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Mitochondrial DNA damage analysis in bronchoalveolar lavage cells of preterm infants

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1. ABSTRACT

In mechanically ventilated preterm infants, the combination of immaturity, volutrauma, oxidative stress, and inflammatory processes can lead to chronic lung injury. Mitochondrial DNA (mtDNA) is more susceptible to oxidative damage than nuclear DNA. We aimed to investigate the level of mtDNA damage (deletions, mutations and changes in copy number) in bronchoalveolar lavage (BAL) cells from 10 preterm infants (27-30 weeks). A first BAL (BAL1) was done within 24 h of endotracheal intubation and BAL2 was performed 30-103 h thereafter. Deletions were analyzed by long range PCR, point mutations by heteroduplex analysis of the D-loop region, and copy number changes by real-time PCR. Using these methods, no deletions were found in any of the BAL samples. When BAL1 and BAL2 samples were compared no new mutations were found. In contrast, a marked decrease in mtDNA copy number was observed in 5 patients. In conclusion, we found that exposure of preterm infants to short term mechanical ventilation did not lead to detrimental consequences for the mtDNA in the form of mutations or deletions.

2. INTRODUCTION

In mechanically ventilated preterm infants, immaturity in combination with oxidative stress, volutrauma, and inflammatory processes can lead to chronic lung injury (1-6). Oxidative damage is found very early in infants subsequently developing chronic lung disease and knowledge is now accumulating on how reactive oxygen species (ROS) trigger cellular and molecular changes that may impair normal pulmonary growth and development (1, 2).

Mitochondrial DNA (mtDNA) is more susceptible to oxidative damage and consequently acquires mutations at a higher rate than nuclear DNA (7). This is due to exposure to high levels of ROS generated during respiration, lack of protective histones, and limited capacity for repair of DNA damage (8). Significant damage to mtDNA will compromise cellular respiration, resulting in elevated levels of ROS that cause further injury to mitochondrial and nuclear DNA and, therefore, potentially contribute to chronic lung disease (8, 9). In this study, we hypothesized that mechanical ventilation would induce

mtDNA damage in the lung of preterm infants. To test our hypothesis, we conducted a pilot study evaluating mtDNA deletions, mutations and changes in copy number in bronchoalveolar lavage (BAL) cells from preterm infants following endotracheal intubation and within 2-4 days of mechanical ventilation.

3. PATIENTS AND METHODS

The study was approved by the Maastricht University Medical Centre Ethics Committee and fully informed, written consent was obtained from the parents of each infant. The study population consisted of ten intubated, mechanically-ventilated preterm infants, whose clinical information is summarized in table 1. The information about the characteristics of the mechanical ventilation used during the study period is summarized in table 2. The ten children received surfactant (Curosurf 200 mg/kg; Chiesi Farmaceutica, Parma, Italy) within 2h after birth and at least 6 h before the first BAL. Patients1, 8, 9, and 10 received a second surfactant dose between the two BAL procedures.

3.1. BAL-cell collection and processing

A first BAL (BAL1) was done within 24 h of endotracheal intubation and a second (BAL2) was performed 30-103 h (median 48.5 h) thereafter. BAL was performed as previously described (10). Briefly, with the infant lying supine and the head turned to the left, a 6 F suction catheter was gently inserted into the endotracheal tube until resistance was felt. Then, 1ml/kg birth weight of sterile isotonic saline was gently infused into the lung. After 5 s, suctioning was performed while slowly retracting the catheter. The procedure was repeated once after which the collected fluid was pooled and placed on ice. From the pooled aspirate, aliquots were taken for total and differential cell count. Haemocytometer counting was used to obtain total cell counts per ml of BAL fluid and a differential cell count was performed on cytospins of BAL cells using May-Grünwald-Giemsa stain. 500 cells were counted on each cytospin. The remainder of the BAL fluid was centrifuged at 300 x *g* for 10 minutes at 4 °C and the supernatant and the cells were stored at -80°C until analysis. All subsequent analyses were done by investigators who were blinded to the clinical features of the patients.

