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Research Paper

Ascorbylperoxide from parenteral nutrition induces an increase of redox potential of glutathione and loss of alveoli in newborn guinea pig lungs

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ABSTRACT

Background: Bronchopulmonary dysplasia is one of the main complications associated with extreme prematurity. Oxidative stress is suspected to be a trigger event of this lung disease, which is characterized by impaired alveolar development. Peroxides, mainly ascorbylperoxide and H_2O_2, are known contaminant of parenteral nutrition. We hypothesize that these oxidant molecules induce bronchopulmonary dysplasia development. The aim was to determine if the infusion of ascorbylperoxide, whether in presence or absence of H_2O_2, is associated with oxidative stress, apoptosis and loss of alveoli in the lungs of newborn guinea pigs.

Method: Three-day-old guinea pigs received parenteral solutions containing 0, 20, 60 or 180 μM ascorbylperoxide in the presence or not of 350 μM H_2O_2 (concentrations similar to those measured in parenteral nutrition). After 4 days, the lungs were collected for determination of glutathione's redox potential, caspase-3 activation (an apoptosis marker), alveolarization index (by histology), activation of Nrf2 and NFκB (biological markers of oxidative stress), and IL-6 and PGJ_2 levels (markers of NFκB activation). Groups were compared by ANOVA, $p < 0.05$.

Results: Loss of alveoli was associated with ascorbylperoxide in a dose-dependent manner, without an influence of H_2O_2. The dose-dependent activation of caspase-3 by ascorbylperoxide was lower in the presence of H_2O_2. Ascorbylperoxide induced an increase of redox potential in a dose-dependent manner, which reached a plateau in presence of H_2O_2. Nrf2 and NFκB were activated by H_2O_2 but not by ascorbylperoxide.

Conclusion: Results suggest that ascorbylperoxide, generated in parenteral nutrition, is involved in the development of bronchopulmonary dysplasia, independently of the increase of the redox potential. This study underlines the importance of developing a safer formulation of parenteral nutrition.

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Introduction

Decreased alveolar number and impaired vascular development are characteristic features of bronchopulmonary dysplasia (BPD) [1,2]. Extremely premature infants (<29 weeks of gestation) are affected by BPD in 50% of cases. Amongst the multiple consequences of BPD, lung function impairment, which can persist until adulthood [3–5], and altered neurodevelopment are the most worrisome [6,7]. Requirement in oxygen supplementation, with its associated oxidative stress, has long been recognized as a component in the aetiology of BPD [1,2,8]. Recently, parenteral nutrition (PN) has been suggested to play an important role in BPD development. It has been reported that peroxides, which contaminate PN, induce oxidative stress in newborn animals [9] as well as in premature infants [10]. Light-protection of PN reduces by half the peroxide generation in intravenous solution [10–12], decreases the incidence of BPD [13] and chronic lung diseases [12] in premature infants and prevents the alveolar loss in newborn guinea pigs [14]. An animal study by Lavoie et al. [15] reported that an exaggerated apoptosis is associated with the loss of alveoli, independently of the H_2O_2 level present in PN. This study also described that the loss of alveoli is reproduced by the infusion of a solution containing vitamin C and riboflavin, components found in PN. Interaction between these two vitamins, in presence of ambient light, generates a new organic peroxide derived from the peroxidation of dehydroascorbate by H_2O_2 [16,17]. We suspect that this molecule (2,3-diketo-4-hydroxyperoxyl-5,6-dihydroxyhexanoic acid), named in the current report as "ascorbylperoxide", participates in the development of BPD.

Peroxides are detoxified by the glutathione metabolism, in which GSH is oxidized into GSSG. The concentration of both forms of glutathione impacts on the cellular redox environment. Because cell

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cycle (proliferation, differentiation and apoptosis) is influenced by the redox environment [18], the presence of peroxides in PN can induce arrest of pulmonary development or even apoptosis. Chessex et al. [19] have recently reported that the level of redox potential of glutathione measured in blood of premature infants was strongly correlated with the severity of BPD [19]. We hypothesize that ascorbylperoxide infused with PN induces oxidative stress characterized by a higher redox potential, which stimulates apoptosis and leads to alveolar loss. Therefore, the aim of this study was to document that the infusion of increasing concentrations of ascorbylperoxide is associated with an elevation of redox potential of glutathione, activation of an apoptosis marker and a reduced number of alveoli in the lungs of newborn guinea pigs. Because PN contains also H_2O_2, which can potentially exacerbate the oxidation of glutathione, the intravenous solutions infused to animals in this study will contain or not H_2O_2 (ascorbylperoxide $\pm$ H_2O_2).

Materials and methods

Sixty Hartley guinea pigs (Charles River Laboratories, Saint-Constant, Quebec, Canada) have been used in eight groups (6–9 animals per group). At 3 days of life, a catheter (SAI Infusion Technologies, Lake Villa, IL) was fixed in the right jugular vein as previously described [13,14,20] to inject the experimental intravenous solutions freshly prepared each day. The solutions, which were continuously infused at a rate of 19 mL/100 g/d [21], contained: 8.7% (w,v) dextrose + 0.3% (w,v) NaCl + 1 U/mL heparin + 0, 20, 60 or 180 μM ascorbylperoxide $\pm$ 350 μM H_2O_2 (concentrations previously measured in total parenteral nutrition) [11,14]. A usual total parenteral nutrition containing 1% of a multivitamin preparation (Multi-12 paediatric, Sandoz, Boucherville, QC, Canada) generates 36 $\pm$ 1 μM ascorbylperoxide (J.C. Lavoie, personal communication). Because ascorbylperoxide does not commercially exist, it was generated *in vitro*, as previously described [21]. After 4 days of infusion, lungs were collected as per the protocol previously explained in references (14) and (15). The right lung was used for biochemical measurements and the left one was used for histology. Urine was collected directly from the bladder for determination of ascorbylperoxide concentration. Forty-eight animals had urine in their bladder at the time of sacrifice. The protocols were approved by the Institutional Committee for Good Practice with Animals in Research, in accordance with the Canadian Council of Animal Care guidelines.

