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Autopsy diagnosis of decompression illness in rats by quantifying pulmonary emphysema

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Abstract: Objectives. In medico-legal autopsy related to decompression illness (DCI) in divers, it is important to differentiate between postmortem decompression and antemortem decompression; however, definitive criteria for autopsy diagnosis of DCI remain to be established. We aimed to clarify autopsy findings that would aid the diagnosis of DCI.

Methods. A total of 27 Wistar and 33 Zucker fatty male rats were categorized into 3 groups: antemortem decompression group (group AD), postmortem decompression group (group PD), and controls. The AD and PD rats were subjected to antemortem compression (both groups) followed by antemortem and postmortem decompression, respectively, in a hyperbaric chamber. Control rats were kept at atmospheric pressure. Intravascular bubbles and histopathological findings in the 3 groups were compared, and pulmonary emphysema was quantified using ImageJ software.

Results. Intravascular bubbles were observed in both AD and PD groups. In group AD, prolonged hyperbaric exposure increased mortality, intravascular bubbles, and pulmonary emphysema, with Zucker fatty rats showing more marked changes than Wistar rats. Further, intravascular bubbles and pulmonary emphysema in the dead rats were quantitatively greater than those in the rats that survived decompression. Moreover, in Zucker fatty rats, pulmonary emphysema was quantitatively significantly greater in group PD than that in the dead rats of group AD.

Conclusion. Our results indicate that the quantitative evaluation of pulmonary emphysema can potentially distinguish postmortem decompression from antemortem decompression. This technique may be useful in autopsy diagnosis of diving-related deaths, including DCI.

Key Words: decompression illness, diving, autopsy, pulmonary emphysema, fat embolism, risk factor.

In recent years, scuba diving has become a popular sport, with increasing numbers of people participating in recreational diving. Consequently, the numbers of diving-related accidents and deaths have also increased [1, 2]. The cause of diving-related deaths varies from drowning, decompression illness (DCI), to endogenous disease; in such diving-related cases, detailed autopsy is necessary to make an accurate diagnosis [3].

DCI is a condition often associated with diving, which is difficult to diagnose based on autopsy findings alone. DCI is a collective term for diseases caused by the presence of intravascular or extravascular bubbles formed due to a reduction in environmental pressure (decompression) [4]. Most incidents of DCI occur with diving, typically caused by inadequate decompression, such as in rapid ascent after diving. One of the best-known autopsy findings associated with DCI is intravascular bubbles; however, past studies have indicated that bubbles

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are formed as a result of postmortem decompression, resuscitation maneuvers, and putrefaction [5-8].

Therefore, autopsy diagnosis of DCI should not be based on the presence of intravascular bubbles alone. Few studies have thus far described autopsy findings that are useful for diagnosing DCI, excluding bubbles; accordingly, the criteria for the autopsy diagnosis of DCI remain to be established [9].

Therefore, in order to clarify the findings that would aid the autopsy diagnosis of DCI, we created a DCI rat model and investigated the histopathological findings, physiological data, and risk factors associated with DCI. Further, to investigate the effect of obesity on the pathogenesis and histopathology of DCI, we included 2 types of rats in the DCI rat model, i.e., Wistar and Zucker fatty rats. Moreover, we focused on the utility of the quantitative evaluation of pulmonary emphysema using software-based image analysis in the autopsy diagnosis for scuba diving-related deaths, including DCI.

MATERIALS AND METHODS

The animals used in this study were handled in accordance with the Guidelines for Animal Experimentation of the University of the Ryukyus, and the experimental protocol was approved by the Animal Care and Use Committee of the institution.

Animals

A total of 27 male Wistar rats (Kyudo Co., Ltd., Saga, Japan; age, 12 ± 0 weeks; weight, 396 ± 22 g) and 33 male Zucker fatty rats with obesity due to overeating (Crlj: ZUC-Lepr^{fa} Genotype: fa/fa, Japan Charles River Co., Ltd., Yokohama, Japan; age, 14 ± 1 weeks; weight, 587 ± 15 g) were used in this study. The body weight of Zucker fatty rats was approximately 1.5 times that of Wistar rats. Almost all excess weight consisted of subcutaneous and perivisceral fat tissue. The percentage of body fat was derived from dividing the subcutaneous and perivisceral fat mass by the total body mass. The percentage of body fat in Wistar rats was less than 10% and that in Zucker fatty rats was more than 30%. We considered the Wistar rats as non-obese rats and the Zucker fatty rats as obese rats in this study.

