Research Article

Correlation between Malondialdehyde (MDA), Hyaluronan (HA), and Alpha-tocopherol (Vit E) in Tracheal Aspirate Fluid (TAF) and Oxygenation Index (PaO_2/FiO_2) in Pediatric Patients with Chronic Lung Disease

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Abstract

Objective: To study the correlation between malondialdehyde (MDA), hyaluronan (HA), total sulfhydryl (TS) group, alpha-tocopherol (Vit E) in blood or tracheal aspirate fluid (TAF) and the PaO_2/FiO_2 ratio in pediatric patients with chronic lung diseases.

Methods: This study was performed in 40 pediatric patients with average age of 5.35±0.625 months (range 0.5-12) whom required intubation for lung dysfunction [Pneumonia (n=10), Bronchopulmonary dysphasia (BPD) with pneumonia (n=12), Heart disease with pneumonia (n=7), Respiratory distress syndrome (RDS) with pneumonia (n=2), Sepsis with pneumonia (n=3), Miscellaneous with pneumonia (n=6)]. Blood and TAF from routine suction were determined for the levels of TS group, MDA, HA, and Vit E by dithionitrobenzoic acid (DTNB), thiobarbituric acid reactive substance (TBARs), enzyme-linked immunosorbance assay (ELISA), and high-performance liquid chromatography (HPLC) methods, respectively. The correlation was analyzed by Pearson correlation statistical analysis with $p< 0.05$ (two-tail test) was considered significant.

Results: The results showed a significantly weak positive correlation between MDA and HA ($r = 0.410$, $p<0.01$); and a negative one between MDA and TS ($r = -0.365$, $p<0.01$) in the blood while significant correlation between MDA, HA, Vit E or TS and the PaO_2/FiO_2 ratio was not found. Whereas, in TAF five significantly correlated couples were shown; Vit E and TS ($r = 0.396$, $p<0.05$); HA and MDA ($r = 0.60$, $p<0.01$); HA and the PaO_2/FiO_2 ratio ($r = -0.573$, $p<0.01$); MDA and PaO_2/FiO_2 ratio ($r = -0.320$, $p<0.05$), and Vit E and PaO_2/FiO_2 ratio ($r = 0.371$, $p<0.05$).

Conclusion: It can be concluded that MDA, HA and Vit E in TAF may be potential biochemical markers for the prognosis of lung damage and lung function. **Bull Chiang Mai Assoc Med Sci 2003; 36: 24-34.**

Key words: Alpha-tocopherol, glutathione, hyaluronan, malondialdehyde, chronic lung disease.

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บทคัดย่อ: ความสัมพันธ์ระหว่างมาลอนไดออลดีไฮด์, ไฮยาลูโรแนน และวิตามินอี ในสารคัดหลั่งจากหลอดลมกับค่า PaO_2/FiO_2 ในผู้ป่วยเด็กที่เป็นโรคปอดเรื้อรัง

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วัตถุประสงค์: ศึกษาหาความสัมพันธ์ระดับมาลอนไดอัลดีไฮด์, ไฮยาลูโรแนน กลุ่มไทออลด์ และวิตามินอีในเลือดและในสารคัดหลั่งจากหลอดลมกับอัตราส่วน PaO_2/FiO_2 ในผู้ป่วยเด็ก

วิธีการศึกษา: ทำการศึกษาในผู้ป่วยเด็กอายุเฉลี่ย 5.35±0.625 เดือน (ช่วงระหว่าง 5-12 เดือน) ที่เป็นโรคปอดเรื้อรังและต้องใช้ท่อช่วยหายใจทางหลอดลม (ได้แก่ โรคปอดบวม 10 ราย, Bronchopulmonary dysplasia (BPD) ร่วมกับปอดอักเสบ 12 ราย, โรคหัวใจร่วมกับปอดอักเสบ 7 ราย, Respiratory distress syndrome (RDS) ร่วมกับปอดอักเสบ 2 ราย, ติดเชื้อในกระแสเลือดร่วมกับปอดอักเสบ 3 ราย และโรคอื่นร่วมกับปอดอักเสบ 6 ราย. รวมทั้งสิ้น 40 ราย นำเลือดและสารคัดหลั่งจากหลอดลมมาตรวจวัดหากลุ่มไทออลด์ มาลอนไดอัลดีไฮด์ ไฮยาลูโรแนนและวิตามิน อี ด้วยวิธี Dithionitrobenzoic acid (DTNB), thiobarbituric acid reactive substance (TBARs), enzyme-linked immunosorbance assay (ELISA), และ high performance liquid chromatography (HPLC) ตามลำดับ. ทำการวิเคราะห์ค่าความสัมพันธ์ระหว่างค่าต่างๆ กับอัตราส่วน PaO_2/FiO_2 โดยใช้ Pearson correlation และใช้ค่านัย สำคัญที่ p น้อยกว่า 0.05