Alveolarization index represents the mean value for each animal of the number of intercepts between a standardized straight-line (1 mm) and histological structures calculated from four different fields of the same lung (200 × magnification) [14,15].

Ascorbylperoxide concentration in intravenous solutions, as well as in urine, was quantified by using an LC/MS methodology previously described [16,21]. Because the internal standard was L-2-oxothiazolidine-4-carboxylate (OTC), results are expressed in OTC equivalent.

Glutathione (GSH and GSSG) was determined by capillary electrophoresis, as previously described [20–22], whereas the redox potential was calculated (25 °C, pH 7) by using the Nernst equation.

Apoptosis was defined by calculating the proportion of active (cleaved caspase-3) on total caspase-3 (cleaved + non-cleaved). These two forms of the protein were quantified by Western blot as described in [22].

Nrf2 and NFκB: In order to qualify the oxidative stress, in addition to glutathione redox state, protein levels of Nrf2 and NFκB have also been measured. Nrf2 is a nuclear factor involving antioxidant defences. It favours the transcription of genes encoding for, among others, glutathione synthesis. NFκB is a nuclear factor favouring inflammation. Determination by Western blot [23] of their cytosolic level and nuclear fraction, using a commercially nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA), allowed calculation of the proportion present in the nucleus (active form). They were reported as activated protein (nuclear level/nuclear + cytosolic levels). The Western blot method described by Hübner et al. [23] was used, except for the antibody against NFκB, for which we have used the MaxPab mouse polyclonal NFκB antibody from Abnova (Taipei, Taiwan). IL-6 and PGJ₂: From lung homogenate (1 in 4 volume of water), concentration of IL-6 was evaluated by the Human IL-6 ELISA kit (Anogen Mississauga, Ontario) whereas the 15-deoxy-$\Delta^{12,14}$-PGJ_2 EIA kit (Enzo, Plymouth, PA, USA) was used to measure PGJ_2 levels after a C-18 extraction [24].

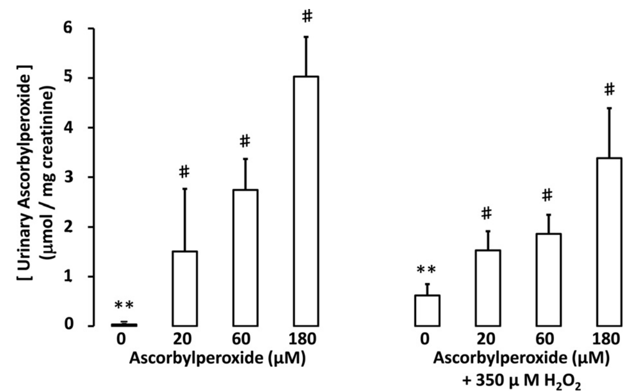


Fig. 1. Influence of ascorbylperoxide and H_2O_2 on the urine concentration of ascorbylperoxide. The level of ascorbylperoxide in urine increased (**0 vs. 20, 60,180 μM; $p < 0.01$) linearly in function of the received dose of ascorbylperoxide (#between 20, 60 and180 μM; $p < 0.01$) and was lower in groups infused with solutions containing H_2O_2 (with vs. without H_2O_2; $p < 0.05$). Mean $\pm$ S.E.M.; $n = 4$–7.

Statistical analysis

Data from each group are presented as mean $\pm$ S.E.M., and have been compared by factorial ANOVA (4 concentrations of ascorbylperoxide × H_2O_2). All comparisons were orthogonal. To meet the homoscedasticity, which was verified by using Bartlett's Chi squared test, data for GSH, Nfr2 and NFκB have been logarithmically transformed. The level of significance was set at p-value <0.05. Comparison between groups receiving the solutions devoid of ascorbylperoxide and groups infused with the three concentrations of this peroxide was used to statistically document the effect of ascorbylperoxide. The use of coefficients for linear and quadratic polynomials allowed analysis of the dose–response effect of ascorbylperoxide. A significant interaction between H_2O_2 and ascorbylperoxide led us to analyze the impact of ascorbylperoxide separately of the presence or absence of H_2O_2.

Results

There was no statistical difference between initial mean body weight among groups (104 $\pm$ 2 g ($n = 60$); H_2O_2 effect: $F_{(1,52)} < 0.1$; ascorbylperoxide effect: $F_{(3,52)} = 1.3$). The urinary concentration of ascorbylperoxide (Fig. 1) increased ($F_{(1,40)} = 12.6$, $p < 0.01$) linearly ($F_{(1,40)} = 37.3$, $p < 0.01$) in function of the dose of ascorbylperoxide received and was 25% lower in animals infused with solutions containing H_2O_2 ($F_{(1,40)} = 5.9$, $p < 0.05$). The total amount of intravenous solution received was similar ($F_{(1,40)} = 0.3$) in the group receiving H_2O_2 (72.3 $\pm$ 1.3 mL) and in the group without H_2O_2 (71.9 $\pm$ 0.8 mL).

