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Ovine plasma dipalmitoylphosphatidylcholine does not predict decompression bubbling

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ABSTRACT

Decompression illness (DCI) is the main risk associated with scuba diving. Some divers ("bubblers") are more sensitive to DCI than others ("non-bubblers"). We found that there are active hydrophobic spots (AHS) on the luminal aspect of ovine blood vessels, which contain the surfactant dipalmitoylphosphatidylcholine (DPPC). DPPC leaks from the lung into the plasma, settling on the blood vessel to create AHS. These are the main source of gas micronuclei from which bubbles develop after decompression. A correlation between bubbling ovine blood vessels and the animal's plasma DPPC might lead to the development of a blood test for vulnerability to DCI. Samples from ovine blood vessels were stretched on microscope slides, placed anaerobically in saline at the bottom of a Pyrex bowl, and exposed to high pressure. Automated photography was used after decompression to reveal AHS by visualising their bubble production. Phospholipids were extracted from the AHS and plasma for determination of DPPC. Bubbling was unrelated to the concentration of DPPC in the plasma (2.15 ± 0.87 μg/ml). Bubble production from the AHS (n = 130) as a function of their DPPC content yielded two groups, one unrelated to DPPC and the other which demonstrated increased bubbling with elevation of DPPC. We suggest this may be related to alternate layering with hydrophobic and hydrophilic phospholipids. This study reinforces the connection between DPPC and DCI. However, a blood test for diver vulnerability to decompression stress is not recommended.

1. Introduction

Decompression illness (DCI) is the main risk associated with scuba diving. It is well known that there are certain divers ("bubblers") who are more sensitive to decompression stress than others ("non-bubblers") (Cialoni et al., 2015; Kaczerska et al., 2013; Lambrechts et al., 2013). However, there is no way of determining beforehand whether a prospective diver will be vulnerable to decompression.

Over the past few years, we have found that there are active hydrophobic spots (AHS) on the luminal aspect of ovine blood vessels. The surfactant dipalmitoylphosphatidylcholine (DPPC) leaks from the lung into the plasma, settling on the blood vessel to create the AHS (Arieli and Marmur, 2014, 2016; Arieli et al., 2015, 2016). We also suggested that these AHS are the main source of gas micronuclei from which bubbles develop after decompression (Arieli, 2017).

Surfactant proteins (SPs) in the lung are necessary to stabilise the films of DPPC: SP-B for the assembly and SP-C to be inserted within the layers for stability. Both proteins were found in the plasma of control subjects in studies of lung injury: SP-B 14.3 ng/ml, and SP-C 1.4 ng/ml (Papaioannou et al., 2016). These SPs, together with the DPPC (2.04 μg/ml) which we identified in ovine plasma (Arieli et al., 2016), enable layering of the DPPC to create the AHS. The AHS we described should coincide with the oligolamellar lining of phospholipids on the luminal aspect of ovine blood vessels described by Hills (1992). Thus lung surfactants, mainly DPPC, play a major role in DCI (Arieli, 2017). The distribution of DPPC in the bubbling blood vessels of sheep, as opposed to non-bubbling vessels, was similar to the number of bubblers as against non-bubblers among divers (Arieli, 2017). It may be that increased leakage of DPPC from the lung into the plasma will result in more AHS, covering a greater surface area, and consequently an increase in bubble production after decompression. Alternatively, variability between sheep could be related to the endothelial sites at which DPPC settles, and this may account for the variability between bubblers and non-bubblers.

In our study demonstrating the presence of DPPC at the AHS (Arieli et al., 2016), the temperature during the hyperbaric exposure was not

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uniform, and more bubbles were produced in warmer saline. So we were unable to compare bubbling of a sheep with its plasma DPPC levels. In that study, we showed that AHS which produced 4 bubbles or more within 30 min contained more DPPC than the less productive AHS. However, due to the temperature differences, a more refined analysis could not be performed. The density of nanobubbles (the source of gas micronuclei) on a hydrophobic surface is highly dependent on the water temperature. A low density was found below 30 °C, with a sharp increase as the temperature rose above 30 °C (Yang et al., 2007). In the present study, the temperature of the saline during the hyperbaric exposure was maintained constant at 7 °C. Immediately after decompression, the saline was warmed up, reaching 32 °C at the end of the photography period. These controlled conditions were intended to enable us to determine whether there was a correlation between DPPC levels in the AHS and their bubble productivity, and between bubbling sheep and their plasma DPPC. A positive correlation of bubbling with plasma DPPC might lead to the development of a blood test for diver vulnerability to decompression.