ผลการศึกษา: พบความสัมพันธ์ชัดเจนระหว่างมาลอนไดออลดีไฮด์และไฮยาลูโนแนน ($r = 0.410$, $p<0.01$) และ มาลอนไดอัลดีไฮด์และกลุ่มไทออล ($r = -0.365$, $p<0.01$) ในเลือด แต่ไม่พบความสัมพันธ์ระหว่างมาลอนไดอัลดีไฮด์ ไฮยาลูโรแนน วิตามินหรือกลุ่มไทออลด์กับ PaO_2/FiO_2 ขณะที่ในสารคัดหลั่งจาก

หลอดลม พบความสัมพันธ์อย่างมีนัยสำคัญระหว่างวิตามิน อี กับ กลุ่มไทออล ($r = 0.396$, $p<0.05$); ไฮยาลูโรแนนกับมาลอนไดอัลดีไฮด์ ($r = 0.60$, $p<0.01$); ไฮยาลูโรแนนกับ PaO_2/FiO_2 ($r = -0.573$, $p<0.01$); มาลอนไดอัลดีไฮด์กับ PaO_2/FiO_2 ($r = -0.320$, $p<0.05$), และ วิตามิน อีกับ PaO_2/FiO_2 ($r = 0.371$, $p<0.05$)

สรุป: จากผลการศึกษาพบว่ามาลอนไดอัลดีไฮด์ ไฮยาลูโรแนน และ วิตามิน อี ที่ตรวจพบในสารคัดหลั่งจากหลอดลม น่าจะเป็นดัชนีบ่งชี้ การทำนายโรคของภาวะปอดถูกทำลายและการทำงานของปอดได้ดี

วารสารเทคนิคการแพทย์เชียงใหม่ 2546; 36: 24-34.

คำหัส: วิตามิน อี กลูตาไธโอน ไฮยาลูโรแนน มาลอนไดอัลดีไฮด์ โรคปอดเรื้อรัง

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หมายเหตุ ได้รับทุนวิจัยจากคณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

Introduction

Lung infection with viral or bacterial microorganisms is mostly found in neonatal or pediatric patients. The severities on lung tissue depend on the type and quality of microorganisms.[1] Alveolar stimulated macrophages produce free radicals such as superoxide radical, hydrogen peroxide, nitric oxide, hypochlorus radical, nitrogen species, and hydroxyl radicals.[2] Inflammation is an important contributor to the pathogenesis of chronic lung diseases because of its pro-inflammatory mediators that have direct effects on protein and lipid membrane degradation. Especially, the activity of myeloperoxidases from neutrophil in tracheal aspirate fluid can promote the lipid peroxidation in pre-termed infants.[3] The products of lipid peroxidation, which can be detected in the serum and bronchoalveolar lavage fluid (BAL), are malondialdehyde and 4-hydroxynonenal (HNE).[4] Hyarulononan (HA), an important substance which absorb water and keep the alveolar distension while expanding. Increased HA is found during remodelling of tissue in normal development as well as in damaged tissue. Inflammation in the lung stimulates fibroblasts to produce more HA by oxygen-induced stimulation and oxygen free radicals.[5,6] Two main antioxidants in the lung were found. The first is a group of enzymes that can primarily reduce free radicals, and the second is antioxidant group of chemical substances such as alpha-tocopherol, beta-carotene, uric acid, ascorbic acid, or

glutathione.[7] Glutathione (L-glutamyl-L-cysteinyl-glycine; GSH) is a tripeptide that is widely distributed in various intracellular and extracellular parts such as blood, nasal lining fluid (NLF) and epithelial lung fluid (ELF). The alpha-tocopherol (Vit E) represents the primary chain-breaking antioxidant in hydrophobic environment as scavenger of peroxyl radicals.[8] Generally, the lung function can be evaluated by physiological markers such as oxygen and carbon dioxide pressure in the artery ($PaCO_2$), pH, base excess (BE), total carbon dioxide (tCO_2), and oxygen saturation. Lung injury score has been reported as clinically physiological markers for classifying the severity of lung injury in acute respiratory distress syndrome (ARDS). The severity of lung injury can be classified into 3 levels, no lung injury, mild to moderate lung injury, and severe lung injury.[9-10]

In this clinical study, the lung injury score was studied in our patients in additon to the levels of GSH, MDA, HA, and Vit E in TAF.