Procedure

The rats were anesthetized by an intraperitoneal injection of urethane (1.5 g/kg). A single rat was placed in a hyperbaric chamber (length, 50 cm and inner diameter, 16 cm; Syn Co., Ltd., Kyoto, Japan). Chamber temperature was maintained at approximately 28°C. Electrocardiogram, heart rate, and respiratory movements were recorded by a monitor (PowerLab® 4/30 with LabChart® 7 software; AD Instruments Japan Inc., Nagoya, Japan). The rats were divided into the following 3 groups: the antemortem decompression group (group AD), postmortem decompression group (group PD), and controls. The rats in group AD were compressed from atmospheric pressure (0.1 MPa) to 0.6 MPa (equivalent to approximately 50 m depth of seawater) in the hyperbaric chamber. The pressure in the hyperbaric chamber was maintained at 0.6 MPa for 1, 2, or 3 h with fresh air supplied at all times. Then, the rats were decompressed antemortem from 0.6 MPa to atmospheric pressure. The rate of compression and decompression was +0.1 and −0.1 MPa/min, respectively, which are considered to be safe for divers. The rats were observed until cardiac arrest. The rats that survived for 20 min after the end of decompression were subsequently euthanized by exposure to CO_2 under atmospheric pressure. Similarly, the rats in group PD were exposed to 0.6 MPa for 3 h. Then, the rats were euthanized by exposure to CO_2 under pressure maintained at 0.6 MPa. The rats were subsequently decompressed postmortem from 0.6 MPa to atmospheric pressure. The control rats were left at atmospheric pressure for 3 h; then, these control rats were euthanized by exposure to CO_2 under atmospheric pressure. Autopsies were carried out on each animal within 10 min after death.

Macroscopic examination and evaluation of bubbles

The thoracic cavity, peritoneal cavity, and cranial cavity were opened to examine the organs. Bubbles in the subcutaneous veins, mesenteric veins, inferior vena cava, and right atrium were observed, and the degree of bubbles was evaluated macroscopically as follows: 0, no bubbles; 1, slight bubbles; 2, bubbles appear as dotted line; and 3, abundant bubbles filling the vessels (Figure 1).

Preparation for microscopic examination

The lungs, heart, liver, kidneys, spleen, and cerebrum were harvested, fixed in 4% formaldehyde, and embedded in paraffin. Then, 3-μm-thick sections were cut and stained with hematoxylin-eosin (HE) for light microscopic analysis. To investigate fat emboli, fixed specimens were frozen and 10-μm-thick sections were cut and stained with oil red O.

Quantitative evaluation of pulmonary emphysema by ImageJ software

To evaluate pulmonary emphysema quantitatively, we selected 2 fields without large vessels or bronchi from the peribronchial area (central area) and subpleural area (peripheral area) of the cross-sectioned inferior lobes of the bilateral lungs (Figure 2). We obtained color pictures using a microscopic camera (×100, photographic sensitivity ISO800, resolution 4140 × 3096 pixels, Olympus Co., Ltd., Tokyo, Japan). Color pictures were converted to black and white images

