Biomarkers for oxidative stress in acute lung injury induced in rabbits submitted to different strategies of mechanical ventilation

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Mechanical ventilation, the main supportive treatment in ARDS (11), can cause inflammatory lung injury due to large inspired tidal volumes or high ventilator inspiratory pressures, a typical drawback with conventional mechanical ventilation (CMV) (22, 45). An attractive alternative, high-frequency oscillatory ventilation (HFOV), uses a lower tidal volume at a higher frequency than normal breathing, thus avoiding higher alveolar pressures and volume excursions (3, 18, 43).

Total antioxidant performance (TAP) assay is used to determine overall antioxidant defense/oxidative stress by evaluating the biological antioxidant network between water- and fat-soluble antioxidants and their interactions (49) against oxidative stress. This method has been validated by Beretta et al. (6) and applied to tissues (13, 15, 36). It is based on a lipophilic radical generator (MeO-AMVN) and a lipophilic oxidizable substrate (BODIPY) that specifically measures the lipid compartment oxidizability related to fat-soluble antioxidants, and to water-soluble antioxidants, acting through a synergistic/cooperative mechanism (6).

Single-cell gel electrophoresis (SCGE), also known as comet assay, is a standard method for assessing DNA damage. It is a fundamental research tool (9, 10, 16, 23, 32, 33) for assessing DNA double-strand breaks, single-strand breaks, alkali-labile sites, and incomplete repair of a-basics and crosslinks. It has significant advantages over other genotoxicity tests, which include its fairly simple methodology, high sensitivity, requirement for a small number of cells, and rapid data production (26, 41). Comet assay routinely uses lymphocytes to assess DNA damage in human biomonitoring (12); however, it can be used with any tissue provided a single cell/nucleus suspension can be obtained (21).

Recently, we have reported that HFOV attenuates oxidative lung injury by comparing CMV and HFOV for oxidative stress, assessed by TAP assay in a rabbit model of acute lung injury (ALI) induced by surfactant depletion (36). Based on our previous research, the objective of the present study was to evaluate the correlation between oxidative stress/antioxidant performance in peripheral blood and target tissue. To the best of our knowledge, there is no study yet available evaluating overall antioxidant performance and oxidative stress determined by TAP and comet assay in an animal model of ALI. Moreover, it is still not clear whether the oxidative damage in peripheral blood can reflect oxidative damage in target tissue. Such an approach would be useful, when target tissue cannot be accessed, to establish prognosis and to apply newer therapeutic strategies.

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MATERIALS AND METHODS

Chemicals

N-lauroyl sarcosinate, dimethyl sulfoxide (DMSO), sodium chloride (NaCl), sodium hydroxide (NaOH), normal and low melting point agarose, Tris, and EDTA were purchased from Sigma Chemical (St. Louis, MO); Triton X-100 and chlorohydric acid (HCl) were from J. T. Baker (Phillipsburg, NJ); Syber Green was from Trevigen (Gaithersburg, MD); and trypan blue was from Merck (Darmstadt, Germany).

Design, Animals, and Instrumentation

Thirty-two male Norfolk white rabbits, weighing 2.0–3.0 kg, were anesthetized with ketamine (50 mg/kg im) and xylazine (2 mg/kg im). A tracheotomy was performed by inserting a tracheal tube (3.0- to 3.5-mm ID; Portex, Hythe, UK), secured in position with umbilical tape. Immediately after tracheotomy, ventilation was initiated with a Galileo Gold ventilator (Hamilton Medical). In addition, five healthy rabbits were studied for oxidative stress.

Anesthesia was maintained with continuous intravenous infusion of ketamine (10 mg·kg⁻¹·h⁻¹). Muscle paralysis was induced by intravenous administration of pancuronium bromide (0.2 mg/kg) and maintained with 0.1-mg/kg doses as needed to control movement. Euthanasia was performed by high doses of intravenous administration of ketamine and xylazine.

Rabbits were cared for, minimizing discomfort, distress, and pain in accordance with the guidelines by the National Institutes of Health. This study was approved by the Experimental Research and Ethics Committee of Botucatu Medical School-Sao Paulo State University and Jean Mayer U.S. Department of Agriculture-Human Nutrition Research Center on Aging at Tufts University.