3.2. Detection of deletions in the mitochondrial genome

Total DNA was isolated according to the manufacturer's procedure using the QIAmp^R DNA mini kit from Qiagen. A deletion PCR was performed for the whole mitochondrial DNA. Two primer pairs were used, yielding two mtDNA fragments of 16.1 and 16.0kb in size, respectively. Primer sequences for fragment one were 5'-CCGCTTCTGGCCACAGCACTTAAACACATC-3', at location 0314-0343, and 5'-GGAGGATGGTGGTCAAGGGACCCCTATCTG-3', at location 16411-16382. For fragment 2 primers 5'-CAAGGTGTAGCCCATGAGGTGGCAAG-3', located at 01330-01355, and 5'-GCTGCATTGCTGCGTGCTTGATG-3', located at 00778-00756, were used. The PCR mix consisted of Expand Long Template buffer (10x; 27.5mM $MgCl_2$), dNTP-mix containing 8.33mM of each base, 100ng per microliter forward and reverse primer, 5U per microliter Expand Long Polymerase and 50ng of DNA in a total volume of 50 microliter. The PCR was performed using a PCR Perkin Elmer type 9700. First denaturation was achieved at 98°C for 30 s, followed by 30 cycles of 10 s of denaturation at 98°C and 8 min 15 s annealing at 72°C, followed by 10 min of elongation at 72°C. The PCR products were stored at 4°C until use. 25 microliter PCR-products were loaded on ethidium bromide stained 0.7% agarose gels in TAE-buffer and run for 16 hours at 32V. After 16 hours a picture was taken of the PCR products in the gel and the gel was run for another 16 hours at 32V. Samples from the DNA bank of our institution were used as controls. The positive control was DNA obtained from muscle tissue of a 45-year-old female, whose mtDNA contained several deletions as a consequence of aging. The negative control was a DNA sample without mtDNA deletions.

3.3. Mutation detection in the mitochondrial D-loop

Another set of PCRs was performed to yield three fragments (fragment 1, 2 and 3 from the MitoScreen^{TM} Assay Kit, Transgenomic, Elancourt, France) spanning the mutation hot spot D-loop region of the mtDNA, that were subjected to denaturing HPLC (DHPLC) analysis. The first primer pair (5'-CTCCACCATTAGCACCCAAAGC-3' and 5'-GAGGATGGTGGTCAAGGGACC-3') was used to amplify the region between 15974-16409bp, the second primer pair (5'TACAGTCAAATCCCTTCTCGTCC-3' and 5'-TCCAGCGTCTCGCAATGCTATC-3') was used to amplify the region between 102-16341bp, and the third primer pair (5'-CTCACGGGAGCTCTCCATGCAT-3' and 5'-ATTAGTAGTATGGGAGTGGGAGG-3') was used to amplify the region between 29-480 bp. The PCR mix contained 10* Optimase® reaction buffer with $MgSO_4$, dNTP mix containing 10mM of each dNTP, Optimase Polymerase 2.5U, 100ng DNA, and 100ng of both primers in a total volume of 50µl. The PCR was performed using a Thermocycler (Perkin Elmer type 9700). Denaturation was achieved at 95°C for 2 min, then the samples were subjected to 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 56°C, 3 min of elongation at 72°C, followed by 5 min of elongation at 72°C. PCR products were stored at 4°C until use. Heteroduplex formation was achieved by heating the PCR products at 95°C for 5 min. Then the samples were cooled at a rate of 1.5°C/min until a temperature of 25°C was reached. Next, the samples were placed in the WAVE® nucleic acid fragment analysis system for analyses. The DNA was initially mixed with tri-ethyl ammonium acetate, which functions as an ion pairing reagent. This enables size based binding of the hydrophilic DNA to the hydrophobic DNASep® Matrix. Heat was used as a denaturant, in conjunction with the hydrophobic eluant acetonitrile, which lead to elution of the DNA from the matrix according to helicity and size. DNA containing a heteroduplex (and thus a mutation) will elute earlier from the matrix than the homoduplex species. In this way the chromatogram pattern changes and the presence of a mutation can be detected. WAVE® Control Standards