2. Methods

The methods were described in detail in our previous reports (Arieli and Marmur, 2016; Arieli et al., 2015, 2016), and will be presented briefly here.

2.1. Tissue preparation

The complete heart and lungs from 15 slaughtered sheep (taken on separate days) were obtained at the abattoir. A blood sample was also taken from the throat incision using a heparinised syringe. In the laboratory, under saline and without any exposure to air, samples from four blood vessels, the aorta, superior vena cava, pulmonary vein and pulmonary artery, were gently stretched on microscope slides, held by metal clips and with the luminal aspect exposed. Four slides were placed anaerobically at the bottom of two Pyrex bowls (diameter 26 cm, height 5 cm), two in each bowl under 2.5 cm saline. One bowl was placed in the high pressure chamber and the second was kept in a refrigerator at 6 °C until the following day. Blood samples were centrifuged in a cooled centrifuge (4 °C) for 10 min at 2500 rpm for plasma separation. DPPC is most probably carried by albumins, because it is not soluble in water. The plasma was kept in a freezer at −20 °C until extraction of the phospholipids.

2.2. Protocol

Active hydrophobic spots can be determined by observing the formation of bubbles after decompression. The bowl containing the two blood vessels was transferred to a 150-litre hyperbaric chamber and placed on a pair of welded aluminum plates, with cooled water circulating in the space between the two plates. Cooling during the hyperbaric exposure was necessary for tissue preservation. The temperature of the circulating water was 6 °C. Saline temperature was 1 °C above that of the circulating water. Pressure was elevated (using air) at a rate of 200 kPa/min to 1013 kPa, 90 m sea water, and remained at that pressure overnight (18.9 ± 3.3 h). In the morning, the chamber was decompressed at a rate of 200 kPa/min. The bowl was placed carefully on a pair of welded aluminum plates for photography, with hot water (70 °C) circulating in the space between the two plates. Saline was delivered over the blood vessel at a flow rate of 100 ml/min using a peristaltic pump. It was shown in our previous study (Arieli et al., 2015) that circulating saline enhanced bubble development by replenishing gas-saturated saline in the vicinity of the AHS. Circulation also helped heat and equilibrate the cold saline during the photography period. The temperature of the saline from the end of decompression to the termination of photography is displayed in Fig. 1. We began automated photographing at 1-s intervals 15 min after the end of decompression,

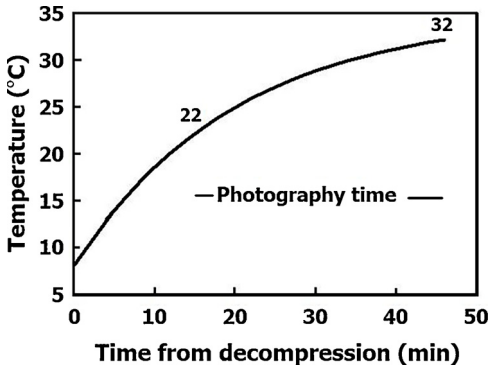

Fig. 1. Temperature of the saline from the end of decompression to termination of the 30 min automated photography.

for a period of 30 min. At the end of this photographic session, the slide was photographed against scaled paper and was transferred to a freezer (−20 °C). The same protocol was followed for the second bowl the next day.

The photographs of each sample were examined in sequence for the appearance of bubbles. Each bubble was observed until detachment. Bubbles produced near the edges of the tissue were not taken into consideration, and therefore the last 1 mm to the edge of the tissue was not included in the analysis. At the end of the observation period, the location of AHS and the number of bubbles produced at each AHS were noted on the photograph that had been prepared for use during tissue sampling. The area of the blood vessel samples was measured using the image processing program Image-Pro-Plus (Media Cybernetics Inc., Bethesda, MD, USA). The mean sampled area for all four blood vessels was 35.3 ± 5.7 cm^2.

2.3. Phospholipid extraction

Internal standard solution of 1 mg of 1, 2-Diheptadecanoyl-PC in 1 ml methanol was prepared.