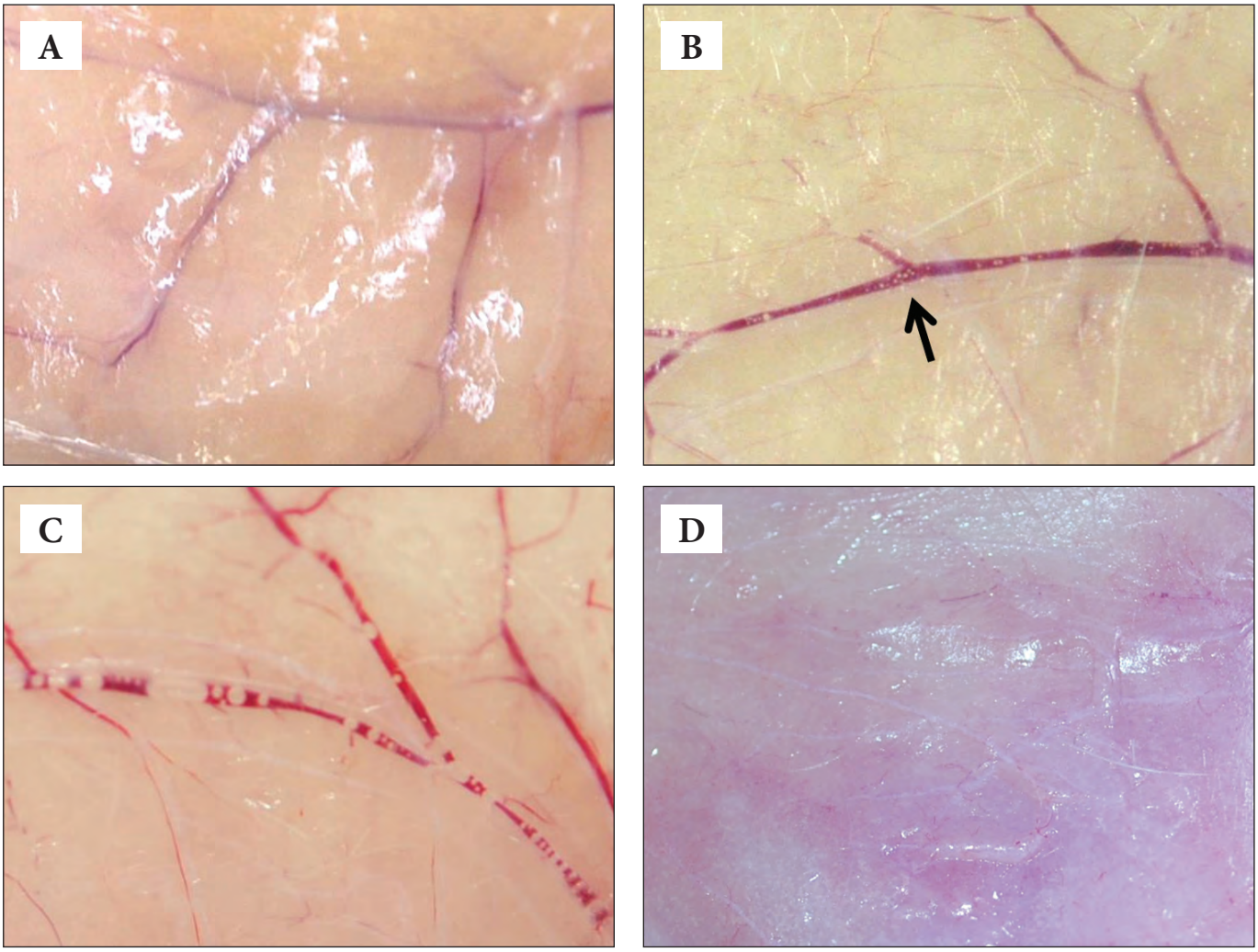

Figure 1. Evaluation of typical cases of bubbles in subcutaneous veins. (A) No bubbles. (B) Slight bubbles (arrow). (C) Bubbles appear as dotted line. (D) Abundant bubbles filling the vessels.

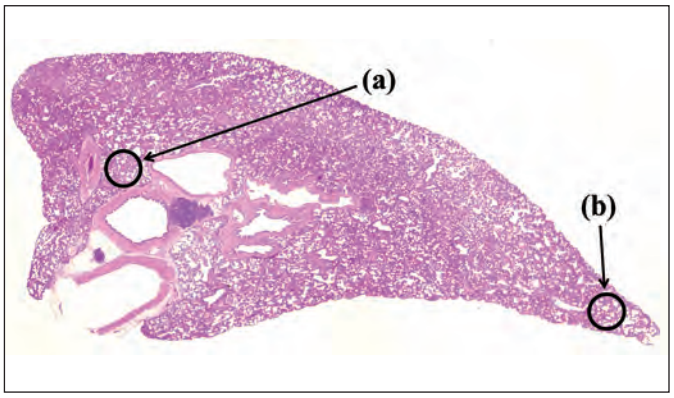

Figure 2. Cross-section of a lung (HE stain, ×12.5). The 2 observed locations are indicated by circles. (a) Central area. (b) Peripheral area.