METHOD

Subjects

The protocol of this study was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Permission was given to study all 40 subjects by their parents and pediatric physicians. The parents were informed before giving written consent for the treatment and the participants were checked for risks of severe anemia, thrombocytopenia and clinical status for the exclusion criteria. Forty patients with an age range of 1-12 months had a mean age±SD of 5.35±0.625 months.

Sample preparation

Blood (1.5 ml) was collected by using acid citrate dextrose (ACD) for analysis of the plasma alpha-tocopherol and total and non-protein sulfhydryl (TS) compound in the red blood cells and it was preserved at -20°C. Another 1.5 ml of clotted blood was collected and serum was separated for analyzing of MDA concentration and hyaluronan. Standardized procedure of tracheal aspirate fluid (TAF) was performed by collecting it from routine suction with 100 mmH_2O of negative pressure. TAF was aspirated and preserved in a sterile specimen trap, mucus extractor #6 (Endomed Co. Ltd.) with 0.9% sodium chloride solution. TA samples were kept at -20°C and analyzed for the TS, MDA, Vit E, and HA within a week.

Malondialdehyde by the thiobarbituric acid reactive substance (TBARs) assay

The technique, TBARs assay, was modified from the method of Smith.[11] In brief, 0.45 ml of normal saline solution (NSS), 0.2 ml of thiobarbituric acid (TBA) (Sigma; St.Louis, MO) reagent and 1.0 ml of 100% trichloroacetic acid (TCA) (Sigma) were mixed with 0.1 ml of serum or TAF. The mixed solution was incubated for 30 min in a water bath at 95 °C. After cooling, 2.0 ml of distilled water was added

before centrifugation (3,000g, 10 min). The pink color of the mixture was measured with a spectrophotometer at 532 nm. Readings were converted into μM using Malonaldehyde bis-(dimethyl acetyl) 99% as standard. (Aldrich Chemical Co. Inc. USA) (5-50 μM).

Total sulfhydryl group determination

The non-protein total sulfhydryl group in whole blood was determined by the modified method of Beutler.[12] A mixture of 0.4 ml of whole blood and 1.6 ml of distilled water was added to 3.0 ml of precipitating reagent (0.02 M glacial metaphosphoric acid, 0.68 mM EDTA-Na_2 and 0.51 M NaCl). After the mixture was incubated and filtered through No.1 Whatman filter paper, 1.0 ml of supernatant was added with 4.0 ml of phosphate buffer (pH 8.0). After adding 0.5 ml of Dithionitrobenzoic acid (DTNB) and shortly mixed, the yellow color developed was read with a spectrophotometer at 412 nm. The quantity of total sulfhydryl compound was read from standard GSH (Sigma; St.Louis, MO) curve (10- 40 μg/ml).

Hyaluronan quantitiation by ELISA-based technique.

The HA in serum and tracheal aspirate fluid were measured using ELISA-based technique.[13] Briefly, 0.175 ml of sample or standard competitor (Haelon) (range 3.9-1,000 ng/ml) was added with an equal volume of biothylated hyaluronan binding protein (B-HABP) (1:200) and incubated for 60 minutes at room temperature. The samples or standard HA in the microtiter plates (Maxisorp NUNC®) were pretreated by coating with umbilical cord HA (Sigma) (100 μg/ml) in coating buffer and blocked with a 150 μl/well of 1% BSA in PBS pH 7.4. The plate was then washed with 150 μl of PBS-Tween buffer. Peroxidase-mouse monoclonal anti-biotin (Zymed Lab, Inc.,CA) (100 μl/well: 1:2000) was added to each well and incubated for 60 min at 25 °C. After washing the plates three times with PBS-Tween buffer, peroxidase substrate, o-phenylenediamine (OPD) (12 mg/10 ml of phosphate buffer pH 5.5 with 5 μl of H_2O_2) was added (100 μl/ml) and incubated at 37 °C in order to develop color. Stop reaction was achieved by the addition of a 50 μl/well of 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using a microtiter plate reader (Multiskan®MCC/340, Northland). The absorbance and concentration were used to construct a standard curve using Deltasoft software on Macintosh.

