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.

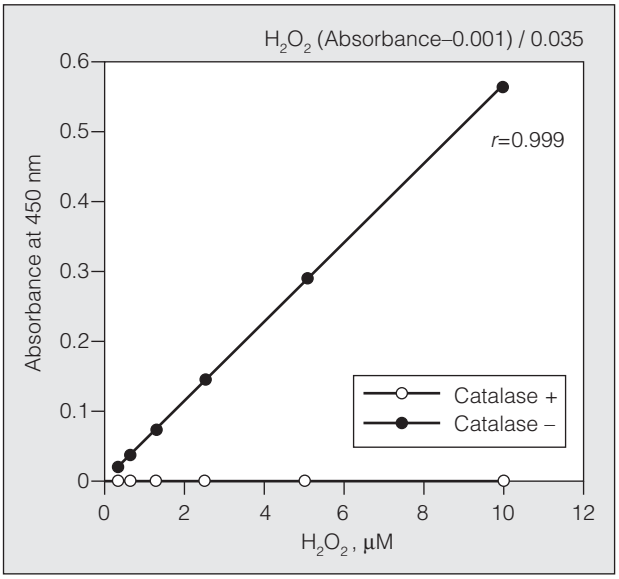

Figure 1. Calibration curve prepared using a standard solution of hydrogen peroxide (H_2O_2). Black circles indicate untreated solution and white circles solutions pretreated with catalase.

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.

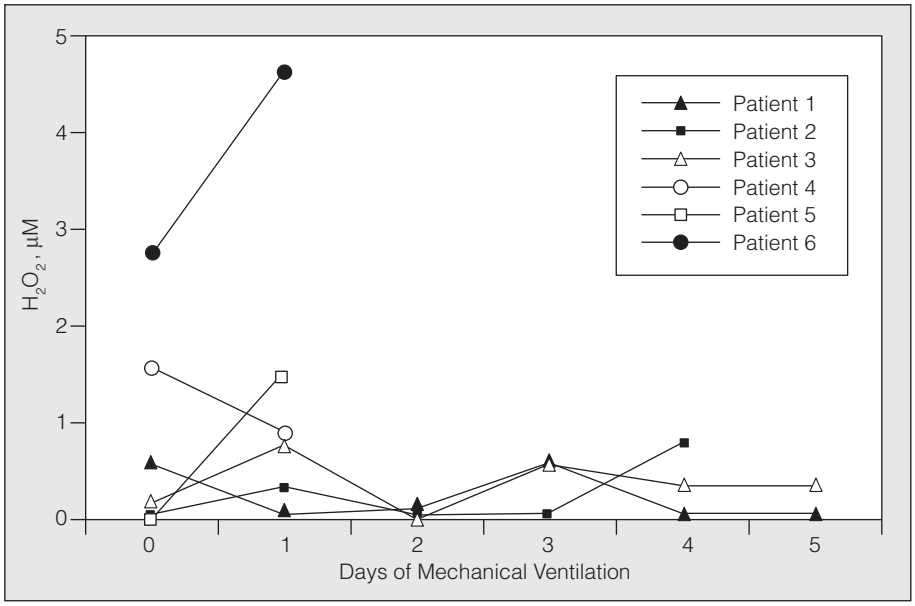

Figure 2. Temporal changes in the concentration of hydrogen peroxide (H_2O_2) in exhaled breath from 6 patients with adult respiratory stress syndrome. Each symbol corresponds to a patient. The results from patients with the highest concentrations of H_2O_2 displayed large differences from one day to the next.

The control group contained 5 mechanically ventilated patients (4 women and 1 man; mean age, 31 [10] years) who had undergone elective surgery under general anesthesia for reasons other than treatment of cancer and who were classified as risk category I on the American Society of Anesthesiology scale. The same system for collecting exhaled breath condensate was used in these patients as in the patients with ARDS. This type of control subject was chosen on the basis that it is very difficult to obtain breath condensate from nonintubated subjects without the sample being contaminated by saliva, an additional source of H_2O_2.[14,15] Furthermore, Wilson et al[18] reported no correlation between general anesthesia and H_2O_2 concentration in exhaled breath condensate.

Statistical Analysis

Comparison of results obtained in control subjects and patients with ARDS was performed with the Student *t* test. One-way analysis of variance (ANOVA) was used to analyze results on H_2O_2 stability and the Spearman correlation coefficient was used to assess the dependence of volume of condensate on levels of patient ventilation. Statistical significance was established at $P<.05$.