Experimental Groups

Animals (n = 37) were assigned to three groups: 1) healthy group (HG, n = 5; without lung injury and mechanical ventilation); 2) ALI + CMV group [CMV, n = 16: tidal volume (VT) 6 ml/kg, positive end-expiratory pressure (PEEP) 10 cmH₂O, plateau pressure limited to (less than or equal to) 20 cmH₂O in the Galileo Gold ventilator (Hamilton Medical); and 3) ALI + HFOV group [HFOV, n = 16: mean airway pressure (Paw) 12–14 cmH₂O, respiratory rate 10 Hz, inspiratory time of 33%, and initial pressure amplitude of 20 cmH₂O, in the SensorMedics 3100A ventilator (Viasys Healthcare, Yorba Linda, CA)]. In the CMV, pressure-regulated volume control mode was used, and respiratory rate was maintained at 40–50 breaths/min to reach targeted arterial Paco₂ (Paco₂) (40–45 mmHg), as pressure amplitude in HFOV was modified to the same Paco₂ level. The fraction of inspired oxygen (P'O₂) was maintained at 1.0 throughout the experiment for all groups. Ventilator settings were chosen according to previous studies using the same model (15, 22, 36, 38).

Lung Injury Induction

Lung injury was induced by lung lavage with 30 ml/kg aliquots of 0.9% warm saline solution (38°C) as previously described (22, 38). This is one of the most commonly used animal ALI models (29). It is primarily a surfactant depletion model, which causes a lung injury very similar to human ARDS in its effects on oxygenation, respiratory system compliance, atelectasis, and edema perivascular/peribronchial.

After lung injury induction animals were ventilated during 4 h using the mechanical ventilators described above. This period of the experiment was chosen taking into consideration of the viability of the rabbits submitted to this protocol (15, 22, 38), as these animals are very sensitive to hypotension.

Sample Collection

Isolated lymphocytes and cells recovered from target tissue were analyzed by comet assay to determine DNA damage (%DNA in tail). TAP assay was applied to measure overall antioxidant performance (%protection) in plasma as well as lung tissue.

Lung tissue collection. Lung was dissected and stored for oxidative stress analysis. Tissue specimens were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Lymphocyte isolation, cryopreservation, and recovery. Lymphocytes from high-frequency oscillatory ventilation group (n = 15 animals) were immediately isolated after the blood collection. Isolation was performed by density gradient sedimentation (Histopaque 1077, Sigma Diagnostics) and frozen in a mixture containing 50% fetal bovine serum, 40% culture medium (RPMI 1640, Sigma Diagnostics), and 10% dimethyl sulfoxide at a freezing rate of −1°C/min to a final temperature of −80°C before being stored in liquid nitrogen (50). Cells were recovered by submersion in a 37°C water bath. Cells were placed in a prechilled 50% RPMI 1640 medium combined with 50% fetal bovine serum, then centrifuged at 200 g for 5 min at 4°C. Cells were resuspended in 250 μl of cold PBS and checked for

Fig. 1. Oxidation kinetics of phosphatidylcholine (PC) liposome control suspension used as the reference biological matrix. DHS, delipidized human serum; HG, healthy group; CMV, conventional mechanical ventilation group; HFOV, high-frequency oscillatory ventilation group.
viability (typically ≥95% viability) and cell number (typically 1 × 10⁵ cells/ml).

Lung tissue preparation for comet assay. Techniques previously described (31, 39) with minor modifications were followed. Briefly, frozen lung tissue (200 mg) was carefully minced and homogenized in 2 ml of cold PBS (20 mM EDTA) using a Potter homogenizer (Wheaton Instruments) for cell preparation. The suspension of cells was collected and left on ice for 10 min, then centrifuged at 200 g for 5 min at 4°C. Cells were resuspended in 250 µl of cold PBS and checked for viability (typically ≥95% viability) and cell number (typically 1 × 10⁵ cells/ml).

Single-Cell Gel Electrophoresis Analysis (Comet Assay)

DNA strand breaks were measured in cells obtained from lung tissue and peripheral blood using comet assay as proposed by Singh et al. (40), Tice et al. (42), and Collins (10), with some modifications. Volumes of 20 µl of cells obtained from lung tissue homogenate or peripheral blood were added to 150 µl of 0.5% low-melting-point agarose at 37°C. From the mixtures, 130 µl was layered onto slides precoated with 1.5% normal agarose, covered with a coverslip, and left for 5 min at 4°C to solidify the agarose. Subsequently, the coverslips were carefully removed and the slides immersed in a lysis solution for 24 h. Then, slides were washed with PBS for 5 min and immersed in a freshly prepared alkaline buffer [1 mM EDTA, 300 mM NaOH (pH 13)] in a horizontal electrophoresis tray. After a 40-min DNA unwinding period, electrophoresis was conducted at 25 V and 300 mA for 30 min, followed by 15 min neutralization with 0.4 M Tris buffer (pH 7.5). Slides were washed in absolute ethanol and stored at room temperature for drying. The gel on each slide was stained with 50 µl SYBRgreen (Trevigen, Gaithersburg, MD) and diluted 1:10,000 in Tris-EDTA buffer. Slides were examined using a fluorescent microscope at 400× magnification (8). Every step was carried out under indirect light. Slides were coded and analyzed without knowledge of the sample identity. DNA damage was measured by percentage of DNA in tail using a computer scoring system (comet assay IV Software, Perceptive Instruments, Haverhill, Suffolk, UK).

