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An attempt to use immunohistochemical methods for semi-quantitative determination of surfactant in bronchial secretion after hyperbaric exposures

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Abstract:

Background

The most significant index of pulmonary oxygen toxicity is a decrease in vital capacity (VC) dependent on the duration of exposure and partial pressure of oxygen. The only method to measure this decrease is spirometry performed directly after exposure.

Objective

The aim of the study was to check whether the extent of lung damage could be assessed by quantitative determination of pulmonary surfactant in bronchial secretion.

Design

Sputum samples were collected before, during and after hyperbaric air or oxygen exposures; histological preparations were prepared and stained immunohistochemically to visualize surfactant.

Amongst 781 samples collected, 209 contained sputum and 126 were included in the study. In this group, only 64 preparations could be paired for comparison.

Results

The semi-quantitative method used and statistical findings have not demonstrated any significance.

Conclusions

The method suggested for assessing the extent of lung damage has been found unsuitable for practical use due to difficulties in obtaining the proper sample; moreover, the study findings do not allow to draw conclusions concerning its effectiveness.

Keywords: oxygen toxicity, lung surfactant, immunochemical methods, detection

Introduction

Toxic effects of oxygen are physiological and pathological reactions of organisms resulting from exposure to partial pressure of oxygen higher than that in the atmospheric air. The problem is becoming increasingly common due to more frequent oxygen use during diving, hyperbaric therapy¹⁻³ and long-term oxygen therapy in patients with respiratory failure. The effect of oxygen during hyperbaric exposure is of particular relevance. Although the exact reactions have not been fully elucidated, oxygen toxicity is assumed to be associated with the generation of its reactive forms⁴ and interactions with the adjacent cell structures, which lead to functional and morphological deficits⁵⁻⁸. The effector organs that are affected by oxygen are the central nervous system (CNS) and lungs. The CNS symptoms arise due to the partial pressure of oxygen⁹ whereas the lung symptoms depend on the partial pressure and duration of exposure¹⁰. When the partial pressure of oxygen in the pulmonary alveoli exceeds the arterial P_aO_2 , the lungs are considered to be exposed to a high oxygen pressure. At oxygen pressures of 0.05 – 0.2 MPa, maximum time of exposure is limited by the development of symptoms of pulmonary oxygen toxicity^{1,11,12}. This limits the time of exposure of a diver under such conditions. In addition to clinical symptoms such as dyspnoea, pain and burning sensation in the throat and/or thorax, cough, retrosternal pain, or easy fatigue¹³⁻¹⁵, the basic measurable clinical index of pulmonary oxygen toxicity is a decrease in vital capacity (VC)¹³⁻¹⁵. When the partial pressure exceeds 50 kPa, the “oxygen clock” kicks in. The following units were introduced to determine quantitatively the toxic effects of oxygen on pulmonary parenchyma: unit of pulmonary toxicity dose (UPTD), cumulative pulmonary toxicity dose (CPTD), and oxygen tolerance unit (OTU), which corresponds to damage resulting from one-minute exposure to 100% oxygen at a pressure of 0.1 MPa (1 ATA)¹³. The OTU plays an essential role in estimation of a decrease in VC related to oxygen toxicity. A decrease in VC is associated with:

- Damage to surfactant structure

- Desquamation of surfactant to the alveolar lumen
- Toxic damage to type II pneumocytes, i.e. inhibition of surfactant production.

All the mechanisms mentioned above can be associated with the function of pulmonary surfactant, which maintains surface tension and alveolar size. The only reliable test determining the extent of lung damage (atelectasis) caused by hyperbaric oxygen is spirometry. Its limitation is the necessity to perform the test immediately after diving. The use of histopathological or cytological methods would enable simultaneous collection and storage of samples from a large group of divers and performing the tests at any time after diving.

Aim

The aim of the study was to design a method for indirect determination of lung damage under conditions of oxygen hyperbarism by examining the amount of surfactant in the bronchial secretion.

Materials and methods

The sample, i.e. bronchial secretion, can be obtained from:

- bronchoaspirate
- bronchial lavage
- sputum

The first two methods are invasive and complex. Therefore, in our study the sputum was examined.