The ANOVA revealed a significant interaction between ascorbylperoxide and H_2O_2 ($F_{(3,49)} = 3.1$, $p < 0.05$) on the redox potential of glutathione in the lungs (Fig. 2A). In the absence of H_2O_2, the ascorbylperoxide has induced an increase of the redox potential ($F_{(1,49)} = 7.7$, $p < 0.01$) that was linearly dependent on the dose

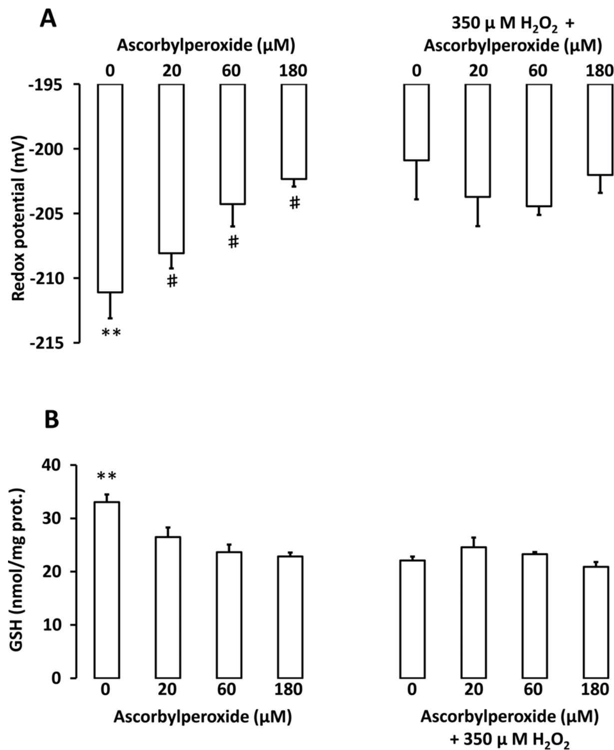


Fig. 2. Influence of ascorbylperoxide and H_2O_2 on the redox potential of glutathione and GSH level in lungs. Due to the significant interaction ($p < 0.05$) between ascorbylperoxide and H_2O_2, the effects of the ascorbylperoxide on the redox potential and on GSH have been analyzed according to the presence or not of H_2O_2. (A) In absence of H_2O_2, the redox potential was lower in the group devoid of ascorbylperoxide (**0 vs. 20, 60,180 μM; $p < 0.01$). The impact of the ascorbylperoxide was linearly dose-dependent (#between 20, 60 and180 μM; $p < 0.05$). There was no modification of redox potential by the ascorbylperoxide in animals infused with solution containing H_2O_2. (B) In absence of H_2O_2, the GSH level was higher in the group devoid of ascorbylperoxide (**0 vs. 20, 60,180 μM; $p < 0.01$). The impact of the ascorbylperoxide was independent of the dose (no difference between 20, 60 and180 μM). There was no modification of the GSH value in animals infused with solution containing H_2O_2. Mean ± S.E.M.; $n = 6$–9.

($F_{(1,49)} = 4.4$ $p < 0.05$), whereas in presence of H_2O_2 the redox potentials were not influenced by ascorbylperoxide ($F_{(3,49)} = 0.7$). The mean value of redox in H_2O_2 groups was of −203 ± 1 mV ($n = 28$). This value was similar to the one measured in the groups 60 and 180 μM ascorbylperoxide without H_2O_2. The modification in redox potential was caused by modification in GSH rather than a change in GSSG value. Indeed, there was no modification in GSSG levels between groups (0.61 ± 0.03 nmol/mg prot, $n = 57$) whereas a significant interaction ($F_{(1,49)} = 4.0$ $p < 0.05$) between ascorbylperoxide and H_2O_2 was observed for GSH values (Fig. 2B). The level of GSH was lower in the lung of animals infused with a solution containing ascorbylperoxide or H_2O_2. The actions of these peroxides were not additive (significant interaction) and the effect of ascorbylperoxide was not dose-dependent ($F_{(1,49)}$ <2.1) (Fig. 2B).

Ascorbylperoxide and H_2O_2 influenced the levels of activated caspase-3 (Fig. 3). The absence of significant interaction ($F_{(3,52)} = 1.0$) suggested an additive effect. In fact, data show that ascorbylperoxide was a linear dose-dependent ($F_{(1,53)} = 5.3$, $p < 0.05$) inducer ($F_{(1,53)} = 24.7$, $p < 0.01$), whereas H_2O_2 had a 15% inhibitory effect ($F_{(1,53)} = 11.3$, $p < 0.01$). The activated caspase-3 is defined as the ratio of cleaved caspase-3 on the total caspase-3 (cleaved + non-cleaved) (Fig. 4). Ascorbylperoxide ($p < 0.01$) and H_2O_2 ($p < 0.05$) had a similar impact on cleaved caspase-3 and on activated caspase-3 whereas they did not influence the level of the non-cleaved enzyme. The low level of non-cleaved caspase-3 in the group 180 μM ascorbylperoxide without H_2O_2 remains without explanation.