Alpha-tocopherol determination by HPLC method

Alpha-tocopherol concentration was determined by the method of Shearer[14], by using the Reverse phase-HPLC C-18 (Spherisorb ODS-2, 5 μm, 4.6 X 250 mm column) (Water). The α-tocopherol in 0.1 ml of plasma or TAF was extracted into n-hexane solution with internal standard α-tocopherol acetate (10 mg/L) (Sigma, St.Louis, MO). After mixing and shaking the mixed solution

for 10 min then centrifuge for 5 min, the upper hexane layer was separated and evaporated at room temperature. Immediately before the HPLC processing the lipid residue was dissolved in 200 µl of absolute ethanol and filtered through 0.45 µm pre-cut membrane Nylon PTFE using syringe, then 50 µl of the filtrate was injected into the HPLC column. The mobile phase was 7% (v/v) dichoromethane (Lab scan, Island) with a flow rate of 1.0 ml/min making the pressure in the column between 2,400-2,600 psi and the retention time of alpha-tocopherol was between 3.75-3.90 minutes. The absorbance of alpha-tocopherol at 292 nm was detected by Conta Meric LDL Analyzer. Standard alpha-tocopherol at 10 mg/L was run along with the samples for calculation.

Statistical Analysis

The correlation between the levels of TS, Vit E, MDA, or HA and the PaO_2/FiO_2 ratio were analyzed with Pearson's correlation test. The significant level, at a $p < 0.05$, was considered.

Results and Discussion

Oxidative stress

From this study, we found that higher oxidative stress occurred in all 40 pediatric patients with high concentration of MDA and HA, and a low level of Vit E in their blood.

The mean±SD of MDA was 17.15±1.0 µM in serum and 33.25±3.45 µM in TAF. In a previous study reported that the MDA level of serum in one year old healthy children should be less than 10 µmol/L.[15] Another report that examined in 32 pediatric patients with lower airway infection was 3.5±1.4 µM.[16] We found also a very high level of HA in plasma (12,000-17,610 µg/L) and TAF (63,900-85,000 µg /L). Surprisingly, no report has documented the level of HA in TAF before. This study used the specific anti-HA antibody for the detection of HA in TAF. Nevertheless, there were fews studies that show the correlation between HA and lung diseases such as interstitial disease and sarcoidosis,[17] acute extrinsic alveolitis,[18] and COPD-induced horses.[19] A previous study of 12 patients with adult respiratory distress syndrome (ARDS) showed higher levels of HA in serum (619 µg/L) compared with those in healthy subjects (353 µg/L).

This study reported a low level of total sulfhydryl (TS) group in erythrocyte (range 27.15- 94.55 mg/dL, mean+SD = 59.7±1.3 mg/dL) compared with the study by Metsvaht and co-workers[20] in 24 neonates after 2-4 days of life (66.4±12.5 mg/RBC dL). The sulfhydryl group in protein is necessary for the protection of oxidative stress from free radicals[21] and maintaining the level of reducing agents, such as Vit E.[4] All pediatric patients had been suffering with a chronic lung disease and/or other disorder for a long time, possibly because of total sulfhydryl group was reduced from prolonged oxidative stress in the lung. We found that the total sulfhydryl group in TAF by DTNB

assay was very low (ranged 0.5-5.9 mg/dL, mean±SD = 2.6±0.4 mg/dL). A low level of alpha-tocopherol in plasma, by HPLC in all cases was also found. Even though, there was no exact reference to the normal value of alpha-tocopherol in one-year old children. Wewan and co-workers[22] suggested that it should be approximately 28 μM, and 18 μM in full-termed infants (2-12 yrs), 9 μM in premature infants, and 5- 20 mg/L in healthy adults. Plasma Vit E found in this study was nearly in the range of the Vit E concentration (6.83-34.65 μM) studied by Shock and co-workers,[23] which also reported a low concentration of Vit E in BAL (0.026 μM) in 124 children (age range 1.6-12.6 yr). Our study found a higher concentration in TAF, which had never been studied before. It is feasible that Vit E in TAF may be secreted from excess degradation and oversynthesis of alveolar type II cells in chronic lung condition in order to control lipid peroxidation in the lung.[24]