The samples were collected between 2000-2011 during hyperbaric exposures in a pressure chamber using air or oxygen. Air exposures were administered in a series of two 30-minute exposures with the first one to 0.4 MPa and the second one to 0.7 MPa with a one-day interval. The exposure profiles are presented in Fig.1 and Fig. 2.

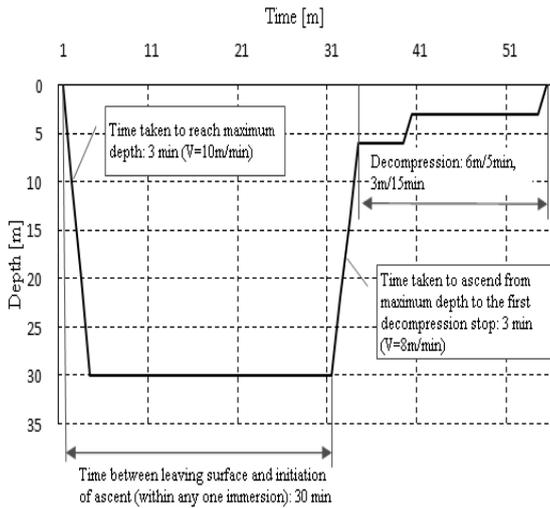


Figure 1. Profile of 30m/30min. exposure¹⁶ Exposure parameters: Time taken to reach maximum depth: 3 min ($V=10\text{m/min}$), time between leaving surface and initiation of ascent (within any one immersion): 30 minutes, time taken to ascend from maximum depth to the first decompression stop: 3 min ($V=8\text{m/min}$), decompression: 6m/5min, 3m/15min.

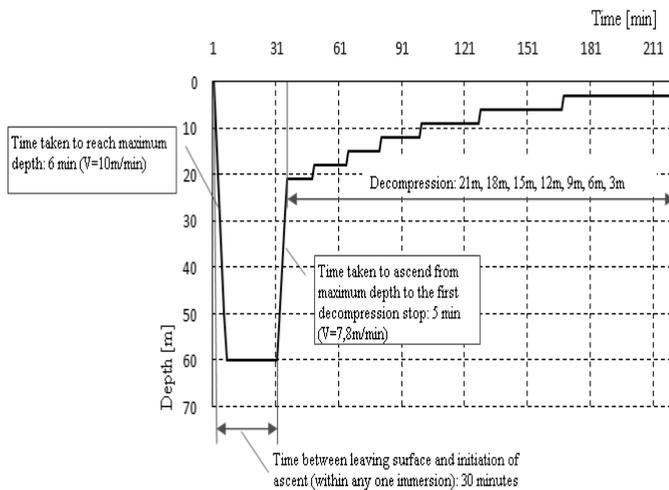


Figure 2. Profile of 60m/30min. Exposure¹⁶ parameters: Time taken to reach maximum depth: 6 min ($V=10\text{m/min}$), time between leaving surface and initiation of ascent (within any one immersion): 30 minutes, time taken to ascend from maximum depth to the first decompression stop: 5 min ($V=7.8\text{m/min}$), decompression: 21m/12min, 18m/15min, 15m/16min, 12m/19min, 9m/28min, 6m/40min, 3m/52min.

The samples from oxygen exposures were collected during oxygen tolerance test. This group was administered a single exposure. The exposure profile is presented in Fig.3.