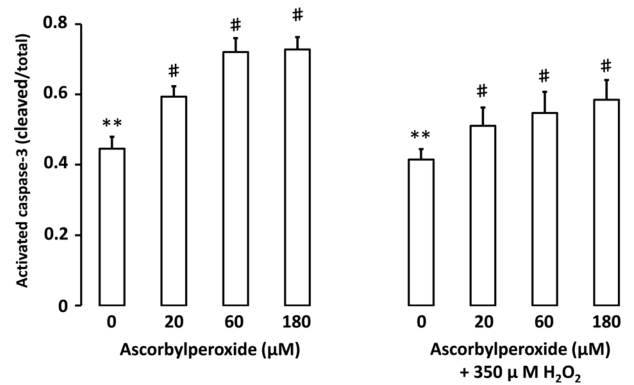


Fig. 3. Influence of ascorbylperoxide and H_2O_2 on the activated caspase-3. The level of activated caspase-3 was increased (**0 vs. 20, 60,180 μM; $p < 0.01$) linearly in function of the received dose of ascorbylperoxide (#between 20, 60 and180 μM; $p < 0.05$) and was lower in groups infused with solutions containing H_2O_2 (with vs. without H_2O_2; $p < 0.01$). Mean ± S.E.M.; $n = 4$–7.

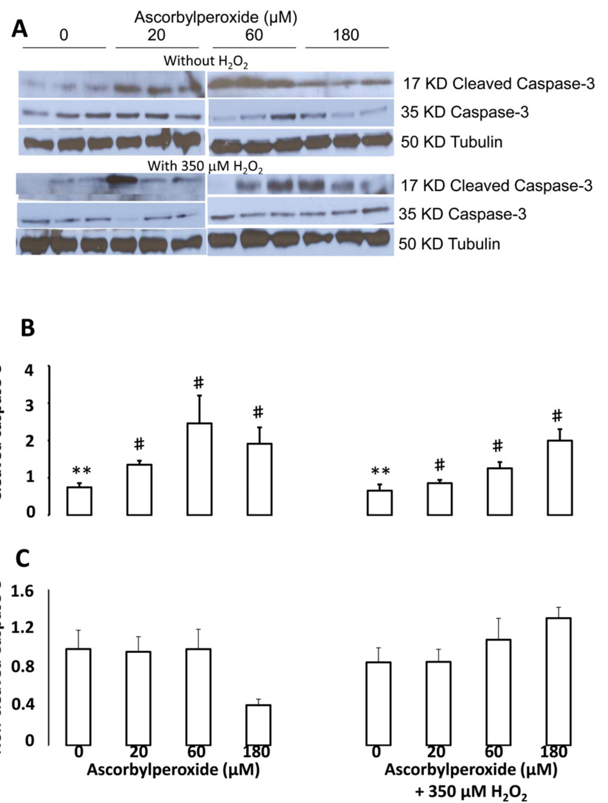


Fig. 4. Influence of ascorbylperoxide and H_2O_2 on cleaved and non-cleaved caspase-3. (A) Representative examples ($n = 3$ per group) of Western blot obtained for the cleaved and non-cleaved caspase-3, relatively to tubulin. (B) The level of cleaved caspase-3 was lower in groups devoid of ascorbylperoxide (**0 vs. 20, 60,180 μM; $p < 0.01$) but increased linearly in function of the dose of ascorbylperoxide (#between 20, 60 and180 μM; $p < 0.05$). The level of cleaved caspase-3 was not affected by the presence of H_2O_2. (C) Non-cleaved caspase-3 was not affected by ascorbylperoxide or H_2O_2, with exception of the 180 μM ascorbylperoxide group ($p < 0.05$). Mean ± S.E.M.; $n = 6$–10.

Despite the negative impact of H_2O_2 on the apoptosis marker (Fig. 3), the alveolarization index (Fig. 5A) was influenced only by ascorbylperoxide ($F_{(1,47)} = 6.8$, $p < 0.05$), not by H_2O_2 ($F_{(1,47)} = 0.1$), without interaction ($F_{(3,47)} = 0.6$). The decreasing in alveolarization index responded to a linear dose-effect of ascorbylperoxide ($F_{(1,47)} = 9.0$, $p < 0.01$). Examples of histology of lungs are shown in Fig. 5B.

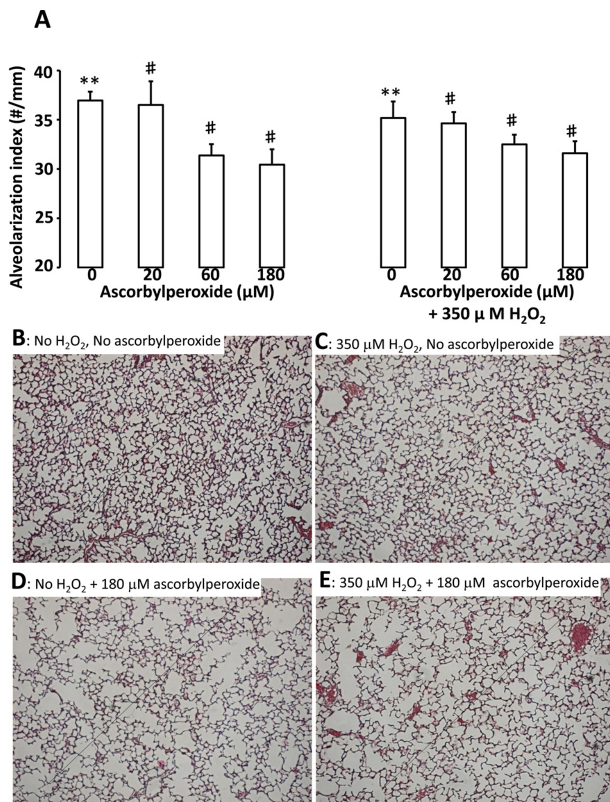


Fig. 5. Influence of ascorbylperoxide and H_2O_2 on alveolarization index. (A) The alveolarization index was higher in groups devoid of ascorbylperoxide (**0 vs. 20, 60,180 μM; $p < 0.05$). The impact of the ascorbylperoxide was linearly dose-dependent (#between 20, 60 and180 μM; $p < 0.01$). The alveolarization index was not affected by the presence of H_2O_2. (B–E) representative examples of histological pictures of lungs from animals infused with a solution (B) devoid of H_2O_2 and of ascorbylperoxide, (C) containing 350 μM H_2O_2 without ascorbylperoxide, (D) containing 180 μM ascorbylperoxide without H_2O_2, or (E) containing 350 μM H_2O_2 and 180 μM ascorbylperoxide. The alveolarization index is based on the number of histological structures crossing the calibrated (1 mm) line. Mean ± S.E.M.; $n = 6–10$.