Total Antioxidant Performance Analysis

TAP assay was used to measure oxidative stress/antioxidant performance of rabbit lung tissue and peripheral blood. TAP was quantified by comparing the area under the curve relative to the oxidation kinetics of phosphatidylcholine liposome control suspension used as the reference biological matrix (Fig. 1). The antioxidant performance ranges from 0% (no protection) to 100% (total protection) (6). Liposomes for control were prepared as previously validated and reported in plasma (6) and animal tissue (13, 15, 36).

Rabbits were ventilated in supine position. Characteristically, lung injury is most prominent in the dependent lung (17); thus a dorsal portion of right lung was weighed (0.4 g), minced, and homogenized for 20 s on ice with 2 ml of PBS (100 mM, pH 7.4) using an IKA, Ultra-Turrax T8 homogenizer (Wilmington, NC). Aliquots of supernatant were collected after lung tissue homogenate centrifugation at 800 g for 15 min in a Sorvall RT 6000 refrigerated centrifuge (Du Pont, Newtown, CT) and used for TAP. Protein concentration was determined using BCA Protein Assay, as previously described (2, 6, 15).

Plasma total antioxidant performance was determined fluorometrically with a 1420-multilabel counter (Wallac Victor 2; Perkin-Elmer Life Sciences, Boston, MA) as described previously (6).
The alveolar edema caused by ARDS inflammatory process and by mechanical ventilation itself may locally dilute pulmonary tissue and affects total antioxidant TAP assay results. Based on this, total antioxidant performance values in lung tissue were adjusted by lung protein. On the other hand, this is not the case when plasma is analyzed because the period of ALI induction (4 h) is too short to influence plasmatic concentration of protein.

**Statistical Analysis**

Statistical analysis was performed using SigmaPlot 11 (Systat Software, San Jose, CA). Data were compared between the different treatment groups by one-way ANOVA with all pairwise comparison procedures (Student-Newman-Keuls test) and results expressed as means ± SD. The correlation was performed using Pearson correlation. Statistical significance was considered when *P* < 0.05.

**RESULTS**

There were no statistical differences between groups for weight (HG 2.6 ± 0.27, CMV 2.5 ± 0.23, HFOV 2.7 ± 0.22 kg; *P* > 0.05) and number of lavages to induce lung injury (CMV 8.4 ± 2.5, HFOV 8.6 ± 2.3; *P* > 0.05). Percentages of fluid recovered from lavaged lungs were 83.6% and 84.3% for CMV and HFOV, respectively (*P* > 0.05).

**Oxygenation and Lung Mechanics**

Both injured groups showed significant hypoxemia after lung injury compared with baseline (Fig. 2). There was also significant increase in mean airway pressure (Paw) and decrease in respiratory system compliance after lung injury induction lasting until the end of the experiment, as shown in Fig. 3.

**Total Antioxidant Performance**

Total antioxidant performance in plasma and lung tissue from rabbits submitted to HFOV showed similar antioxidant defense to those of HG, and TAP was significantly higher compared with those ventilated with CMV. In addition, a strong positive correlation (*r* = 0.58; *P* = 0.0006) between overall antioxidant defense in plasma and lung tissue was observed (Fig. 4).

**DNA Damage Analysis**

Endogenous DNA damage was significantly lower in rabbits ventilated with HFOV compared with those of CMV (Fig. 5). In addition, a statistically significant correlation (*r* = 0.66; *P* = 0.007) was observed between DNA damage in cells recovered from target tissue and isolated lymphocytes (Fig. 6). Moreover, high antioxidant performance was significantly associated with the lower DNA damage (*r* = −0.50; *P* = 0.002) in lung tissue (Fig. 7).

**DISCUSSION**

Two different methods to measure antioxidant defense/oxidative stress were studied in this rabbit model of ALI treated with HFOV and CMV.

The involvement of oxidant-mediated tissue injury is likely to be an important event in ARDS pathogenesis (20), and ARDS patients are reported to have low plasma concentrations of antioxidants (35). Activated leukocyte aggregates in pulmonary microvasculature release ROS, which can attack membrane polyunsaturated fatty acid, thus initiating a peroxidation process. Peroxidation leads to loss of cell membrane functional integrity, culminating in an acute increase of alveolar-capillary permeability. In ALI, activated lymphocytes stimulate tumor necrosis factor and other cytokines. These lymphokines augment free radical generation by polymorphonuclear leukocytes, macrophages, and other cells, which may ultimately lead to ARDS (25).
We have evaluated oxidative DNA damage by comet assay. This assay is a highly sensitive method to distinguish different types of DNA fragmentation caused by genotoxic compounds (34) and can be applied to any tissue in vivo model (21). The use of freshly isolated or cryopreserved lymphocytes to determine DNA damage using comet assay has been well established (9, 12, 27, 50). However, the use of target tissue to evaluate DNA damage by comet assay in animal models remains unclear and has not been correlated with peripheral blood cells.