Correlation study

There was no study that showed a correlation of MDA, GSH, Vit E, and HA concentrations in the blood or TAF with the PaO_2/FiO_2 ratio before. From our study, we found a statistical correlation between serum MDA with serum HA ($r=0.410$, $p<0.01$); serum MDA and erythrocytes GSH ($r=-0.365$, $p<0.01$). It was very interesting that a higher level of HA, MDA and Vit E in tracheal aspirate fluid (TAF) was found. Five significantly correlated couples were seen: Vit E and GSH ($r=0.390$, $p<0.05$); HA and MDA ($r= 0.600$, $p<0.01$); and Vit E and the PaO_2/FiO_2 ratio ($r= 0.371$, $p< 0.05$) (Fig 1.); HA and the PaO_2/FiO_2 ratio ($r= -0.573$, $p<0.01$) (Fig.2); and MDA and the PaO_2/FiO_2 ratio ($r= -0.320$ $p<0.05$) (Fig. 3).

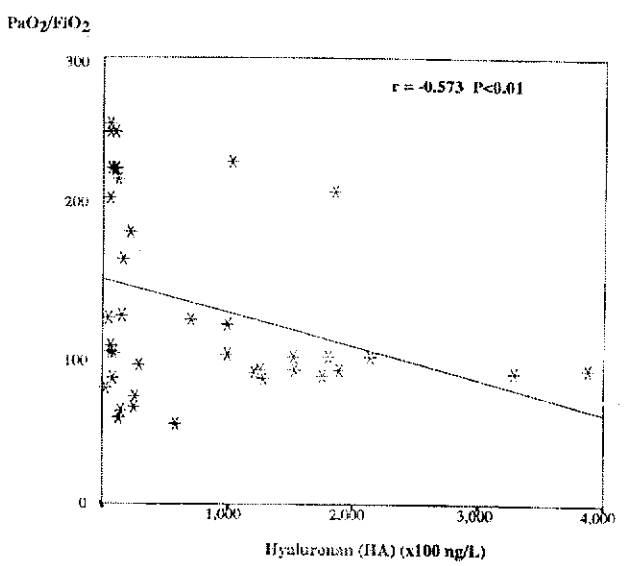

Fig.1. Relationship between hyaluronan (HA) (x100 ng/L) in tracheal aspirate fluid (TAF) and oxygenation index (PaO_2/FiO_2 ratio) in 40 patients.

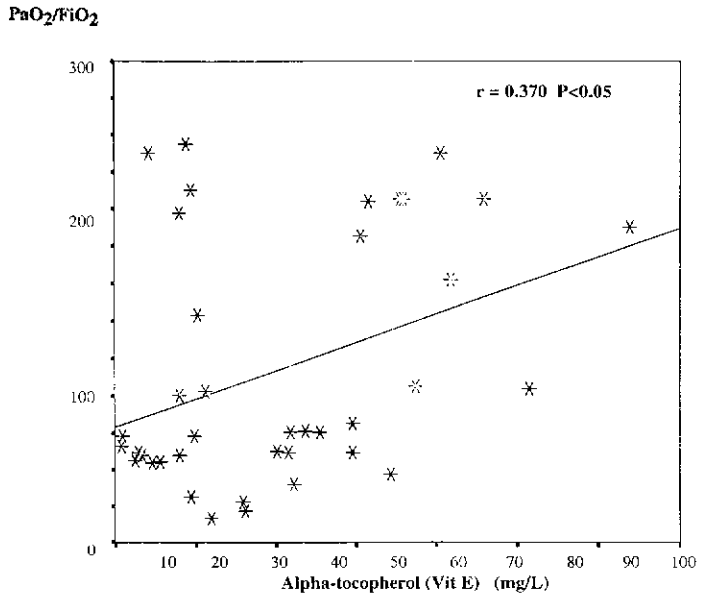

Fig.2. Relationship between alpha-tocopherol (Vit E)(mg/L) in tracheal aspirate fluid and oxygenation index (PaO_2/FiO_2 ratio) in 40 patients.

