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DIFFERENCES IN OXIDATIVE STATUS, LUNG FUNCTION, AND PULMONARY SURFACTANT DURING LONG-TERM INHALATION OF MEDICAL OXYGEN AND PARTIALLY IONIZED OXYGEN IN GUINEA PIGS

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Inhalation of partially ionized oxygen may have less adverse effects on lung functions than medical oxygen. Guinea pigs inhaled air, 100% molecular medical oxygen (O_2mol), partially negatively (O_2neg) or positively (O_2posit) ionized oxygen during 17 and 60 h. After 17 h, dityrosines, markers of oxidative injury, in lung homogenate increased in O_2neg and decreased in O_2posit groups vs. controls. After 60 h, dityrosines rose after inhalation of O_2mol and O_2neg, but not in the O_2posit group. Lysine-LPO products increased and lung wet/dry weight ratio decreased in O_2mol and O_2neg, and not in O_2posit group. Relative neutrophil count in BALF was elevated in all oxygen-treated groups with lower numbers in O_2posit vs.O_2mol and O_2neg groups. After 60 h, surfactant activity was better in O_2posit *vs*. O_2mol group. In conclusion, long-term inhalation of partially positively ionized oxygen is associated with less oxidative stress, milder lung inflammatory response, and better surfactant activity than molecular or negatively ionized oxygen.

Key words: *ionized oxygen therapy, lung function, oxidative damage, oxygen, pulmonary surfactant, reactive oxygen species*

INTRODUCTION

Supraphysiological concentrations of oxygen are routinely used to treat hypoxemia and respiratory failure. During prolonged exposure to high oxygen concentrations, the lungs are the organ most affected and pulmonary surfactant may be a target in hyperoxia-induced lung toxicity (1). Oxygen toxicity is believed to be mediated by excessive production and accumulation of reactive oxygen species (ROS), which exceeds the capacity of the lung antioxidant defence mechanisms (2). Excessive ROS can directly damage cellular macromolecules, resulting in cell death (3). In addition, exposure to hyperoxia triggers an inflammatory response, which exacerbates oxidative toxicity (3), and the release of mediators that induce surfactant gelation (4).

In order to take advantage of positive effects and to reduce adverse effects of oxygen therapy, partial ionization of medical oxygen for therapeutical purposes was introduced in 1980 (5). Medical oxygen is partially ionized in a high-voltage plasma chamber of an ionization device. By ionization, the number of electrons is changed in electrically neutral environment and the mixure of different oxygen forms containing extremely low doses of radicals is applied by inhalation in a short periods of 12-20 min with favorable responses (6).

Based on the promissing results with clinical application (7) and *in vitro* studies on ionized oxygen (8, 9), in the present study we aimed to prove the hypothesis that the long-term application of ionized oxygen is a safe method with fewers adverse effects on lung function in comparison with classical oxygen therapy.

MATERIAL AND METHODS

The experimental protocol was approved by the Ethics Committee of the Jessenius Faculty of Medicine (EK 308/2007) and by the State Veterinary and Food Executive of the Slovak Republic (Ro-430/07-221).

The experiments were carried out in 40 adult Trik guinea pigs weighing 327 ±57 g. The animals were placed in a sealed metabolic cage, as described previously (10) with some modifications and exposed to 100% medical molecular oxygen (O_2mol), or partially negatively (O_2neg) or positively (O_2posit) ionized oxygen, using a unipolar device Oxygen Ion 3000/by Dr.Engler (CStronic GmBH, Austria, No.SN 1512-140765 VER 13-13) for 17 and 60 h. The oxygen passed from the device to the animal cage through 60 cm long silicon antistatic tube. At the oxygen flow 8 l/min, the device generated 200 000 positively or negatively ionized oxygen ions/ml of oxygen (Ions Measurement Device, Kathrein-Werke KG, Germany). Oxygen concentration was periodically monitored by an oxygen analyzer (Permolyt 3, Veb Junkalor, Germany). The control animals inhaled atmospheric air. Biophysical parameters of the cage environment were maintained as follows: temperature 23-25°C, humidity 60-70 %, concentration of $CO_2 \approx 0.2$ vol %.

At the end of experiment, a lethal dose of anaesthetics was injected intraperitoneally. A part of the right lung was cut, strips of the tissue were weighed and dried at 60°C for 24 h. The wet/dry weight (W/D) ratio was determined to evaluate the degree of lung edema. Other parts of the lungs were homogenized and the products of lipid and protein oxidation were determined as described elsewhere (11). Briefly, the concentration of lipid peroxidation (LPO) products (thiobarbituric acid-

reactive substances, TBARS) was determined from the absorbance at 532 nm and expressed in nmol/mg protein (12). The accumulation of dityrosine (13) and lysine-LPO products (14) demonstrating oxidative modification of proteins were determined in lung homogenate by fluorescence method using spectrofluorometer (RF-540, Shimadzu, Japan). Protein assay was performed using bovine serum albumin as a standard (15).