Due to the difference in effect between both peroxides on alveolarization index, a doubt persisted on the value of the observed redox potentials and on the real oxidative capacity of each peroxide infused during the 4 days of the experiment. Thus, biological markers of oxidative stress, such as NFκB and Nrf2, were measured. Both peroxides were associated with a similar impact on activated NFκB and Nrf2 (Fig. 6A and B, respectively). Their levels were higher in animals receiving H_2O_2 ($F_{(1,49)} = 14.9$, $p < 0.01$) and ascorbylperoxide ($F_{(1,49)} = 8.2$, $p < 0.01$), without a dose-effect ($F_{(1,49)} = 1.0$). The interactions did not reach statistical significance ($F_{(3,49)} = 1.8$).

Examples ($n = 3$ for each group) of Western blot of proteins measured in cytosolic and nuclear fractions are shown in Fig. 7. Complete values are reported in Figs. 8 and 9. NFκB levels in nucleus (Fig. 8A) and in cytosol (Fig. 8B) were higher in animals infused with solutions containing ascorbylperoxide ($F_{(1,49)} = 4.9$, $p < 0.05$), independently of the presence of H_2O_2 (no interaction, $F_{(3,49)} = 2.8$). The effect of ascorbylperoxide was without dose-effect ($F_{(1,49)} = 2.0$). NFκB was lower in the cytosolic fractions of lungs from animals infused with solution containing H_2O_2 ($F_{(1,49)} = 23.1$, $p < 0.01$). H_2O_2 had no effect on values in nucleus ($F_{(1,49)} = 1.5$).

In the nucleus, the interaction between peroxides for Nrf2 was significant ($F_{(3,49)} = 3.0$, $p < 0.05$) (Fig. 9A). The levels of Nrf2 were higher in animals receiving solutions containing both peroxides ($F_{(1,49)} = 9.1$, $p < 0.01$) without dose-effect of ascorbylperoxide ($F_{(1,49)} = 3.9$). Without H_2O_2, ascorbylperoxide was without significant effect ($F_{(1,49)} = 1.6$). In the cytosol, the levels of Nrf2 were

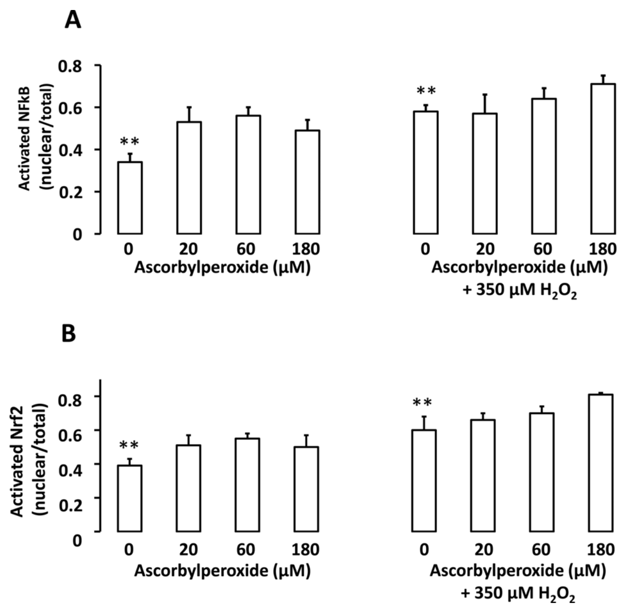
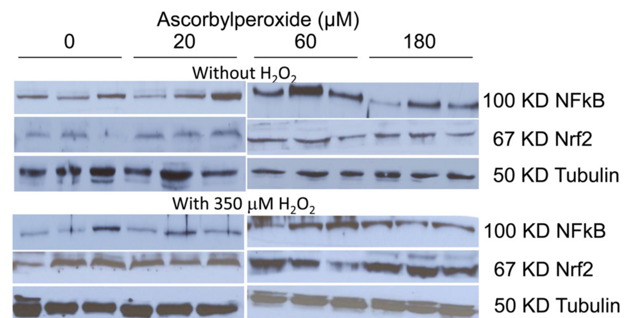


Fig. 6. Influence of ascorbylperoxide and H_2O_2 on the activated NFkB and Nrf2 in lungs. Activation of NFkB (A) and of Nrf2 (B) were lower on groups without ascorbylperoxide (**0 vs. 20, 60,180 μM; $p < 0.01$). However, the impact of ascorbylperoxide was without dose-effect (no difference between 20, 60 and180 μM). There was an additive effect of H_2O_2 ($p < 0.01$) on activation of both factors. Mean ± S.E.M.; $n = 6–8$.



