorbate alone).

Discussion

Our present findings are in agreement with previous work showing that neonatal PN solutions containing multivitamins generate high concentrations of OOHs when exposed to light [5,6], which is largely (but not completely) attenuated by light protection. Experimental neonatal lung injury is reported to be induced by light-exposed PN containing multivitamins or high concentrations of OOH [10,11]. Previous work has confirmed the pro-oxidant role of ascorbate in the generation of peroxides in PN solution either by acting as an electron donor [14] or by formation of ascorbylperoxide [17,18,20]. Ascorbate has also been

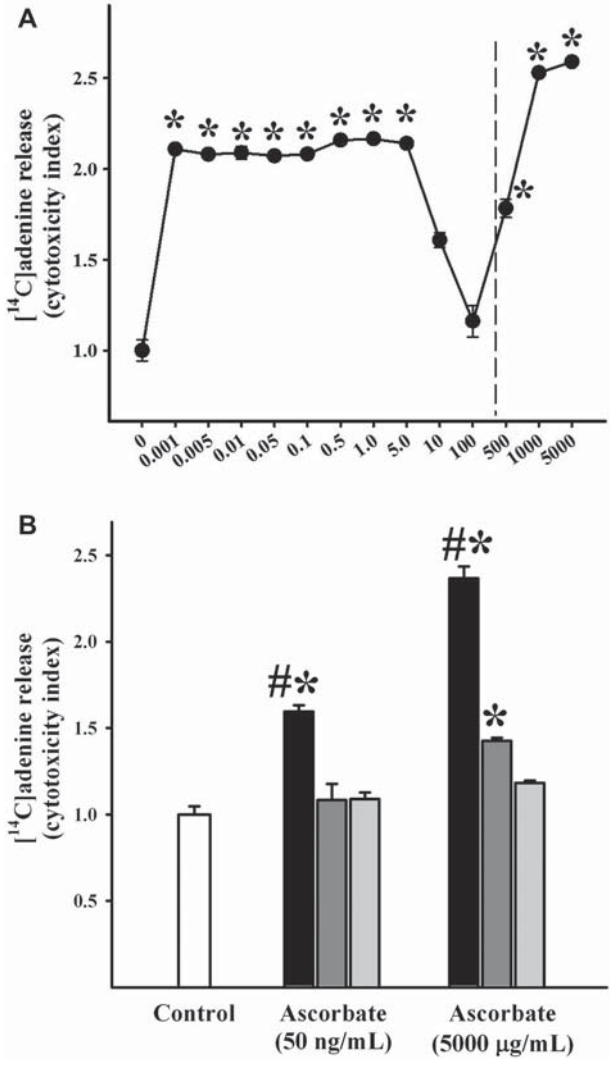

Figure 3. (A) Concentration-toxicity curve for ascorbate. Dashed line indicates ascorbate amount added to standard Primene® 3% PN with multivitamins for comparison. $^{*}p < 0.01$, by ANOVA, compared to control (0 dose) group. (B) Cytotoxicity index secondary to treatment of cells with low- (50 ng/ml) or high-dose (5000 µg/ml) ascorbate in DMEM alone (closed bars) or in DMEM containing DPPD (dark grey bars) or Desferal (light grey bars). $^{*}p < 0.01$, by ANOVA, compared to control (DMEM only) group. $^{\#}p < 0.01$, by ANOVA, compared to respective DPPD and Desferal groups.

described to promote metal ion-dependent hydroxyl radical formation through Fenton-like reactions [31–33]. Accordingly, our aim was to explore the role of ascorbate in generation of peroxides and to provide a mechanistic basis for PN-induced cell toxicity *in vitro*. Collectively, our findings implicate a direct role for ascorbate which, when present in low concentrations due to photodegradation, leads to increased generation of OOHs and cell death.

Hydroperoxide generation in PN containing multivitamins has been suggested to result from a synergistic reaction between riboflavin and ascorbate [14], with ascorbate acting as an electron donor to reduce riboflavin in the presence of light and oxygen, producing H_2O_2 and other peroxides [12,34,35]. Our finding that more OOH was generated in PN solutions with multivitamins than with ascorbate alone suggests that this model is at least partly correct. However, as shown in the present study, large quantities of OOH may also be generated in the presence of ascorbate alone. We speculate that the OOH species generated in the presence of ascorbate were predominantly ASCOOH, but this remains to be confirmed [36].

We observed that light-exposed PN was more toxic to lung cells than baseline and light-protected PN, which could be consistent with an important role for locally-generated OOHs in cell death. Hydroperoxides are highly toxic, leading to cell death at concentrations much lower than that found in light-exposed PN. However, our finding that progressive dilution of PN led to increased toxicity is the opposite of what would be expected had OOH generated *within PN* been the major culprit. Furthermore, we found that dilution-toxicity curves for baseline and light-protected PN were right-shifted when compared to light-exposed PN. Both observations suggest that light exposure caused a change in concentration of a factor in PN which led to cellular toxicity. Given that ascorbate loss is known to occur rapidly upon exposure of PN to light [17,30] and that ascorbate was toxic to epithelial cells at very low concentrations (1–5000 ng/mL), it appears likely that ascorbate loss was a major contributor to light-exposed PN-mediated cell death either through a loss of antioxidant properties at normal concentrations or an increase in pro-oxidant effects at lower concentrations.

Ascorbate is known to cause concentration-dependent cell death in the presence of divalent transition metal ions such as Cu^{2+} and Fe^{2+} through hydroxyl radical generation [13,31–33,35]. Our findings that an OOH scavenger, DPPD, and Fe^{2+} chelating agent, Desferal, both attenuated ascorbate-induced oxidative stress and cytotoxicity together suggest that ascorbate-induced hydroxyl radicals accounted for increased locally-generated OOH, which in turn may have contributed to cell death. This theory is partially supported by previous work on promyelocytic HL60 leukaemia cells, whereby in the presence of verteporfin (photosensitizer) and light, ascorbate increased H_2O_2 production, leading to increased myeloperoxidase activity and consequent cytotoxicity [37]. In cultured chick embryo fibroblasts, addition of ascorbate to the cell medium also induced toxicity, which was prevented by the addition of catalase to the medium, suggesting that H_2O_2 was the cytotoxic agent [38].

We acknowledge our use of epithelial cells as a limitation of our study, since direct contact of epithelial cells with PN *in vivo* will be less likely to occur than with other cell types, such as endothelial cells. We emphasize that we employed this cell type, with which we have considerable experience, to test the

effects of various components of the PN solution on cell integrity. Although lung epithelial cells will not be directly exposed to PN solutions, the harmonic mean thickness of the air–blood barrier is only <1 μm, so the epithelium may be expected to be within diffusing distance of PN contents or their products within the pulmonary capillaries.

In conclusion, our findings suggest that an important mechanism of cytotoxicity from light-exposed PN may be ascorbate loss and generation of ascorbate related peroxides, which contributes to oxidant-mediated cytotoxicity. Together, these observations provide a further rationale for protecting PN from light in the NICU as a potential means of reducing neonatal morbidity. It remains to be confirmed in human infants whether infusion of light exposed vs light-protected PN alters serum and tissue ascorbate levels and whether such changes might be linked to *in vivo* generation of OOH and morbidities, such as BPD.

Declaration of interest

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