The left lung was lavaged with 0.9% NaCl (37°C) 3x10 ml/kg and bronchoalveolar lavage fluid (BALF) was centrifuged at 1500 rpm for 10 min. The relative number of cells was evaluated microscopically in BALF sediment and peripheral blood. Remaining fluid was centrifuged at 40000 x g for 1 h at 4°C. The pellet was lyophilized and resuspended in 10 µl of sample buffer (3.5 mM $CaCl_2$, 10 mM HEPES, and 0.5 mM EDTA, pH = 7) (16). The final phospholipid concentration was 1 mg/ml. A capillary surfactometer (CS) (Calmia Medical, Toronto, Canada) was used for the assessment of BALF´s ability to maintain the patency of the narrow capillary that simulated the terminal conducting airways. The airflow through capillary was maintained for 120 s after the liquid was first extruded and the percentage of the total study time for which the capillary was open was calculated (open%) by CS. The open% value for each animal was taken as the mean of 5 assays of BALF.

The results are expressed as means ±SD or as median and range. The between-group differences were examined by the analysis of variance (ANOVA) followed by Student-Neuman-Keuls´test. Data which were not normally distributed were analyzed by Kruskal-Wallis test (BALF cells and surfactant data). A P value ≤ 0.05 was regarded as statistically significant.

RESULTS

Markers of lipid and protein oxidation

Changes in the oxidative status are shown in *Fig. 1* (*Panels A* and *B*) and in *Table 1*. After 17 h, the level of dityrosines (in arbitrary units) in the lungs increased in O_2neg group (19 ±0.8) and decreased in O_2posit group (16.1 ±1.2) *vs*. controls (17.5 ±0.8) (both P<0.01). In the group inhaling molecular oxygen (18.1 ±0.8), the increase was not statistically significant. After 60 h, the fluorescence of dityrosines significantly rose in the O_2mol and O_2neg groups *vs*. controls (20.2 ±1.6 and 21.7 ±7 *vs*. 17.5 ±0.8; both P<0.01), while in the O_2posit group there was no increase (17.3 ±0.8) (*Fig. 1A*).

The level of lysine conjugates with LPO products (in arbitrary units) in the lung homogenate significantly increased after 17 h of inhalation of O_2neg (8.2

Table 1. Plasma values of TBARS and SH-groups after 60 h of oxygen inhalation in the experimental and control groups.

Group	TBARS (nmol/mg of protein)	SH-groups (mmol/mg of protein)
O_2neg	0.170 ±0.027	0.028 ±0.007*
O_2mol	0.173 ±0.023	0.026 ±0.007*
O_2posit	0.194 ±0.041	0.034 ±0.005
Control	0.189 ±0.021	0.040 ±0.006

Values are means ±SD. Statistical analysis of between-group differences: *P<0.05 *vs*. control.