using Otsu's method [10], and the percentage of alveolar spaces in each microscopic field (%Area) was calculated using the image-processing software, ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA) (Figure 3). An average %Area of 4 fields was evaluated as the level of pulmonary emphysema for quantitative evaluation. Elevated values of %Area indicated severe pulmonary emphysema.

Statistical analysis

Values are shown as mean ± standard deviation. All statistical analyses were performed using a Microsoft Excel® statistical program file, ystat2008 (developed by Yamazaki S, Igaku-Tosho-Shuppan Co., Ltd., Tokyo, Japan). Statistical analysis for evaluation of the

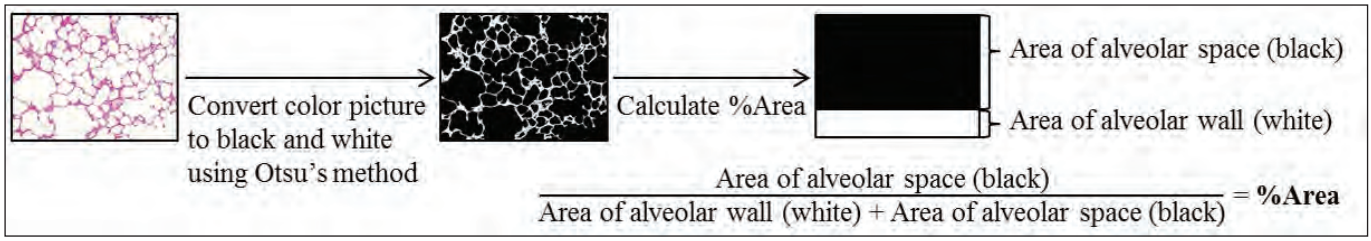

Figure 3. ImageJ processing procedure.

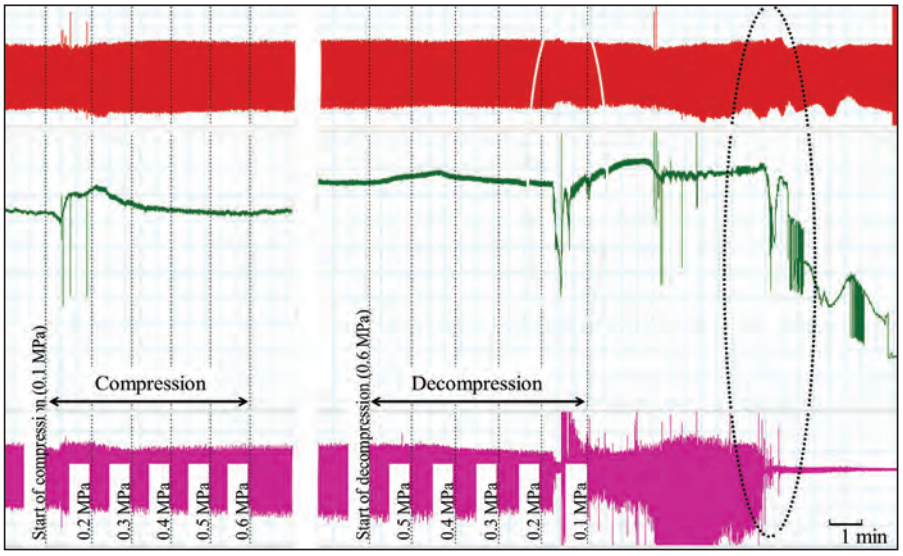

Figure 4. Changes in electrocardiogram (ECG), heart rate (HR), and respiratory movements (RM) (typical dead case in group AD, non-obese rat, 3 h). In the course of decompression from 0.2 MPa to atmospheric pressure (0.1 MPa), abnormal physiological data was observed frequently (solid circle). Shortly before death, the respiratory rate increased and RM became larger; subsequently, RM were smaller and slower, culminating in respiratory arrest. Around the time of respiratory arrest, marked arrhythmia and decrease in HR continued until cardiac arrest (dotted circle). *bpm, beat per min.

intravascular bubbles was performed using the Kruskal-Wallis H-test followed by Mann-Whitney U-test with Bonferroni's correction. Statistical analysis for %Area was performed by 1-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. Significance was defined as $P < 0.05$.