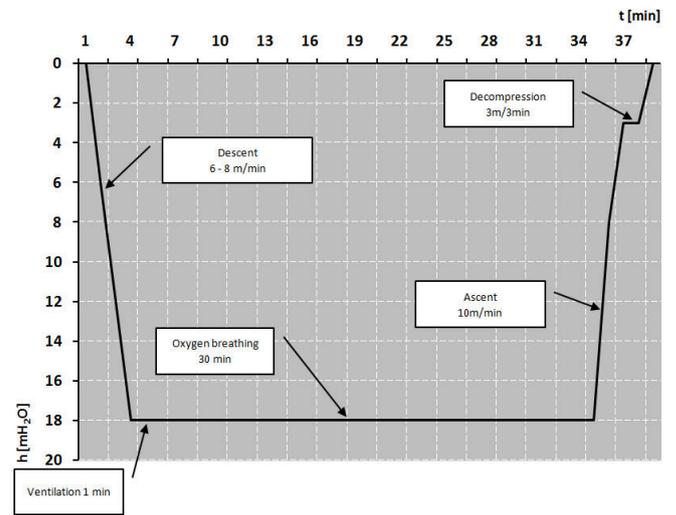


Figure 3. Oxygen exposure profile¹⁷

The study encompassed 270 individuals undergoing training or experimental exposures in the hyperbaric complex DGKN 120, Department of Diving and Underwater Work Technology, Naval Academy.

In total, 781 samples were collected, including 84 from oxygen exposures. All participants were instructed on how to expectorate to limit the number of non-diagnostic samples such as the saliva.

The sputum was expectorated into a polyethylene container filled three quarters with 80% ethyl alcohol after – rinsing mouth.

The samples were marked in the following way:

- “a” – before exposure
- “x” – during exposure (only the exposures in the hyperbaric chamber)
- “b” – after exposure
- “c” – before the second exposure
- “y” – during the second exposure (only the exposures in the hyperbaric chamber)
- “d” – after the second exposure.

The sputum was fixed in 80% ethyl alcohol for about 7 days and subsequently in 10% neutralized formalin directly before embedding in paraffin blocks.

Histopathological specimens were prepared using the paraffin method and sliced into 4-micron sections with a sledge microtome; 586 paraffin blocks were obtained from

781 samples. The remaining samples (195) formed a suspension in the fixer, from which the samples were not recovered despite the use of methods for cytological material recovery. Similarly, some samples were lost during embedding in paraffin.

Histological specimens from paraffin blocks were stained with haematoxylin-eosin and selected for further tests. Two pathologists assessed the same preparations independently. Pulmonary macrophages were used to indicate the origin of sample from not only the oral cavity but also the bronchial tree. During the second stage of selection, the surface occupied by the sample identified as sputum was evaluated. When ten non-overlapping fields were observed under 100 x magnification, the material qualified for further tests.

Two hundred and nine preparations were identified as "sputum", including 126 selected for further tests based on the surface occupied by the bronchial tree material.

The selected paraffin blocks were re-sliced and the assay for the presence of human surfactant using SURFACT PROTEIN A, RB X (Millipore) was performed. A red colour denoted the presence of the reaction in the preparation.

The microscopic picture of an example reaction in the preparation is shown in Fig.4.

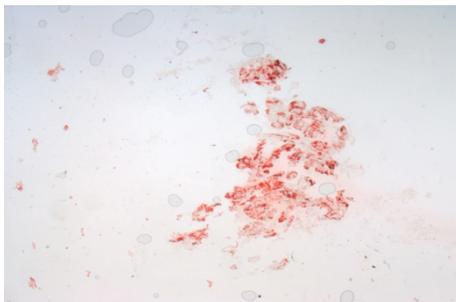


Figure 4. Human surfactant immunohistochemical reaction

Preparations were assessed semi-quantitatively as follows:

0 – no reaction

1 – Single colour fields in the preparation

2 – Reaction involving over 25% of surface

3 – Reaction involving over 75% of surface.

The results were compiled in tables; whenever possible, they were paired and statistically analysed.

Results

Thirty-two cases (64 preparations) were found in which comparisons were possible as they formed pairs from the same individual and from the same exposure. The following pairs were used for comparisons:

- "a" and "b"
- "a" and "x"
- "c" and "d"
- "c" and "y"

First, the normality of variable distribution was tested. All the variables showed the normal distribution.

Subsequently, descriptive statistics were used. High values of standard deviations were of interest, which is presented in Fig.5.