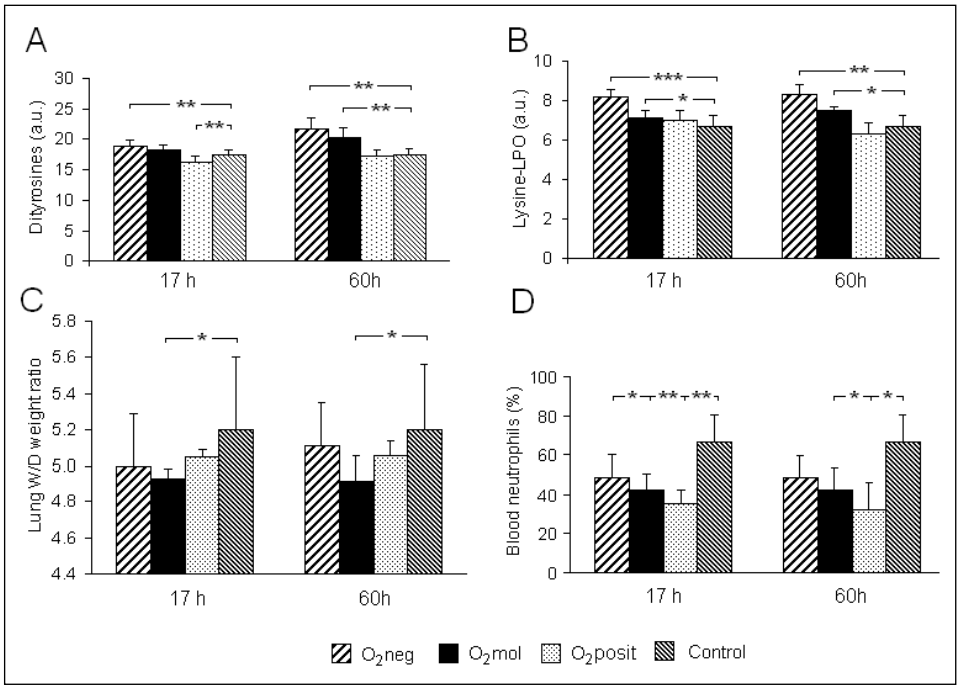

Fig. 1. A - Fluorescence of dityrosines; B - Lysine conjugates with LPO products; C - Values of lung wet/dry weight ratio; and D - Relative number of neutrophils in the blood, all after 17 and 60 h in animals with different forms of oxygen and in controls. a.u. - arbitrary units; statistical analysis of between-group differences: *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2. Relative number of neutrophils in BALF (%) and activity of pulmonary surfactant (% of capillary open time) after 17 and 60 h of oxygen inhalation in the experimental and control groups.

Group	Neutrophils in BALF (%)		Activity of pulmonary surfactant (open%)	
	17 h	60 h	17 h	60 h
O_2neg	7.7 (6.1-12.7)**§	15.1 (8.3-21.8)**	23.4 (21.7-46.9)$^{+}$	15.9 (2.1-41.6)
O_2mol	9.4 (3.1-17.1)*	6.0 (3.8-12.2)*	6.1 (0.5-9.9)	14.9 (2.2-19.9)
O_2posit	4.0 (3.4-4.8)	7.7 (7.3-8.0)*	1.3 (0.1-20)	24.5 (20.4-40.2)§
Control	2.4 (1.0-5.3)	2.4 (1.0-5.3)	96.5 (95.6-98.2)*	96.5 (95.6-98.2)*

Values are medians and (range). Statistical analysis of between-group differences: neutrophils in BALF - after 17 h *P<0.05 and **P<0.01 vs.control; §P< 0.01 *vs*. O_2posit; after 60 h *P<0.05 and **P<0.01 *vs*. control; activity of pulmonary surfactant - after 17 h *P<0.05 vs.all oxygen-inhaling groups, $^{+}$P<0.05 *vs*. O_2mol and O_2posit; after 60 h *P<0.05 *vs*. all oxygen-inhaling groups, §P<0.05 *vs*. O_2mol.

±0.4; P<0.001) and O_2mol (7.1 ±0.4; P<0.05) in comparison with controls (6.7 ±0.6). In the O_2posit group, the fluorescence of lysine-LPO products did not rise significantly (7.0 ±0.5; P>0.05 *vs*. controls). After 60 h, the changes were comparable with those after 17 h (*Fig. 1B*).

The level of TBARS and the activity of CuZn superoxiddismutase (SOD) significantly rose in all oxygen-inhaling groups after 17 and 60 h in comparison with controls (data not shown). After 60 h of oxygen therapy, the plasma level of TBARS did not significantly change, while the SH-groups were reduced in the O_2neg and O_2mol groups in comparison with air-breathing animals (*Table 1*).

Inhalation of partially negatively and positively ionized oxygen did not significantly change the W/D ratio either after 17 or 60 h. Inhalation of molecular O_2 significantly reduced the W/D ratio *vs*. controls (at 17 and 60 h, both $P<0.05$) (*Fig. 1C*).

The relative number of blood neutrophils decreased after 17 h in all oxygen-inhaling groups; after 60 h, the decrease was significant in the O_2mol and O_2posit groups (*vs*. control all $P<0.05$ or 0.01) (*Fig. 1D*). The number of neutrophils in BALF was significantly elevated only in animals with O_2neg and O_2mol, but not in the O_2posit group. After 60 h, the number of neutrophils in BALF rose in the O_2posit animals as well (*Table 2*).

The activity of pulmonary surfactant, expressed as the open%, was significantly reduced in all animals inhaling oxygen in comparison with air-breathing guinea pigs after 17 and 60 h. Moreover, after 17 h the open% was significantly higher in the O_2neg group in comparison with O_2mol and O_2posit, while after 60 h it was significantly higher in the O_2posit animals compared with the O_2mol group (*Table 2*).

DISCUSSION

In the present study we evaluated the effects of 17 and 60 h lasting inhalation of molecular and partially negatively and positively ionized oxygen on lung functions. We used a guinea pig animal model for hyperoxic lung injury, which has been succcesfully used earlier (10). It is known from animal and human studies that exposure to high oxygen concentration causes direct oxidative cell damage through increased production of reactive oxygen species (3). Hyperoxia induces lung injury results from direct oxygen toxicity and from the accumulation of inflammatory cells and mediators within the lungs (3). Influx of leukocytes into the lungs is mediated by the degree of chemotactic activity, which is well controlled under physiological conditions. In hyperoxic lungs, chemotactic activity and leukocyte infiltration are highly elevated.