RESULTS

Mortality rate and cause of death

The mortality rate of rats from group AD for 1, 2, and 3 h of hyperbaric exposure time was 33% (4/12), 75% (9/12), and 67% (8/12), respectively. The mortality rate of non-obese rats and obese rats from group AD were 17% (1/6) and 50% (3/6) for 1 h, 50% (3/6) and 100% (6/6) for 2 h, and 33% (2/6) and 100% (6/6) for 3 h (Table 1). In the non-obese rats, the mortality rate of rats from group AD was greater for 2 and 3 h than that for 1 h; in the obese rats, all the rats from group AD for 2 and 3 h died within 20 min after decompression. The cause of death for the group AD rats was determined to be DCI on the basis of autopsy findings and the course of death.

Physiological data

All the rats in group AD exhibited arrhythmia and respiratory abnormalities in the course of decompression. Particularly, in the course of decompression from 0.2 MPa to atmospheric pressure (0.1 MPa), abnormal physiological data was observed frequently. This change was observed in both—the rats that survived (surviving rats) as well as the rats that died (dead rats). The heart rate increased slowly and the respiratory rate remained almost unchanged in the stable state at 0.6 MPa. In almost all of the dead rats, shortly before death, the respiratory rate increased and respiratory movements became larger, which subsequently became slower and smaller, respectively, culminating in respiratory arrest. Around the time of respiratory arrest, marked arrhythmia and decrease in the heart rate was noted, which continued to cardiac arrest (Figure 4).

Table 1. Mortality, degree of bubbles, and %Area of non-obese rats (left) and obese rats (right)

Groups of non-obese rats		n	Mortarity (%)	Degree of bubbles	%Area (%)
AD	1 h	6	17	0.8	54.0
	Survival	5	-	0.6	48.3
	Death	1	-	2.0	82.5
	2 h	6	50	1.3	60.0
	Survival	3	-	0.3	51.1
	Death	3	-	2.3	68.9
	3 h	6	33	1.2	62.2
	Survival	4	-	0.5	52.1
	Death	2	-	2.5	82.3
PD		3	-	1.7	80.5
Control		3	-	0.0	40.9

Groups of obese rats		n	Mortarity (%)	Degree of bubbles	%Area (%)
AD	1 h	6	50	1.0	51.8
	Survival	3	-	0.0	49.3
	Death	3	-	2.0	54.2
	2 h	6	100	2.5	61.5
	Survival	0	-	-	-
	Death	6	-	2.5	61.5
	3 h	6	100	3.0	58.9
	Survival	0	-	-	-
	Death	6	-	3.0	58.9
PD		6	-	2.3	77.3
Control		6	-	0.0	45.4

Data are presented as mean values.
AD, antemortem decompression; PD, postmortem decompression; %Area, the percentage of alveolar spaces in each microscopic field.

