ORIGINAL ARTICLES

Limitations of the Technique to Determine Hydrogen Peroxide Levels in Exhaled Breath Condensate From Patients With Adult Respiratory Distress Syndrome

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OBJECTIVE: Exhaled breath condensate represents an alternative to bronchoalveolar lavage for the analysis of markers of inflammation and oxidative stress in patients with adult respiratory distress syndrome (ARDS). However, analysis of hydrogen peroxide (H_2O_2) yields variable results that do not correlate with severity of the clinical presentation. In an attempt to explain this variability, the aim of the present study was to assess the possible limitations of the most commonly used technique for analyzing H_2O_2 in breath condensate.

PATIENTS AND METHODS: H_2O_2 levels were analyzed using the Gallati technique (linear range between 0.3 and 10 μM, r=0.99; P<.05) in serial samples of condensate taken from the expiratory tube of a mechanical ventilator in 6 patients with ARDS.

RESULTS: The volume of condensate obtained correlated to minute ventilation (r=0.96; P<.05). In 11 out of 23 samples, a spectrophotometer reading was obtained at 450 nm despite the absence of the characteristic color of the reaction and in some of these samples a spontaneous reading was obtained that was indicative of contamination. The absorbance spectrum of these samples did not contain the characteristic peak for H_2O_2 at 450 nm and pretreatment of some samples with catalase did not affect the absorbance at 450 nm.

CONCLUSIONS: The spectrophotometric method commonly used to measure H_2O_2 levels in breath condensate lacks specificity in ARDS due to the presence of variable levels of contaminants in the samples, which lead to false positives.

Key words: *Hydrogen peroxide. Adult respiratory distress syndrome. Oxidative stress. Exhaled breath condensate. Inflammatory markers. Gallati technique.*

Limitaciones de la técnica de determinación de peróxido de hidrógeno en el condensado del aire espirado de pacientes con síndrome de distrés respiratorio del adulto

OBJETIVO: El condensado del aire espirado es una alternativa al lavado broncoalveolar para estudiar marcadores de inflamación y estrés oxidativo en pacientes con síndrome de distrés respiratorio del adulto (SDRA). Sin embargo, el estudio del peróxido de hidrógeno (H_2O_2) ofrece resultados variables que no se relacionan con la gravedad del cuadro clínico. El objetivo del presente estudio ha sido identificar las posibles limitaciones de la técnica más utilizada para medir el H_2O_2 en condensado que expliquen esta variabilidad.

PACIENTES Y MÉTODOS: Se analizaron muestras seriadas de condensado de la vía espiratoria del ventilador de 6 pacientes con SDRA mediante la técnica de Gallati (lineal entre 0,3-10 μM, r = 0,99; p < 0,05) para H_2O_2.

RESULTADOS: El volumen de condensado se relacionó con la ventilación minuto (r = 0,96; p < 0,05). En 11 de 23 muestras se obtuvo lectura a 450 nm sin el color característico de la reacción y en algunas se obtuvo también lectura espontánea indicativa de contaminantes. El espectro de absorción de estas muestras no mostró el pico característico del H_2O_2 a 450 nm y el pretratamiento de algunas muestras con catalasa no modificó la absorbancia a 450 nm.

CONCLUSIONES: El método espectrofotométrico frecuentemente empleado para medir el H_2O_2 en condensado es inespecífico en el SDRA por la presencia en las muestras de cantidades variables de contaminantes que determinan falsos positivos.

Palabras clave: *Peróxido de hidrógeno. Síndrome de distrés respiratorio del adulto. Estrés oxidativo. Condensado del aire espirado. Marcadores de inflamación. Técnica de Gallati.*

Introduction

Exhaled breath condensate has been proposed as an alternative to bronchoalveolar lavage for the analysis of inflammatory phenomena and markers of oxidative stress in the airways and lung parenchyma of patients with a variety of respiratory diseases. One such condition is adult respiratory distress syndrome (ARDS), the pathogenesis of which involves inflammatory phenomena that lead to lung damage caused by free radicals.

The presence of hydrogen peroxide (H_2O_2) in exhaled breath from patients with ARDS suggested the hypothesis that free radicals participate in the pathogenesis of the increased pulmonary capillary permeability that is

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characteristic of ARDS.[1] A number of researchers have speculated that activated inflammatory cells sequestered in the lung in this disease are responsible for the oxidative stress that is indicated by the presence of H_2O_2. This hypothesis is supported by 2 observations: *a)* the results of analyzing bronchoalveolar lavage in these patients show a high proportion of oxidized glutathione and proteins, as well as high levels of isoprostanes and hypoxanthine[2-6]; and *b)* plasma from these patients contains products of lipid and protein oxidation, an increased concentration of hypoxanthine, and reduced levels of some antioxidants, such as α-tocopherol, β-carotene, selenium, and vitamin C.[7-13]