Results

Stability of Standard Solutions of H_2O_2 at –80°C

We observed a progressive reduction in the concentration of H_2O_2 in standard solutions stored at –80°C that was already apparent after 24 hours and was accentuated at 48 hours (Table). These results indicated that immediate processing of samples was required.

Collection of Condensate

Connection of a Teflon-coated tube to the expiratory tube of the mechanical ventilator did not lead to changes in patient breathing pattern. We obtained between 2 and 8 mL of clear liquid. The volume of the condensate displayed a linear relationship with time of collection and minute ventilation ($r=0.96$; $P<.05$).

Estimated Concentrations of H_2O_2

We analyzed 28 samples of exhaled breath condensate: 23 samples from the 6 patients diagnosed with ARDS, in whom a variable number of samples were taken over the course of the study (depending on the clinical condition of the patient), and 5 samples from control subjects. In 3 out of 5 control samples and in 3 out of 6 samples taken on day 0 from patients with ARDS, H_2O_2 was not detected with the method used. In the cases in which a reading was obtained, the concentration of H_2O_2 calculated from the standard curve was 0.36 (0.05) µM for the samples from control subjects and 1.62 (1.1) µM in the samples taken from patients with ARDS on the first day of mechanical ventilation ($P=.181$). Analysis of changes in H_2O_2 concentration over the period of mechanical ventilation revealed a high degree of variability (Figure 2). The subjects from whom fewer

TABLE
Stability of a Standard Solution of Hydrogen Peroxide at –80°C

t=0		24 Hours		48 Hours	
µM	%	µM	%	µM	%
0.625	100	0.35 (0.06)*	56	0.28 (0.03)†	45
1.25	100	0.99 (0.05)*	79	0.63 (0.26)†	50
2.5	100	1.68 (0.02)*	67	1.45 (0.09)†	58
5	100	3.65 (0.64)*	73	3.46 (0.07)†	69
10	100	7.85 (0.22)*	79	7.67 (1.10)†	77

*$P<.05$ for t=0 versus 24 hours. †$P<.05$ for t=0 versus 48 hours.

samples were taken corresponded to patients who died or in whom mechanical ventilation was withdrawn during the course of the study.

Specificity of H_2O_2 Analysis in Condensate Samples

Of the 23 samples from patients with ARDS, 11 presented high absorbance at 450 nm following the reaction that was not accompanied by the color expected according to the standard. A number of those samples exhibited spontaneous absorbance at 450 nm (without reaction), indicating the presence of a contaminant that affected the reading.

Examination of the absorbance spectrum of the samples revealed high and variable absorbance in the range of 350 to 550 nm, without a characteristic peak for H_2O_2 at 450 nm (Figure 3). This absorbance profile prevented subtraction of background absorbance. Finally, pretreatment of some samples with catalase did not alter the absorbance at 450 nm (0.055 [0.58] vs 0.051 [0.051]; $P>.05$).

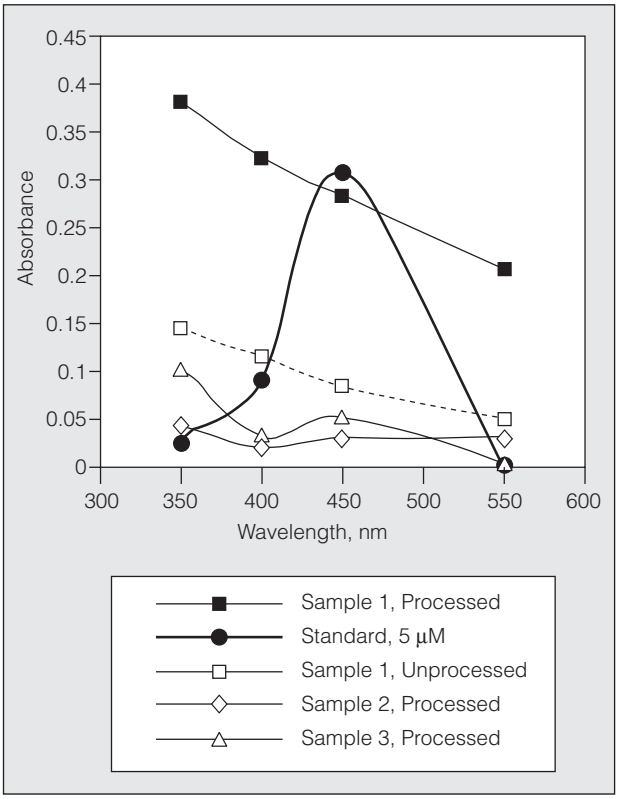

Figure 3. Absorption spectrum following processing of a 5 µM standard solution of hydrogen peroxide and 3 patient samples. The spontaneous absorption spectrum of an unprocessed sample is also shown. Only the standard solution displays an absorption peak at 450 nm.