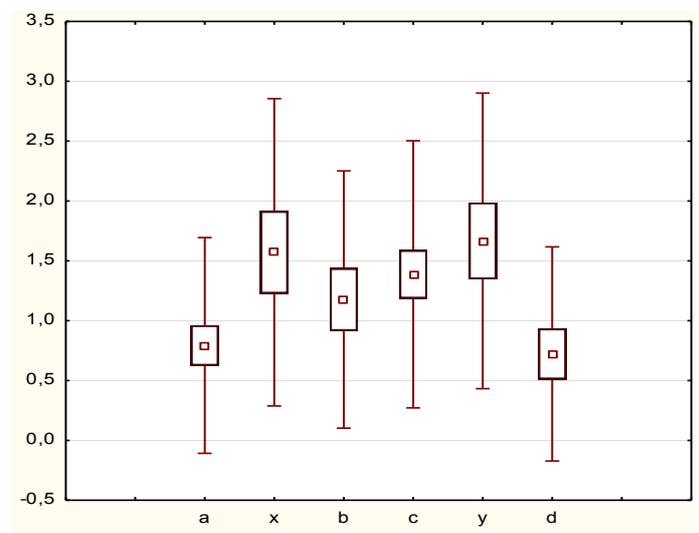


Figure 5. High values of standard deviations in all groups. ◻ – average, ◻ – average ± SE (standard error), T ⊥ average ± SD:(standard deviation)

Statistical significance was tested using the t test for dependent samples.

Based on the results indicated in the table below, we concluded that there were no statistically significant intergroup differences. The findings and significance level are depicted in Tables 1 and 2.

Table 1. The Student's t- test for dependent samples; $p > 0.05$ for each pair

group	a	x	b
A	1.000000	0.476179	0.087693
X	0.476179	1.000000	0.194171
B	0.087693	0.194171	1.000000

Table 2. The Student's t- test for dependent samples. The differences in bold are significant at $p < 0.05$

group	C	y	d
C	1.000000	0.234745	0.047892
Y	0.234745	1.000000	0.284931
D	0.047892	0.284931	1.000000

The findings demonstrated that there were no statistically significant differences between the pairs of groups, except for groups c-d. However, the level of significance p is worth noticing, which is close to $p=0.05$.

Assessment of the efficiency of the method revealed that only 589 paraffin blocks were prepared from 781 samples. According to the first selection, sputum – non-sputum, 209 preparations were qualified; 126 preparations were suitable for semi-quantitative examinations, including 64 preparations that formed pairs enabling comparisons. Percentage distribution is presented in Table 3.

Table 3. Percentage distribution

Number	Material	percentage
781	Collections	100
586	Blocks	75
209	Sputum	27
126	for analysis	16
64	preparations forming pairs	8
32	Pairs	

Discussion

Our study showed that pulmonary surfactant is desquamated, is present in the sputum and can be detected using immunohistochemical methods. Moreover, the presence of surfactant in the preparation can be semi-quantitatively analysed.

Mouth rinsing was found essential for material collection. After careless mouth rinsing, food residues constituted a substantial part of the preparation; hard parts, when present (e.g. parts of nuts), hindered or prevented cutting of the paraffin block.

The designed method for material collection fulfilled the conditions of simplicity and longer storage of material for further analysis. However, it did not fulfil the basic condition of probability of obtaining the diagnostic sample. Although the procedures were followed strictly, only 27% of the sample was usable sputum. For comparisons, pairs of preparations were required and only 8% of preparations formed pairs.

Obtaining only 8% of preparations suitable for use disqualifies the method in question. Furthermore, low efficiency of the method prevents its routine use for assessment of lung parenchyma damage after hyperbaric exposures.

The authors plan to undertake further studies on the group of patients, who have been receiving a long-term oxygen treatment in intensive care units. The results obtained from their sputum will be compared with the results obtained from the BAL.

In spite of low efficiency of the method, semi-quantitative determinations and statistical analysis were carried out, which confirmed that the method was not useful. Comparisons of pairs did not demonstrate statistical significance; extremely high scattering of results and high standard deviations in all groups hindered interpretation of results.

In our opinion, the only reliable method of determining lung damage caused by breathing oxygen, is the current method of spirometry.

Conclusions

1. There are no significant differences in the amount of surfactant in sputum before, during and after hyperbaric exposure.
2. The immunohistochemical method for determination of surfactant in sputum is not suitable for assessment of pulmonary oxygen toxicity due to difficulties in obtaining the diagnostic sample.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest in the research.

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