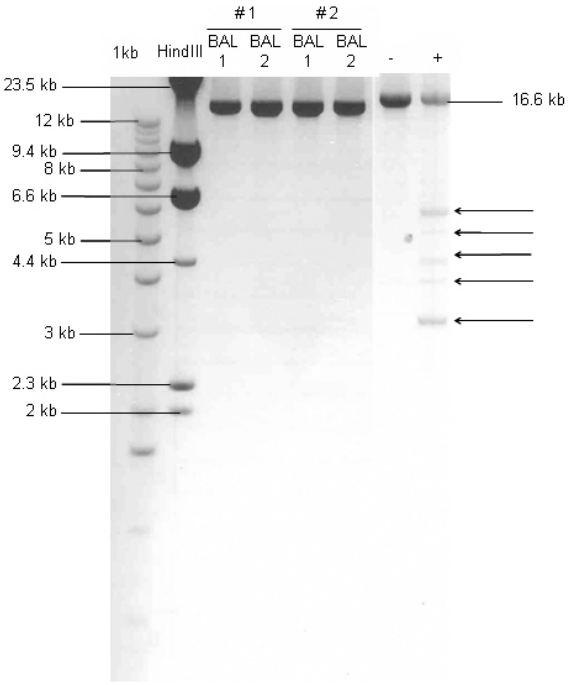

Figure 1. Representative examples of deletion PCR products from patient 1 (lane 3 and 4) and 2 (lane 5 and 6). Positive and negative controls are shown in lane 7 and 8 respectively. A 1 kb DNA ladder (lane 1) as well as a HindIII ladder (lane 2) were used to identify the PCR product size. A size of about 16kb is a PCR product from DNA without deletions. The positive control (muscle tissue from a 45-year-old female with known deletions from aging) shows several products (arrows), apart from the 16kb fragment, indicating deletions in part of the mtDNA. *BAL1:* first BAL*; BAL2:* second BAL

were used to ensure quality during the analysis. This includes the WAVE DNA Sizing Control Sample, the Low Range Mutation Control Standard, the Mid Range Mutation Control Sample and the High Range Mutation Control Standard. The temperatures at which 8µl of the PCR products was analyzed by DHPLC were optmized for each individual fragment: 50°C and 58°C for fragment 1, 50°C and 60°C for fragment 2 and 50°C, 57°C and 59°C for fragment 3. The resulting chromatograms from BAL1 and BAL2 were compared between each other and with standards.

3.4. mtDNA copy number analysis

To analyze potential changes in copy number of mtDNA as a consequence of mechanical ventilation, Real-Time PCR was performed for the nuclear RNase P gene and the mtDNA encoded 12S-RNA gene. The PCR mix contained Taqman Universal PCR Master Mix, TaqMan^{R} RNase P Control Reagents Kit (VIC^{TM} Probe), primers 12S (3'-CCCCAGGGTTGGTCAATTT-5' and 3'-CTATTGACTTGGGTTAATCGTGTGA-5') and Taqman^{R} probe 12S (3'-TGCCAGCCACCGCG-5') 6pmol. The PCR was performed in an ABI Prism 7000 Sequence Detection System. Amplitaq Gold activation was obtained at 95°C for 10 minutes, followed by 40 cycles of denaturation for 15 s at 95°C and annealing and extension at 60°C for 1 min. A standard DNA dilution was made to plot a standard curve to determine the efficiency of the PCR. Every sample was analyzed in duplicate. The efficiency was calculated by the formula Eff = $10^{-1/slope}$. Relative copy number (Rc) was calculated by expressing threshold cycle number differences of the nuclear gene and the mitochondrial gene PCR, as described by Ritov *et al* (11): $Rc=2^{(Ct\ nDNA-Ct\ mtDNA)}$, where Ct is the threshold cycle number.

4. RESULTS

Total and differential BAL cell counts are presented in Table 3. More detailed BAL cytospin data from patient 3, who experienced severe pneumonia, have previously been reported (12). When the mtDNA of the BAL cells was screened for deletions none of the samples showed positive results. Figure 1 shows an example of the PCR products that were exposed to gel electrophoresis for 32 h. The positive control shows several bands, which indicate deletions in the DNA of this sample (muscle tissue from a 45-year-old female, whose mtDNA contained several deletions as a consequence of aging). In contrast, no extra bands are observed in the mtDNA of BAL1 and BAL2 samples, as well as in the negative control.

When BAL1 and BAL2 samples were compared, no newly formed mutations in the D-loop of the mtDNA were observed. Figure 2 shows an example of the DHPLC-output from BAL1 and BAL2 DNA of two children. As can be seen in the figure, no new heteroduplexes were found in the analyzed products.

The results of the mtDNA copy number analysis are presented in Table 4. One of the samples was not successfully amplified despite the availability of ample genetic material. Five of the BAL2 samples analyzed showed a decrease in the relative mtDNA copy number when compared to the corresponding BAL1. Two of the samples showed no change and two showed an increase in mtDNA copy number. The efficiency of this PCR was 1.97.