In our experiments, the relative number of inflammatory cells, neutrophils, in BALF increased already after 17 h in animals inhaling molecular and negatively ionized oxygen, but not in those with positively ionized oxygen. However, hyperoxia lasting for 60 h evoked the elevation of neutrophils in all three oxygen-treated groups and a higher percentage of neutrophils in BALF was associated with a lower neutrophil count in the blood. Similarly, in the rat lungs, exposure to 95% O_2 for 48 h induces substantial neutrophil infiltration which coincided with

the massive damage to endothelial and epithelial cells (17). Recruited inflammatory cells, mainly leukocytes, accumulated within the airspaces are a significant source of additional ROS. As reactive oxygen species are of key importance in the oxygen-induced lung injury, we investigated oxidative damage to lipids and proteins in the lung homogenate and plasma. Peroxidation of lipids by free oxygen radicals leads to the release of lipid peroxidation products, *e.g.*, TBARS (12). We found no changes in the plasma level of TBARS in comparison with air-breathing animals, while TBARS increased in lung homogenate in all oxygen-treated animals.

Aromatic amino acids, like tyrosine and tryptofan, are modified by oxidation and can be estimated by fluorescence of dityrosine and lysine-LPO products (13, 14). After 17 h, the accumulation of dityrosines in lung homogenate was increased only after inhalation of negatively ionized oxygen, and was reduced in the group with positively ionized oxygen. After 60 h, dityrosines rose in animals with molecular O_2 and negatively ionized O_2, but not in O_2posit group. Lysine-LPO products increased only after inhalation of molecular and negatively ionized oxygen.

Another promissing result of othis study was a lower degree of lung dessication (higher lung wet/dry weight ratio) in animals with positively and negatively ionized oxygen in comparison with molecular oxygen. Drying of the respiratory system is an adverse effect of oxygen therapy and our finding could indicate no need for humidification of inhaled partialy ionized oxygen.

Oxidative stress on the endogenous surfactant represents an important mechanism contributing to the surfactant dysfunction associated with hyperoxia-induced lung injury (1, 18). Surfactant lipids and proteins may be directly affected by ROS (19) or by activated neutrophils (20, 21). Synthesis and secretion of pulmonary surfactant may be further impaired by hyperoxia-induced changes in the type II alveolar epithelial cells manifested by apoptosis and DNA damage (22). Important role in surfactant inactivation is played by plasma proteins leaking into the airspaces due to increased alveolar-capillary permeability (23). Moreover, oxidative stress to alveolar epithelial cells is linked to the release of mediators that induce surfactant gelation. Interfacial rheology of gelled surfactant is changed and it becomes functionally inactive (4).

In our study, the results on surfactant activity differs after 17 and 60 h hyperoxia. While after 17 h, the activity of pulmonary surfactant was best preserved in animals with negatively ionized oxygen, after 60 h the activity of surfactant also was improved in animals with positively ionized oxygen. At both time points, lowest surfactant activity was in the group with molecular oxygen. Based on the observations of Baker *et al* (24), differences in the surfactant activity may reflect changes in O_2 adaptation. In the context of the above-discused results, we speculate that negatively ionized O_2, which seems to be more harmful, might stimulate the surfactant metabolism relatively early (17 h) in comparison with the other oxygen forms. With a prolonged exposure time (60 h),

this compensatory reaction may be present also after inhalation of the other oxygen forms.

The mechanisms leading to differences in the response of biological system to different forms of oxygen are not exactly known. Both negatively ionized oxygen, with a negative electrical charge possessing one electron, and positively ionized oxygen, lacking one electron, are reactive oxygen species. They differ only in electrical loading. Even when negatively ionized oxygen is used in small amounts (see Material and Methods), in several parameters measured (markers of protein oxidation, inflammatory cells), it seems to potentiate the effect of long-term exposure to neutral (molecular) oxygen. By contrast, positively ionized oxygen seems to be a better radical scavenger as evidenced by reduced accumulation of markers of protein peroxidation.

There has so far been only one published clinical study dealing with long-term effects (2 years) of positively ionized oxygen inhalation in patients with progressive multiple sclerosis (7) and a few *in vitro* experiments whose results conform with those of the present study. Resistance of red blood cells to hemolysis is increased after exposure to positively ionized O_2, while negatively ionized oxygen has the opposite effect (8). Similarly, positively ionized oxygen significantly increases membrane resting potential in human embryonal pulmonary fibroblasts after their exposure to radon (9) and causes selective destruction of the same cells after their viral malignant transformation (9). These effects and those found in our experiments may be explained on the basis of hormesis theory. While high doses of radiation or ROS are biologically destructive, small doses are proven to have protective effects (25).

In conclusion, long-term inhalation of partially positively ionized oxygen is associated with less oxidative stress, better ability to secure airway patency, and milder lung inflammatory response than molecular or negatively ionized oxygen.

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