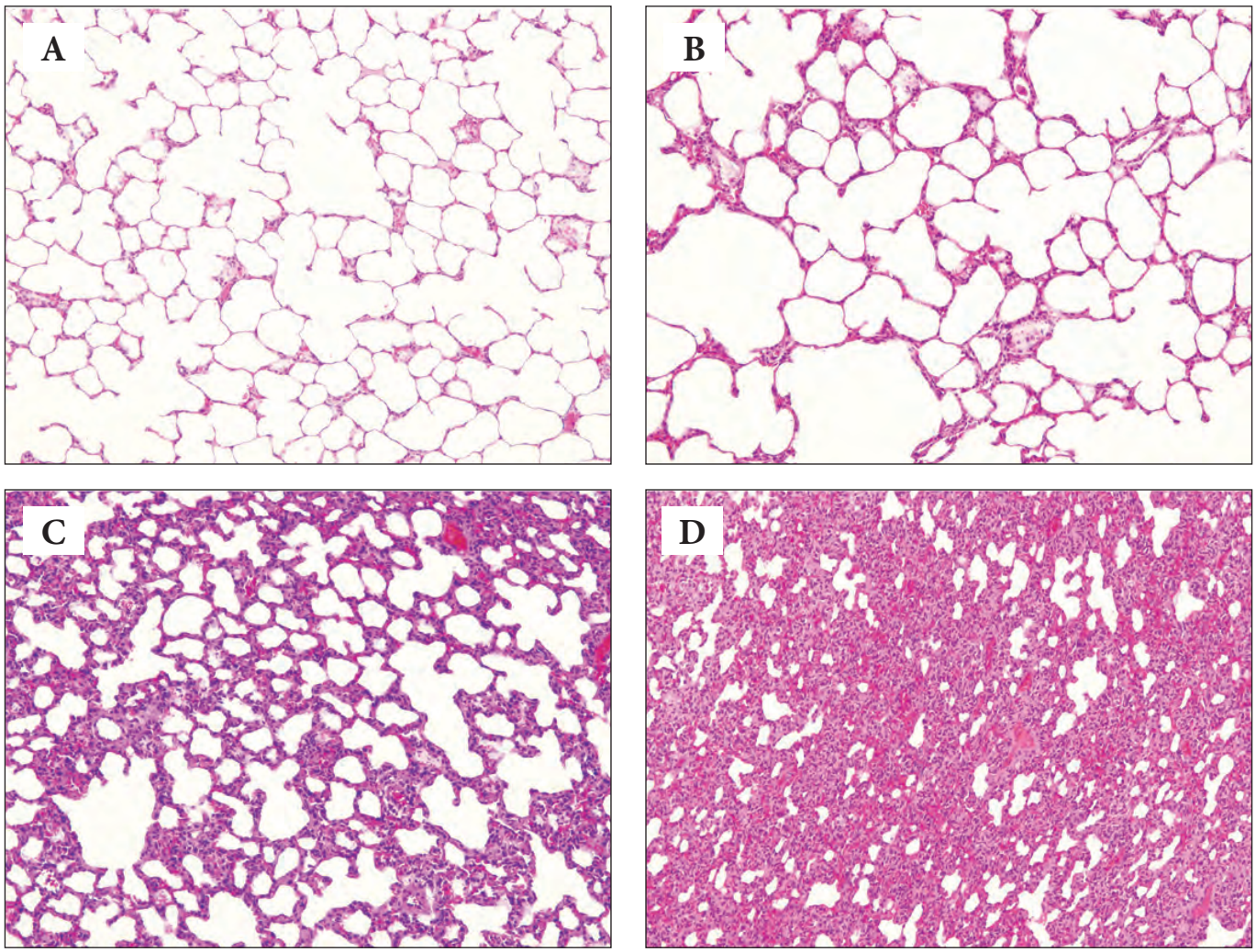

Figure 5. Typical histopathological findings from the lungs (non-obese rats, HE stain, ×100). (A) The dead rat from group AD (3 h). (B) The surviving rat from group AD (3 h). (C) The rat from group PD. (D) Control rat.

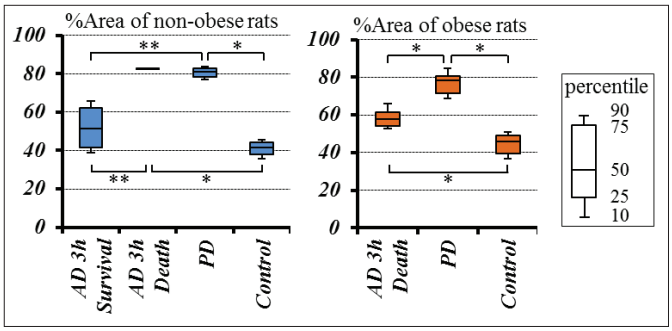

Figure 6. Comparison among the rats from group PD and surviving and dead rats from group AD under the condition of exposure to hyperbaric pressure for 3 h. *P < 0.01, **P < 0.05.