In clinical practice, the early use of HFOV has shown beneficial effects on oxygenation ARDS patients not responding to CMV (30), although improved clinical outcomes with HFOV have not yet been demonstrated. However, while there are many studies evaluating the effects of mechanical ventilation on oxygenation, lung inflammation, and histological injury in animal models of ALI, only a few have analyzed oxidative stress (3, 38). Rotta et al. (38) in a similar model of ALI in rabbits studied oxidative stress by measuring malondialdehyde, which is a nonspecific biomarker of lipid peroxidation (44). Although HFOV did show lower lipid peroxidation than CMV, this conventional assay has been criticized due to its low specificity and sensitivity (19, 48). Using another oxidative stress marker (myeloperoxidase), authors showed reduced lung oxidative damage in HFOV in a rabbit model of gastric juice aspiration (3). Even though myeloperoxidase has been reportedly associated with tissue damage involving inflammatory cells, it only implicates oxidant activity of neutrophils and other myeloperoxidase-containing cells (47). Our group, in a recently experimental study (36), found that HFOV showed better oxygenation, reduced inflammatory and histopathological injury, and lower oxidative stress (evaluated by TAP) compared with CMV using this model of ALI.

To our knowledge, there is no study available correlating overall antioxidant defense and oxidative stress in peripheral blood and target tissue in an animal model of ALI.

In the present study, the homogenization technique with cold PBS using a Potter homogenizer was applied, obtaining a good number of cells. Moreover, we found that homogenization technique was much quicker and simpler than enzyme treatment under this condition. Comparable results have been reported by others (31, 39) using similar technique, which isolates nuclei to apply comet assay in liver, lung, spleen, kidney, and bone marrow cells.

From our results, antioxidant defense and oxidative stress showed a comparable degree of oxidative injury evaluated in plasma and in lung tissue. It was interesting to note that target tissue had a significant correlation with plasma relating to antioxidant performance and DNA damage. These results suggest that oxidative damage analyzed in peripheral blood can be correlated with target tissue for DNA damage analyzed by comet assay and overall antioxidant defense using TAP assay. In agreement, Wiencke et al. (46) reported a positive correlation between DNA adduct levels in blood mononuclear cells and lung tissue using the 32P-postlabeling assay, measuring aromatic hydrophobic DNA adducts in 143 patients with lung cancer.

A few studies have reported the effect of oxygen exposure on DNA damage evaluated by comet assay in lung cells. Barker et al. (5) using comet assay found that mice exposed to 95% oxygen more than 8 h increased DNA base damage in the entire population of pulmonary cells compared with mice in room air (5). Similar results for DNA fragmentation in tissue...
sections in situ and type II cells were observed by others using TUNEL assay and comet assay (4, 37). In the present study, animals in the healthy group were not exposed to oxygen, whereas animals subjected to the CMV or HFOV were exposed to 100% oxygen for a short period. Considering that TAP and DNA damage in animals assigned to the HFOV group were comparable to those in the healthy group, it is probable that the low tidal volume and mean airway pressure used in the HFOV method a exert protective effect against oxidative stress.

Study Limitations and Future Implications

First, mechanical ventilation protocol in animal models is different from settings typically needed in adults, as adults need higher amplitude pressure (14). In addition, 1.0 FiO2 during 4 h may lead to lung parenchyma damage and interfere with oxidative metabolism. However, it should be pointed out that the same concentration of oxygen was used for all groups throughout the experiment to avoid variation in oxidative damage caused by hyperoxia. Considering total antioxidant assay measures overall antioxidant/oxidative stress, it is difficult to delineate the individual mechanism that leads to oxidative damage in lung tissue. Second, isolated lymphocytes were only collected in the high-frequency oscillatory ventilation group for DNA damage analysis, the correlation between DNA damage in lung tissue and peripheral blood thus being limited to the data from high-frequency oscillatory ventilation group. Extrapolation to other groups should be taken with caution.

Conclusions

In summary, this study demonstrates that DNA damage analysis by comet assay and overall antioxidant defense by TAP assay can identify the oxidative stress in this experimental model of ALI. The significant correlation between comet and TAP assay in target tissue and peripheral blood suggests that peripheral blood could be used for large epidemiologic studies conducted in the field, when target tissue is not possible to be accessed, although further studies warrant evaluating the correlation between target tissue and peripheral blood cells for DNA damage in humans. HFOV showed substantial benefits in this animal model of ALI compared with CMV.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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