5. DISCUSSION

Documentation of mtDNA damage *in vivo* after acute insults is sparse compared with the number of *in vitro* studies that have demonstrated oxidative injury to mtDNA (13). To the best of our knowledge, this is the first study addressing the putative mtDNA damage induced by mechanical ventilation in humans. We observed, in a population of ten preterm infants, that 2-4 days of mechanical ventilation with oxygen concentrations up to 100%, and in some cases in combination with NO, did not induce any detectable mutation or deletion in the mtDNA isolated from BAL cells. However, in five of the samples a decrease in mtDNA copy number was detected.

Our findings raise several points of discussion. First of all, whether the methods used are sensitive enough to detect mtDNA deletions and mutations. The deletion PCR we constructed is considered as a sensitive method

Table 1. General characteristics of the patients

Nr	Sex	Gestational age (wks)	Birth weight (g)	HC	Prenatal steroids	Cord pH	Oxygen 28 days	Oxygen 36 wks	Days on MV	Died	Cause of Death
1	M	28+5	1100	-	+	7.29	-	-	6	-	
2	M	30+2	880	-	+	7.23	died	died	12	day 11	Sepsis
3	F	28+0	490	-	+	7.28	+	+	110	day 112	BPD
4	M	29+2	1290	-	+	7.35	-	-	3	-	
5	M	27+3	1110	+	-	NA*	-	-	9	-	
6	F	27+4	940	-	-	7.02	+	died	37	day 50	IVH
7	F	28+6	1240	NA	+	7.06	-	-	5	-	
8	M	29+3	1180	NA	+	7.10	+	-	6	-	
9	F	27+2	980	NA	+	7.39	+	+	10	-	
10	M	28+0	1135	-	+	7.31	+	+	8	-	

HC=histologic chorioamnionitis; MV=mechanical ventilation; BPD=bronchopulmonary dysplasia; IVH=intraventricular haemorrhage; Cord pH = umbilical artery blood pH; NA=not available. * This child was born at home and transported to the hospital with cyanosis and hypothermia. Blood lactate was 7.9 mmol/l.

Table 2. Ventilatory requirements during admission and between BAL procedures

	Before BAL1	Between BAL procedures							
Nr	Max FiO_2 (%)	Max FiO_2 (%)	FiO_2>40% (% of time)	NO (% of time)	Ventilator mode	MAP (mean, cmH_2O)	MAP (max, cmH_2O)	Mean peak pressure* (cmH_2O)	Max peak pressure* (cmH_2O)
1	100	25	0	70	HFV	12	14	16	20
2	100	30	0	0	HFV	8	10	14	17
3	100	65	20	0	IMV	9	10	16	19
4	50	30	0	0	IMV	9	10	15	20
5	100	35	0	100	HFV	8	9	16	21
6	35	30	0	0	IMV	9	10	16	20
7	45	25	0	0	IMV	10	11	18	20
8	30	35	0	0	IMV	11	14	19	25
9	80	35	0	0	HFV	11	13	24	27
10	65	45	10	0	HFV/IMV	10	14	24	29

For HFV ventilation, peak pressures represent amplitude values. HFV=high frequency ventilation; IMV= intermittent mandatory ventilation; MAP=mean airway pressure. *amplitude for HFV

Table 3. Cell content in BAL-fluid

	BAL1	BAL2		
	median	range	median	range
cell count (*10^{-6} cells/ml)	0.93	0.30-4.27	0.80	0.46-8.82
Macrophages (%)	25	5-88	58	25-72
Neutrophils (%)	73	11-95	38	10-73
Lymphocytes (%)	0.7	0-2.0	0.8	0-19
Eosinophils (%)	0	0-1.0	0	0-0

Table 4. Relative mtDNA copy number

#	BAL1	BAL2
1	-	-
2	248.24	466.45
3	133.90	195.76
4	1243.33	130.33
5	311.32	190.71
6	455.50	201.39
7	251.75	275.03
8	406.42	426.29
9	208.69	74.96

Relative copy number was calculated by the formula $2^{(Ct\ nDNA-Ct\ mtDNA)}$ where Ct is the threshold cycle number of the nuclear gene and the mitochondrial gene PCR (see methods for details).