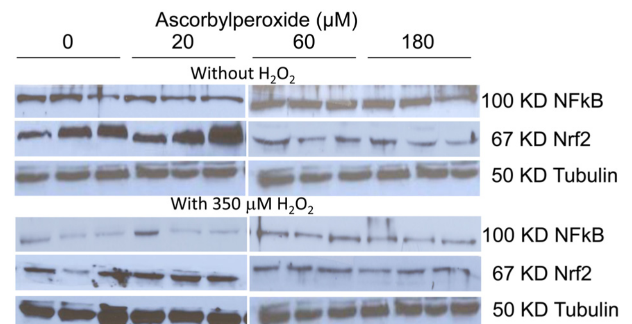


Fig. 7. Representative examples of Western blot results for NFkB and Nrf2 determined in cytosol and nucleus. Three examples of Western blot results (relative to tubulin) for each group of animals infused with 0–180 μM ascorbylperoxide ± 350 μM H_2O_2 on the nuclear (A) and cytosolic fraction (B) of Nrf2 and NFkB in lungs. Full data are shown in Fig. 8-9.

lower in animals infused with solutions containing H_2O_2 ($F_{(1,49)} = 4.5$, $p < 0.05$), whereas ascorbylperoxide was without significant effect

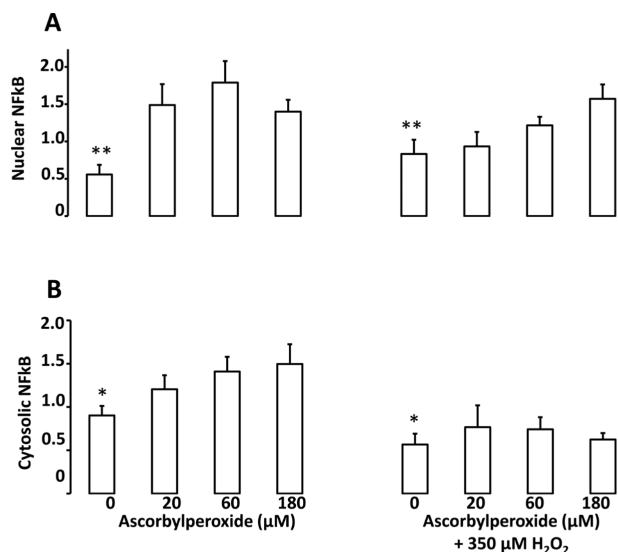


Fig. 8. Influence of ascorbylperoxide and H_2O_2 on nuclear and cytosolic NFkB. (A) Nuclear levels of NFkB were lower on group without ascorbylperoxide (**0 vs. 20, 60,180 μM; $p < 0.01$). The impact of ascorbylperoxide was without dose-effect (no difference between 20, 60 and180 μM). The presence of H_2O_2 had no impact on results. (B) Cytosolic levels of NFkB were lower on group without ascorbylperoxide (*0 vs. 20, 60,180 μM; $p < 0.05$). The impact of ascorbylperoxide was without dose-effect (no difference between 20, 60 and180 μM). Cytosolic levels of NFkB were lower in presence of H_2O_2 ($p < 0.01$). Mean ± S.E.M.; $n = 6$–10.

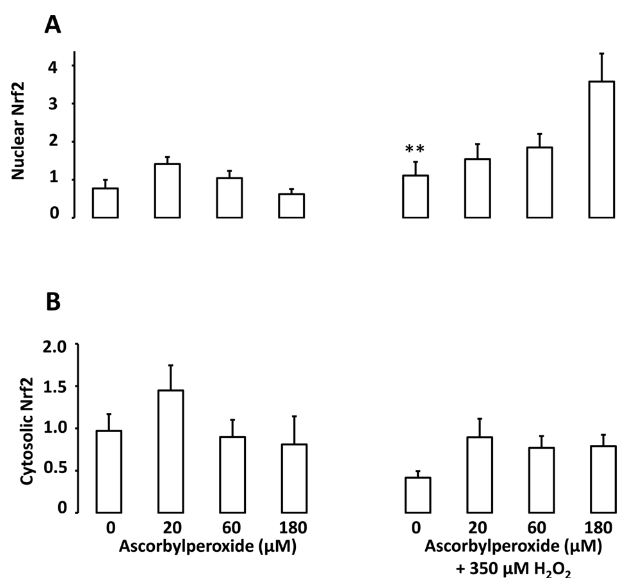


Fig. 9. Influence of ascorbylperoxide and H_2O_2 on nuclear and cytosolic Nrf2. (A) Due to the significant interaction ($p < 0.05$) between ascorbylperoxide and H_2O_2, the effects of the ascorbylperoxide on nuclear levels of Nrf2 have been analyzed according to the presence or not of H_2O_2. In absence of H_2O_2, there was no difference between groups. In presence of H_2O_2, the nuclear level of Nrf2 was lower in the group devoid of ascorbylperoxide (**0 vs. 20, 60,180 μM; $p < 0.01$). (B) Ascorbylperoxide was without effect on cytosolic Nrf2 levels whereas they were lower in animals infused with solutions containing H_2O_2 ($p < 0.05$). Mean ± S.E.M.; $n = 6$–10.

on Nrf2 levels ($F_{(1,49)} = 2.0$). There was no interaction between H_2O_2 and ascorbylperoxide on the cytosolic Nrf2 levels ($F_{(3,49)} = 0.9$).