2.3.1. Tissue sampling

While viewing the photograph of a blood vessel with its AHS, a section of the blood vessel containing the AHS was dissected out, weighed (0.028 ± 0.025 g) and placed in a test tube. For comparison, similar control samples were taken from blood vessels which produced no bubbles at all. Two µl of internal standard solution, 1 ml of chloroform and 2 ml methanol were added. After homogenisation for 2 min, another 1 ml of chloroform was added. Following another 30 s homogenisation, 1 ml double distilled water was added and 30 s homogenisation was performed. The homogenate was centrifuged (4 °C, 2000 rpm, 10 min) and the lower chloroform phase was collected.

2.3.2. Plasma sampling

Two µl internal standard solution, 2 ml of chloroform and 1 ml methanol were added to 0.5 ml of plasma and vortexed for 2 min. The solution was centrifuged at 2000 rpm for 10 min at 4 °C. The lower chloroform phase was collected. Another 1 ml of chloroform and 0.5 ml methanol were added to the upper phase, vortexed for 2 min, centrifuged (4 °C, 2000 rpm, 10 min), and the chloroform phase obtained was added to the previous phase.

2.3.3. Common procedure

The following procedure was common to both the tissue and plasma chloroform phase. After the addition of ~250 mg sodium sulfate and vortexing for 30 s, the test tube was kept still for 10 min and then filtered through cotton. The solvent was dried using a flow of nitrogen, and the test tube containing phosphatidylcholine was stored in a freezer at −20 °C for further analysis. Tissue extraction samples (n = 142) and

plasma samples (n = 15) were prepared for determination of DPPC.

2.4. Determination of DPPC

Samples were re-dissolved in 0.5 ml of methanol, vortexed and centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was then collected and injected into a QTOF LC/MS (Agilent Technologies, Santa Clara, CA, USA) for the analysis of DPPC and the Internal standard (IS) 1,2-Diheptadecanoyl-PC. 10 µl of the supernatant was injected into a 1290 infinity LC system (Agilent Technologies, Santa Clara, CA, USA) connected to a C-18 reverse-phase column, XTerra C18 3.5 µm, 4.6 × 20 mm (Waters Corporation, Milford, MA, USA). The solvents used for separation of DPPC and the IS were solvent A (DDW with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). Solvent B remained at 93% for 17 min, then increased from 93% at 17 min to 98% at 20 min, remaining at 98% for another 5 min, with a flow rate of 0.5 ml/min. The LC eluent was introduced directly into the electrospray ionisation (ESI^+) source connected to a UHD accurate-mass Q-TOF LC/MS 6540 (Agilent Technologies, Santa Clara, CA, USA). The ESI capillary voltage was set at 3500 V, fragmentor 150 V, gas temperature 350 °C, gas flow 8 ml/min, and nebuliser 35 psi. The mass spectra (m/z 100–1700) were acquired in a Positive-ion mode. The calibration curve of DPPC and IS was prepared in methanol at a concentration range of 0.05 to 5 ppm and injected under identical conditions. A linear curve was obtained with R^2 = 0.9971 and 0.9955 for DPPC and the IS, respectively. Results were expressed in concentration of DPPC in the plasma and total DPPC in the AHS.

2.5. Statistical analysis

Pearson correlation test will be used for the correlation of total bubble production and DPPC in the plasma and for the relationship between AHS bubble production and its DPPC content.

3. Results

Standardisation of the course of saline temperature enabled us to conduct an analysis of the results. The mean concentration of DPPC in the plasma of 15 sheep was 2.15 ± 0.87 µg/ml. Bubble production in the four blood vessels of the sheep is presented in relation to DPPC concentration in the plasma in Fig. 2. No correlation could be seen between bubbling level and DPPC concentration in the plasma.

Of the 142 samples taken from blood vessels, eight had an erroneous internal standard solution and were discarded. A further four samples had an undetermined bubble count and were also discarded. DPPC was found in all samples, including those from blood vessels which produced no bubbles at all. The relationship between AHS bubble production and their DPPC content is shown in Fig. 3. The data appear to fall into two populations: dependent and independent groups. After deletion of the common section of both populations, linear regression was performed for the dependent part: bubble production > 4, including AHS which produced 4 bubbles and contained 3 µg or less DPPC. This relationship is represented by the sloping line and the equation (r^2 = 0.2, P = 0.008). For the independent part, the mean bubble count for AHS which contained > 3 µg DPPC and had a bubble count < 5, was used to calculate the independent line (slope = 0, Fig. 3). The sloping line represents the group in which bubble production was positively related to DPPC. The horizontal line represents the group which had low bubble production unrelated to DPPC content.