with a detection limit of 0.5-1% (14, 15). Single base mutations in mtDNA can not be detected by deletion PCR. Therefore we performed heteroduplex analysis using the DHPLC method (16, 17) to screen the D-loop region (a mutation hot spot region in the mtDNA) (18). This method has a 97-100% efficiency of mutation detection (confirmed by sequencing the abnormal PCR-products) and a threshold of 1-5% for detection of a specific mutation (16, 17). Mutations can randomly occur across the DNA fragments and it can be expected that each mutation would eventually lead to a heteroduplex and an altered migration pattern producing altered peak or peaks, which would contain mixtures of different mutations. Differences in these peak patterns were not observed when BAL1 and BAL2 samples were compared, indicating that new mutations were not present. Other techniques, with very high sensitivity for mutation detection have been recently described. One mutation among 109 bases can be detected with the random mutation capture (RMC) method (19, 20). Whether this method could detect mutations that we did not find with the DHPLC method remains to be investigated

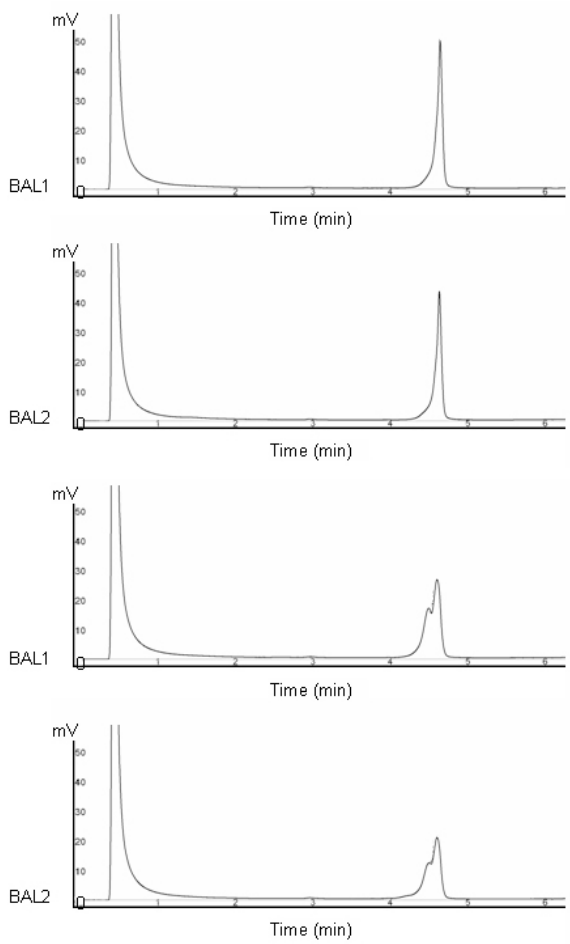

Figure 2. Representative examples of the DHPLC analysis for mutations in the mtDNA. The *x*-axis represents the retention time in minutes, which is dependent on the length of the product analyzed; the *y*-axis is a measure of absorbance (converted to mV). The two upper panels show an example of a normal homoduplex pattern in both BAL1 and BAL2 of patient 7. The two lower panels show the presence of similar heteroduplexes in both BAL1 and BAL2 of patient 9, indicating the presence of mutations that did not change between the two BAL procedures.

Also the intensity and duration of the insult in the studied population could be related to our negative results. It can be argued that higher levels of or longer exposures to oxidative stress would be necessary to induce mtDNA deletions or mutations. The majority of the patients in our population were exposed to high concentrations of oxygen at birth, when resuscitation was performed. A preceding hypoxic period appeared to be present in four cases (Table 1) Afterwards, between the two moments of sampling, patients were generally exposed to gentle mechanical ventilation and the majority did not need oxygen-rich gas. With the advent of surfactant therapy and the widespread use of antenatal steroids, the clinical picture of infant RDS changed considerably (1). Nowadays, preterm newborns often require little supplemental oxygen during their initial postnatal course, and it is uncommon for such infants to receive mechanical ventilation with high inflation pressures or large tidal volumes (1). Interestingly, it has been suggested that the inflammatory response is more likely than hyperoxia to lead to pulmonary oxidative damage in mechanically ventilated preterm infants (5). On the other hand, our understanding of the ill effects of brief oxygen exposure at birth has been increased significantly and it has been shown that hyperoxia, even for short periods and particularly following a previous hypoxic insult, contributes to the development of several neonatal morbidities (1, 2, 21-23). In this regard, oxygen administration in the delivery room has become a matter of discussion in the last years (21-24). Moreover, an association has been suggested between the DNA damage induced by post-hypoxic neonatal exposure to high oxidative stress and childhood cancer (23, 25, 26). In the present work, we analyzed mtDNA, which is more susceptible to oxidative damage than nuclear DNA (7). However, our data suggest that in a normal clinical setting of mechanical ventilation DNA deletions or mutations are not produced or, alternatively, they have been repaired, although repair systems are less efficient for the mtDNA than for the nuclear DNA (7, 8).