Discussion

This study reveals significant limitations to the technique used in the majority of studies in which H_2O_2 concentration is measured in exhaled breath from patients with ARDS. The main limitation of the Gallati technique for this type of sample centers on the presence of variable background absorbance that prevents reliable estimation of H_2O_2 concentration (Figure 3).

Studies measuring H_2O_2 concentration in exhaled breath from patients with ARDS have yielded variable results that do not correlate with clinical and physiological parameters, a finding that limits the clinical usefulness of this measurement.[1,14,15,17-20] To date, this has been linked to a number of factors: *a)* variable levels of antioxidants that metabolize H_2O_2 in different ways in the airways and lung; *b)* different methods for the collection and processing of the condensate; and *c)* heterogeneity of the diseases encompassed by a diagnosis of ARDS. The possibility that the composition of the condensate is altered by the properties of materials used in the collection system should also be added to these factors, since some plastics give rise to contaminants in the range over which spectrophotometric readings are made in this technique (unpublished observations). However, in our study, this factor did not influence the background absorbance of the samples, since samples prepared from nebulized standard solutions of H_2O_2 introduced into the collection system did not display background absorbance of this type, thereby allowing reliable measurement of H_2O_2 concentration.

The origin of the background absorbance observed in the samples in this study could be linked to the presence of particles in suspension and substances other than H_2O_2 that can react with 3,3',5,5'-tetramethylbenzidine, as occurs, for instance, with chloride ions.[14-16,22] Apart from condensed water vapor and volatile substances, exhaled breath condensate also contains various nonvolatile solutes, such as proteins, lipids, and electrolytes, that reach the condensate in aerosol particles released from the fluid that covers the bronchial mucosa and alveoli.[14-16,22] This is an area of increasing interest, since the number of droplets containing aerosol particles is highly variable. In healthy spontaneously breathing subjects, the number of aerosol particles can vary between 0.1 and 4 particles per milliliter and the mean diameter of these particles is 0.3 µm.[14] This means that in healthy subjects the proportion of the volume of condensate accounted for by respiratory fluid varies between 0.01% and 2%.[22] This proportion increases in mechanically ventilated patients, since the number of aerosol particles formed in the respiratory tree depends on the rate of airflow and the presence of turbulence.[14] It is possible that the variable background absorbance observed in samples of condensate from patients with ARDS is linked to particles in suspension arising from respiratory fluid, the levels of which vary according to rate of airflow. Furthermore, this phenomenon could be present in other pulmonary conditions with similar conditions of turbulence.

Various studies have applied the Gallati technique to samples of exhaled breath condensate from spontaneously breathing patients with asthma or

chronic obstructive pulmonary disease and from smokers.[19,23,24] Those studies did not report background in their samples and found that the Gallati method was specific for H_2O_2.[19] It is likely that in those studies the number of particles in suspension was much lower than in the samples from mechanically ventilated patients used in our study. Attempts to estimate the dilution of the particles in suspension[26] could help to confirm or reject this hypothesis.

In contrast to other studies showing that H_2O_2 remains stable for a number of days at –20 or –80°C,[20,23,25] we found that the concentration was reduced by 30% after storage at –80°C for 24 hours. In 2 of the studies that reported stability of H_2O_2 at –80°C, the concentration was measured in the samples themselves.[20,25] In contrast, in our study the stability of H_2O_2 was assessed with known concentrations and in the absence of background absorbance. Based on our results suggesting that the determination of H_2O_2 concentration using the Gallati technique is highly nonspecific, we hypothesize that the finding in some studies that H_2O_2 concentrations remain stable in samples of condensate stored at –80°C may be explained by the detection of other substances or particles in suspension.

In summary, the Gallati technique has significant limitations to its use in samples of exhaled breath condensate from patients with ARDS, due to the presence of variable background absorbance in a large number of samples. The findings of this study help to explain some of the sources of variability in the measurement of H_2O_2 in exhaled breath condensate from patients with ARDS. The use of techniques that do not suffer from background absorbance that interferes with readings is an indispensable requirement if the results of analysis of exhaled breath condensate are to reflect the biochemical changes in the airways and air spaces. Our results highlight the need to standardize the methods used for the analysis of exhaled breath condensate in different diseases.

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