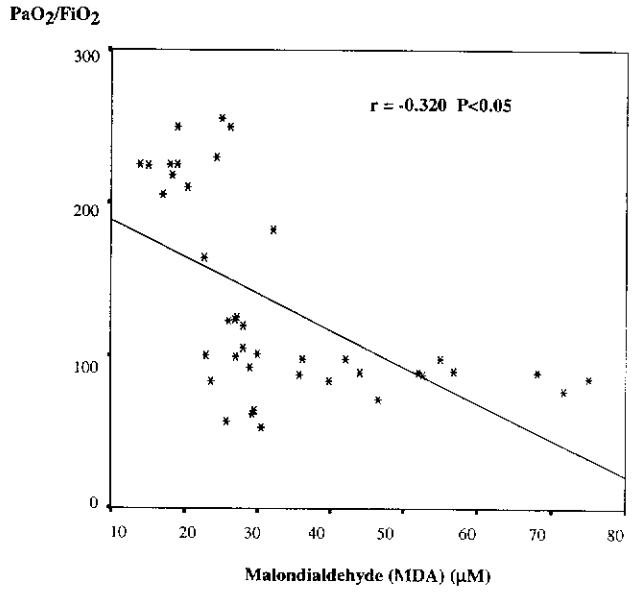

Fig.3. Relationship between malondialdehyde (MDA) ((M) in tracheal aspirate fluid and oxygenation index (PaO_2/FiO_2 ratio) in 40 patients.

Many studies have documented the correlation between MDA levels and lung infection. For example, studying in 144 tracheal aspirate fluids from 86 pre-termed infants with oxygen dependence, a strong positive correlation between MDA level and myeloperoxidase (MPO) activity was found.[25] MPO is the specific enzyme that over expresses lung during infection. The previous study of Moison and co-workers[26] showed a correlation between

MDA in the blood and severity of lung injury in early 21 pre-termed babies with respiratory distress syndrome (RDS).[25] But in our study couldn't find a correlation between serum MDA and lung injury score. There were some controversial reports that show the opposite result such as the study of Yagit and co-workers[15] which didn't find a correlation between MDA in the blood and either FiO_2 level or arterial oxygen tension (PaO_2). Surprisingly, we found a significant correlation between MDA in TAF and the FiO_2/PaO_2 ratio (Fig 3). The reason for the high level of MDA in TAF is still unclear. However we found a good correlation between MDA in TAF and the FiO_2/PaO_2 ratio. The lesion of lipid peroxidation was possibly localized in the infected airway space making a higher MDA concentration in TAF than in serum.

A significant correlation between Vit E in TAF and the PaO_2/FiO_2 ratio in our study was the same as in the previous study by Yoder and co-workers[27] who reported a negative correlation between Vit E and oxygenation index (PaO_2/FiO_2) in BAL. Physiologically, alpha-tocopherol is a breaking-chain antioxidant that stabilizes the phospholipid membrane structure. A low level of Vit E found in our study may be the biological response to the inefficiency of tissue oxygenation.

Some reports demonstrated a significantly high level of HA in the serum and the BAL of patients with adult respiratory distress syndrome (ARDS)[5] or sarcoidosis.[18] From this study, we found a negative correlation between HA in TAF and the PaO_2/FiO_2 ratio. Normally, HA is produced from fibrous tissue and composed in the alveolar epithelial cell for stabilizing lung expansion or distension. We found a high level of HA in the airway reflected a higher degree of destruction and that statistically correlation between HA in TAF and the PaO_2/FiO_2 ratio were found.

However, many other cofactors may affect, for example : food, ventilator setting, or medical treatment. At least, all subjects received only bronchodilator drugs, Berodual and Ventoline with occasionally paracetamol. There is no evidence that show the relationship between drug effects on these markers. Some evidences has shown that dexamethasone could reduce neutrophils and elastase activity in tracheobroncheal lavage fluid,[28] so it might also reduce the injury in the lung of BPD patients. Further studies should be continued for distinguishing the effect of severity of lung disorder, drug intake or other variables.

Conclusion

This study demonstrated that alpha-tocopherol, MDA, and HA in tracheal aspirate fluid (TAF) from routine suction in the hospital are possibly useful as oxidative stress markers that reflect or predict lung oxygenation.

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