There is overwhelming evidence from *in vitro* studies that oxidative stress damages DNA (7, 8, 27). Also, *in vivo* experimental studies using exposure to hyperoxia in premature baboons (28) neonatal rats (29), adult rats (30, 31) and adult mice (32) demonstrated pulmonary oxidative nuclear DNA damage. The urinary levels of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative DNA damage, were significantly higher in preterm infants requiring supplemental oxygen and ventilator support than in non-mechanically ventilated preterm and healthy term neonates (33). To the best of our knowledge, only one study has analyzed pulmonary mtDNA integrity after *in vivo* hyperoxic exposure (13). In consonance with our results, Lightfoot *et al.* observed that hyperoxia (24-48 h of 95% oxygen in adult rats) failed to induce significant injury to mtDNA and only when combined with NO inhalation transient mtDNA damage was produced. They speculate that efficient removal of ROS by mitochondrial antioxidant systems or upregulation during hyperoxia of mtDNA repair systems may be the explanation for the lack of injury in the mtDNA (13).

In five cases, we found a decrease in the relative mtDNA copy number between BAL1 and BAL2. With our limited sample size, we can only speculate about this finding. As different cell types contain different amounts of mtDNA, our finding may simply reflect the differences in BAL cell composition between the two moments of sampling. The majority of the studies dealing with the effects of oxidant stress on mtDNA copy number have been focused on chronic exposure to the insult and they demonstrated an increase in the copy number (see 8 for a review). This has been suggested to be a compensatory mechanism for defective mitochondria bearing impaired respiratory chain or mutated mtDNA (8). Because ROS generation by the mitochondrial respiratory chain is a continuous physiological occurrence, mitochondria have an efficient antioxidant system composed of superoxide

dismutase, glutathione peroxidase, glutathione reductase, glutathione, NAD(P) transhydrogenase, NADPH, vitamins E and C, thiol peroxidases and mitochondrial respiration itself (34, 35). However, when the capacity of antioxidant system is compromised, the exposure to higher oxidative stress may result in an increase of defective mitochondria beyond a non-compensable level and thus, a cyclic increase in ROS production leading to mitochondrial lipid peroxidation and a decrease in mtDNA copy number (8, 27, 36). This could drive the cell into an irreversible cell death process (8, 37) that would lead to an increase in extracellular mtDNA with proinflammatory effects, thereby creating a vicious circle of inflammation and cell destruction (38). Although it is generally accepted that extracellular and cytosolic antioxidant defenses are reduced in preterm infants (2, 4), this has not been demonstrated for the mitochondrial antioxidant system. Therefore, the putative maturational changes in the mitochondrial antioxidant and DNA reparation systems and their possible role in chronic lung disease require further investigation.

Although the pathogenesis of chronic lung disease of the premature infant remains unclear, much evidence suggests that reflects abnormal growth and repair of the immature lung exposed to the continuous stress of repetitive inflation with oxygen -rich gas in a setting of chronic inflammation, often aggravated by recurrent infection (1). An important part of these pathogenetic insights has been obtained through the analysis of BAL fluid and cells (3-6, 10, 12, 39). Our pilot study proposes a new approach for the evaluation of lung injury and shows that the analysis of BAL cell mtDNA is feasible tool for testing the putative effects of oxidative stress during mechanical ventilation. However, and against our initial hypothesis, we could not find any detectable mtDNA mutation or deletion. The validity of our findings is limited by several factors such as the sample size, the relative inhomogeneity of the sample, the lack of parallel measurement of other oxidative stress markers, or the relative short exposure to the oxidative stimulus. The idea of our study was based on three generally accepted paradigms: I) Preterm newborns are very susceptible to oxidative stress (2, 4, 21, 23, 24, 33); II) Oxidative stress plays a significant role in the pathophysiology of ventilator-induced injury (1, 2, 4); and III) mtDNA is highly vulnerable to oxidative stress (7-9). Further studies including larger samples, which include longer periods and different strategies of mechanical ventilation, as well as different methods to evaluate mtDNA damage and function will be needed to assess whether the above paradigms also apply to the real clinical situation of mechanically-ventilated preterm infants.

6. ACKNOWLEDGEMENTS

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Abbreviations: BAL: bronchoalveolar lavage; mtDNA: mitochondrial DNA; ROS: reactive oxygen species

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