The efficiency of activation of NFκB by peroxides was evaluated by measuring two molecules derived from his activation. The 4-day exposure to the peroxides could induce inflammation. Thus, IL-6 level (a pro-inflammatory marker) was measured, as well as and PGJ_2 level (an anti-inflammatory marker). There was no significant effect on IL-6 (mean ± S.E.M. = 0.69 ± 0.06 pg/mg prot; $F_{(1,20)} < 3.9$), whereas PGJ_2 levels (Fig. 10) were higher in the H_2O_2 groups ($F_{(1,51)} = 9.4$, $p < 0.01$). Ascorbylperoxide was without significant effect ($F_{(1,51)} = 0.02$) on PGJ_2 level. There was no significant interaction ($F_{(1,51)} = 0.3$).

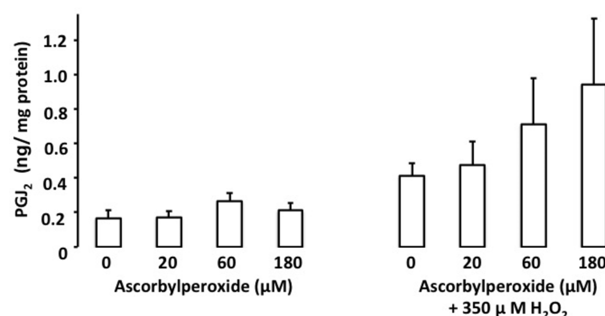


Fig. 10. Influence of ascorbylperoxide and H_2O_2 on PGJ_2. Ascorbylperoxide was without effect on PGJ_2 levels whereas they were higher in animals infused with solutions containing H_2O_2 ($p < 0.01$). Mean ± S.E.M.; $n = 6$–10.

Discussion

This study highlights the toxic effect of ascorbylperoxide, a by-product of peroxidation of dehydroascorbate generated in the solution of parenteral nutrition, on the lungs of our animal model. After 4 days of infusion, this molecule has induced one of the main features of BPD (a lower alveoli number), independently of the presence of H_2O_2.

Consistent with our hypothesis, this study demonstrated that ascorbylperoxide has produced (1) an oxidation of the redox potential, (2) an activation of caspase-3, a marker of apoptosis, and (3) a lower alveolarization index. The second part of the hypothesis was that H_2O_2, a contaminant of parenteral nutrition, exacerbates the oxidative stress and, consequently, influences the impact of ascorbylperoxide. Indeed, H_2O_2 has induced an oxidation of the redox potential that has reached a plateau. The mean redox value measured in animal infused with solutions containing H_2O_2 was similar to that observed in animals infused with solutions containing the two greatest concentrations of ascorbylperoxide (without H_2O_2). This observation suggests that the redox potential might not be the main trigger of the apoptosis. Indeed, although the animals infused with H_2O_2 had the highest redox potential, independently of the presence of ascorbylperoxide, the activation of caspase-3 by ascorbylperoxide was lower by 15% in H_2O_2 groups. Despite this negative effect of H_2O_2 on caspase-3, the activation remained sufficient to induce a decrease in the alveolar number. The effect of ascorbylperoxide on alveolarization index was similar to the values previously reported in studies investigating the effect of parenteral nutrition [14,15].

The discrepancy between redox potential values and activation of caspase-3, especially in H_2O_2 groups, suggests that the modification of the redox potential could be not enough to induce biologically pertinent oxidative stress. The study of the impact of both peroxides on the activation of NFκB and Nrf2 is instructive. The effects of ascorbylperoxide on redox potential, caspase-3 and alveolarization index were linearly dependent to the dose infused. In contrast, the positive impact of this peroxide on activation of NFκB and Nrf2 is not dose-dependent and reached a plateau at the lowest concentration. The levels of NFκB in cytosol and nucleus also reached a plateau, whereas no activation of Nrf2 by ascorbylperoxide was documented in cytosol and nucleus. One may question the relevance of the activation of NFκB by ascorbylperoxide considering the absence of dose-effect between those two molecules. Indeed, levels of IL-6 and PGJ_2, two products of this activation, were not affected by ascorbylperoxide. Thus, the biological oxidative capacity of infused ascorbylperoxide in lungs is

limited. In addition, the action of ascorbylperoxide on redox potential of glutathione is linked to a lower level of GSH rather than elevation of GSSG, an oxidized form of glutathione.

The statistically significant effect of H_2O_2 on activation of NFκB resulted from a lower level of NFκB in the cytosol rather than from a higher level in the nucleus. However, the activation is suggested by the positive impact of H_2O_2 on PGJ_2 levels. Thus, in contrast to ascorbylperoxide, the oxidative effect of infused H_2O_2 is confirmed by the activation of NFκB, the increase of PGJ_2 and the activation of Nrf2. The similarity between the histogram of nuclear Nrf2, shown in Fig. 9A, and the results for PGJ_2 level (Fig. 10) suggest that the activation of Nrf2 in H_2O_2 groups is induced by PGJ_2 [25] rather than by the direct oxidative property of H_2O_2. Thus, independently of its effects on the redox potential of glutathione, ascorbylperoxide seemed to be without biological oxidative capacity in the lungs. Ascorbylperoxide has induced alveolar loss following activation of caspase-3. H_2O_2 exerted a biological oxidative stress, but without an effect on alveolarization index. Those results put into question the relation between oxidative stress and the effect of PN on BPD.