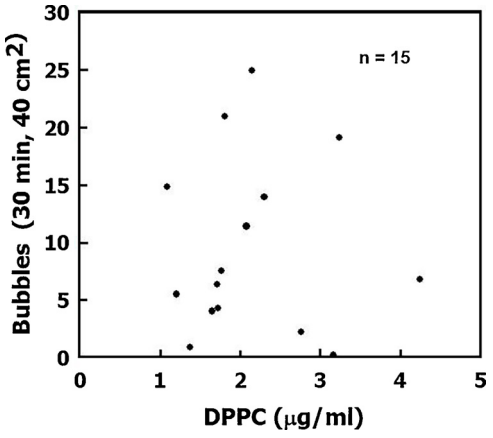

Fig. 2. Total bubbling from the sheep blood vessels plotted against plasma DPPC concentration.

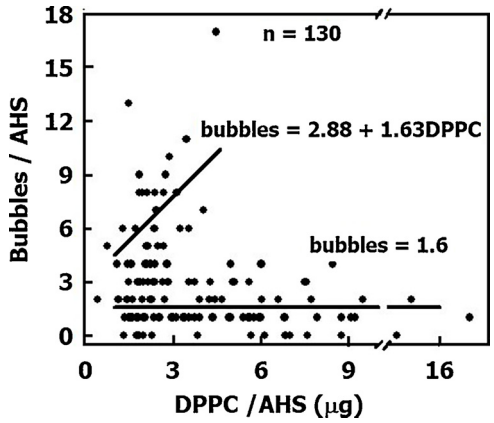

Fig. 3. Number of bubbles produced at an active hydrophobic spot over a period of 30 min plotted against its DPPC content. Lines are suggested to represent two groups of data.

4. Discussion

This study of DPPC in the blood and blood vessels has extended our ability to analyse the data compared with our previous investigation (Arieli et al., 2016), due to standardisation of the saline temperature, the inclusion of 15 sheep as opposed to 11, and of 130 tissue samples as against 36. The mean concentration of DPPC in plasma, 2.15 µg/ml, was similar to our previously determined value of 2.04 µg/ml.

The absence of a correlation between bubbling sheep and the concentration of DPPC in plasma (Fig. 2) suggests that the variability between bubblers and non-bubblers is not due to differences in the leakage of DPPC from the lung into the blood. Rather, it may be related to differences at the sites on the endothelial membrane where the phospholipids begin to adhere and accumulate. Thom et al. (2013) showed that gas-containing microparticles (MP) are rich in iNOS. We suggested that the source of these MP was venous bubbles which detached together with a piece of endothelial membrane, losing most of their gas in the lung (Arieli, 2017). Sites on the endothelial membrane which are rich in iNOS may serve as spots of preference for settling of DPPC. It may therefore be the case that variability in iNOS distribution between sheep (and divers) is the source of variability between bubblers and non-bubblers. The outcome of the present study does not encourage a blood test for DPPC as a means of detecting vulnerability to decompression.

The presence of DPPC in all the samples from the blood vessels, including those which were non-bubbling, is similar to our previous report (Arieli et al., 2016), indicating that DPPC settled over a wide distribution area. The relation between bubbling level and the amount of DPPC at the active hydrophobic spots appears to diverge into two groups. One group of AHS was seen to be low-bubbling, without any relation to the amount of DPPC. In the second group, bubbling was related to the amount of DPPC (Fig. 3). We were unable to observe this phenomenon in our previous report due to the smaller range of DPPC

(4 μg) and bubbling (12/AHS). In the process by which oligolamellar layers of phospholipids are formed on the luminal aspect of the blood vessel, one layer settles with the hydrophobic tails facing the lumen of the vessel, followed by a layer with the phosphate head facing the lumen. Thus one layer will be hydrophobic, the next hydrophilic, and the process will continue with this alternate settling of layers. It is possible that the two groups of AHS are related to either one of these two phases of phospholipid deposition. In an AHS which is mainly hydrophobic, bubble production will be related to the amount of DPPC. In an AHS which is mainly hydrophilic, only a few bubbles will be produced if just a small section is left hydrophobic. In these AHS, bubble production will be unrelated to the amount of DPPC.

5. Conclusion

1 This study reinforces the role of DPPC in the formation of decompression bubbles.
2 Variability in bubbling between sheep is not related to DPPC leakage from the lung.
3 Alternate layering with hydrophobic and hydrophilic phospholipids may provide a basis for our hypothesis that there are two kinds of AHS.

Conflict of interest

The authors declare that they have no conflict of interest.

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