Although bronchoalveolar lavage can be used to assess alterations in alveolar fluid that are considered to be more specific indicators of oxidative lung damage,[14] it is not devoid of risks in critically ill patients with ARDS, who often display hemodynamic instability and do not tolerate repeated tests due to the invasive nature of the technique. Analysis of exhaled breath condensate could provide similar information to bronchoalveolar lavage but without the associated drawbacks. The simple and noninvasive nature of the method would allow serial analyses to be performed in an effort to identify correlations between H_2O_2 levels and indicators of clinical or physiological change, or altered gas exchange. However, the concentrations of H_2O_2 reported in the literature are highly variable and are not correlated with disease course in patients with ARDS.[1,14-20]

Although various approaches are available for the determination of H_2O_2 concentration in exhaled breath condensate, the most widely used in samples from patients with ARDS is the spectrophotometric technique described by Gallati and Pracht.[21]

The aim of this study was to identify the possible limitations of the Gallati technique in samples of exhaled breath condensate from patients with ARDS that could explain the heterogeneity of the results reported in the literature.

Patients and Methods

Condensate Collection

Exhaled breath condensate was obtained by connecting a Teflon-coated tube (100 cm long; internal diameter, 1.2 cm) to the expiratory tube of the mechanical ventilator after removing the filter that is normally placed at the outlet of the endotracheal tube. The tubing was kept submerged in iced water during the collection period. This period ranged from 30 to 60 minutes, depending on the minute ventilation of the patient, in order to obtain between 2 and 8 mL of condensate. The sample was kept on ice for a maximum of 60 minutes prior to analysis.

Determination of H_2O_2 Concentration

We used the spectrophotometric technique described by Gallati and Pracht,[21] a technique that has been widely used in a number of studies. A reaction mixture was prepared as follows: 1.25 mL of condensate, 0.25 mL of 63 µM 3,3',5,5'-tetramethybenzidine, 0.2 M sodium citrate buffer (pH 3.95), and 10 µL of horseradish peroxidase (1.25 U/mL). The reaction was stopped after 30 minutes by adding 50 µL of 5N sulfuric acid and the concentration of the reaction product 3,3',5,5' tetramethyl-1,1' diphenoquinone-4,4' diimine was determined by spectrophotometry at a wavelength of 450 nm. The absorbance at 450 nm is directly proportional to the concentration of H_2O_2 in solution.

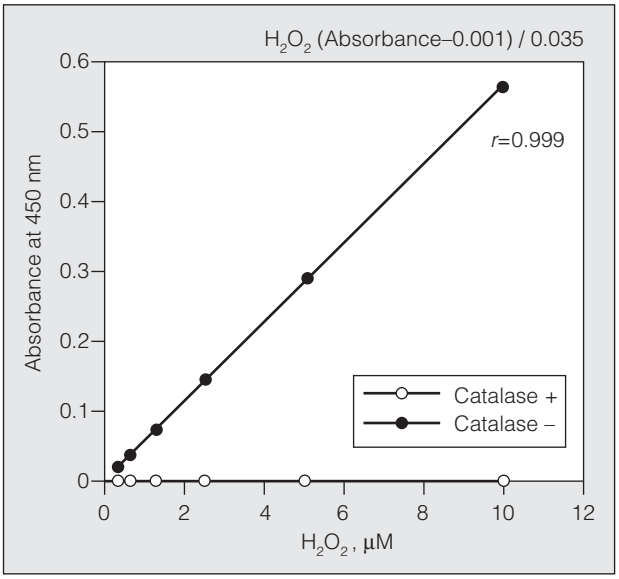

Figure 1. Calibration curve prepared using a standard solution of hydrogen peroxide (H_2O_2). Black circles indicate untreated solution and white circles solutions pretreated with catalase.

To calculate the concentration of H_2O_2 present in the samples we used a calibration curve generated with serial dilutions of a 30% solution of H_2O_2 (Merck, Santiago, Chile) on each day that measurement of patient samples was performed. The calibration curve was linear and highly reproducible in the range of 0.31 to 10 µM H_2O_2 (Figure 1). To confirm the specificity of the method, some samples and standards were pretreated with catalase.

The stability of H_2O_2 was analyzed in both commercial standard solutions and samples following freezing and storage at –80°C. The results from aliquots stored at –80°C for 24 or 48 hours were compared with those from fresh aliquots.

Patients

We studied 6 patients, 5 of whom were men, who had a mean (SD) age of 49 (17) years, were diagnosed with ARDS, and were receiving invasive mechanical ventilation in the intensive care unit of our hospital.

ARDS was defined as acute respiratory failure requiring intubation and mechanical ventilation, accompanied by *a)* diffuse infiltrates seen in both lungs in chest radiographs; *b)* a ratio of PaO_2 to fraction of inspired oxygen less than or equal to 200 mm Hg; and *c)* pulmonary capillary wedge pressure less than or equal to 18 mm Hg, or the absence of signs of left ventricular dysfunction. Patients were enrolled in the study within the first 24 hours of initiating mechanical ventilation and their condition was classified according to the Acute Physiology and Chronic Health Evaluation (APACHE II) severity scale. The etiology of ARDS in the different patients was as follows: pneumonia in 4 cases, complication following abdominal surgery in 1 case, and chest injury in 1 case.