From a previous report by our group, it is known that H_2O_2 infused with parenteral nutrition inhibits the hepatic pathway of transformation of methionine into cysteine, of which the availability is a rate-limiting step in the glutathione synthesis [20]. This is in accordance with the current study in which the increase of redox potential of glutathione was related to a lower GSH rather than to a higher GSSG. The inhibition of hepatic transformation of methionine by PN containing H_2O_2 led to a lower glutathione concentration in liver and in blood [20]. Glutathione in blood circulation is an important source of substrates for the *de novo* cellular synthesis of GSH. Indeed, by-products of the γ-glutamyltranspeptidase action on the glutathione that is in blood circulation are captured by the cell and are processed to a new synthesis of glutathione. A similar inhibition by ascorbylperoxide could also explain the lower level of GSH in lungs from animal infused with ascorbylperoxide alone.

The discrepancy between the positive effect of H_2O_2 on Nrf2 and its negative effect (increase) on redox potential is surprising. Nrf2 is well known to be the nuclear factor that favours a new synthesis of GSH [26]. The absence of concordance between them could be explained by a limiting availability of substrate, specifically cysteine, for the *de novo* synthesis of glutathione. The experimental design involved the use of a solution containing only ascorbylperoxide $\pm$ H_2O_2, without amino acids or lipids. However, glucose was the only carbon substrate added as an energy source. The levels of glutathione in animals infused with these solutions, which were devoid of substrates for GSH synthesis, ranged from 21 to 33 nmol/mg prot (Fig. 2). These levels were similar (16–23 nmol/mg prot) to those reported in a previous study where animals received total parenteral nutrition that included amino acids [14]. Thus, in the present study, the GSH pool seems in equilibrium. Lack of amino acids in nutrition of animals may still limit the *de novo* protein synthesis following gene transcription.

The association between the redox potential of glutathione (measured in lungs from animals infused with solutions containing ascorbylperoxide) and the alveolarization index is in accordance with the association reported by Chessex et al. [19]. Indeed, the aforementioned study described a relationship between the severity of BPD and the redox potential of glutathione measured in whole blood of premature newborns. However, our results suggest that these two parameters are parallel events rather than being interconnected. The discrepancy between pulmonary redox potential and alveolar count has already been reported in newborn guinea pigs infused with parenteral nutrition, despite presence or absence of photo-protection [14].

Activation of caspase-3 leads to cleavage of cellular proteins, a process of irreversible apoptosis [27]. This protease is activated by caspase-8, caspase-9 or caspase-10. Following impairment of mitochondria, cytochrome c is released and transforms the pro-caspase-9 in an active protease [28]. Caspases-8 and 10 are activated following stimulation of receptors such as Fas receptor or Tumour necrosis factor receptor 1 [29]. The redox potential does not seem to play a key role in the activation of caspase-3 and H_2O_2 reduces the activation of caspase-3. These observations suggest that ascorbylperoxide effect on caspase-3 is through the death receptors [29]. From activation of these receptors until the final endpoint of apoptosis: caspase-8 is firstly activated, followed by the activation of caspase-3 by caspase-8. The fact that the later is inhibited by H_2O_2 [30] might explain the negative effect of H_2O_2 on caspase-3 activation, as found in our animal model.

Levels of ascorbylperoxide in urine were lower in animals infused with solutions containing H_2O_2. These animals received intravenous solutions in similar amount to the ones who were infused with solutions devoid of H_2O_2. These findings suggest that animals receiving solutions containing H_2O_2 had a better capacity to detoxify ascorbylperoxide and that the concentration of ascorbylperoxide is important for the activation of caspase-3. The detoxification of ascorbylperoxide could be due to a higher activity of glutathione S-transferases, secondary to the greater activation of Nrf2 in the H_2O_2 group. Further studies are needed to clarify the interactions between ascorbylperoxide, Nrf2 activation, glutathione S-transferases and their effects on alveolar loss.

Ascorbylperoxide is considered a xenobiotic due to its *in vitro* formation (in the parenteral nutrition solution) [17]. However, in animals infused with a solution devoid of ascorbylperoxide but containing H_2O_2, the level of ascorbylperoxide is measurable in the urine (Fig. 1). This could be explained by the interaction between H_2O_2 and dehydroascorbate, elements that are both present in the urine and are known to be precursor of ascorbylperoxide production [16]. The relation between the urinary concentrations of ascorbylperoxide in function of the quantity infused could be useful for a further clinical investigation on the impact of ascorbylperoxide on BPD development.

Conclusion

Ascorbylperoxide generated in parenteral nutrition appears to be related to the loss of alveoli associated to apoptosis induction. Although this molecule induces an increase of the redox potential, findings of the current study suggest that this is a parallel phenomenon to apoptosis. Indeed, the shift in the redox potential toward a more oxidized status, as observed with ascorbylperoxide and with H_2O_2, is perhaps not sufficient to induce apoptosis [18]. However, this modification of the redox value should not be taken lightly. The increase of the redox potential obtained with ascorbylperoxide or H_2O_2 could influence the proliferation and the differentiation of cells [18]. Pulmonary development, such as in premature newborns, must go through cellular proliferation, differentiation and apoptosis for the remodeling of the lungs, until they reach their final maturity. Bronchopulmonary dysplasia may result from the perturbation of several of these cellular states. In our experimental model, we have exposed the association between the loss of alveoli, a feature of bronchopulmonary dysplasia, and ascorbylperoxide. The impact of ascorbylperoxide on other cellular stages and on the nature of the affected cells should be the focus of future investigations. Nevertheless, since the generation of ascorbylperoxide is dependent on light exposure [17], the present study supports the beneficial effect of photo-protection of parenteral nutrition on chronic lung disease [12], such as BPD [13] in neonates. Our results add an important piece of information regarding the mechanism by which parenteral nutrition, as presently compounded, can induce BPD development.

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