Macroscopic examination, evaluation of bubbles, and microscopic examination

Macroscopic examination revealed diffuse, numerous bubbles in the subcutaneous veins, mesenteric veins, inferior vena cava, and right atrium in all the dead rats from group AD and in all the rats from group PD. No bubbles were observed in the control rats (Table 1). The degree of bubbles in the rats from group AD for 1, 2, and 3 h was 0.9 ± 1.0, 1.9 ± 1.1, and 2.1 ± 1.2, respectively.

The degree of bubbles in the surviving rats and dead rats were 0.4 ± 0.7 and 2.0 ± 0.0 for 1 h, 0.3 ± 0.6 and 2.4 ± 0.5 for 2 h, and 0.5 ± 0.6 and 2.9 ± 0.4 for 3 h. The degree of bubbles in the non-obese and obese rats were 0.8 ± 1.0 and 1.0 ± 1.1 for 1 h, 1.3 ± 1.2 and 2.5 ± 0.5 for 2 h, and 1.2 ± 1.2 and 3.0 ± 0.0 for 3 h (Table 1).

In the group AD rats, the greater the number of bubbles observed, the greater was the overinflation of the lungs in macroscopic examination and the pulmonary emphysema in microscopic examination. The degree of bubbles observed in the non-obese and obese rats from group PD for 3 h were 1.7 ± 0.6 and 2.3 ± 0.8 (Table 1). The overinflation and emphysema of the lungs in the rats from group PD was great enough that some alveolar walls were noted to be ruptured on microscopic examination (Figure 5). No bubbles were observed in the parenchyma of any other organs except the lungs.

Quantitative evaluation of pulmonary emphysema by ImageJ software

We compared the %Area among rats from group PD and the surviving and dead rats from group AD under

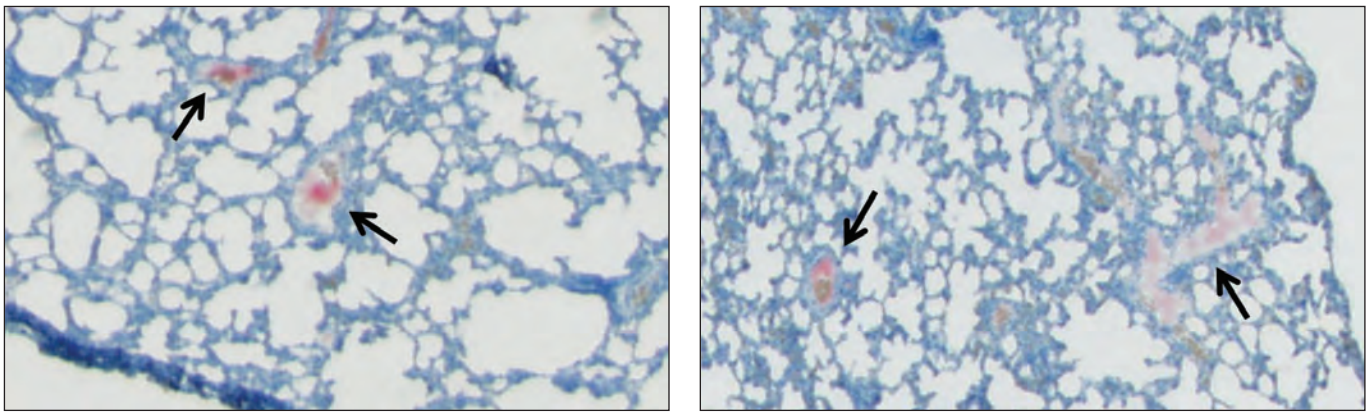

Figure 7. Fat emboli in alveolar capillaries (arrows, obese rats, oil red O stain, ×100). (a) The dead rat from group AD (2 h). (b) Control rat.

the condition of exposure to hyperbaric pressure for 3 h. An elevated value of %Area, which is the quantitative evaluation of pulmonary emphysema, indicated severe pulmonary emphysema. In the non-obese rats, the %Area for rats from group PD (80.5%) and for the dead rats from group AD (82.3%) were significantly greater than that for the surviving rats from group AD (52.1%) and for the controls (40.9%). There were no significant differences between the %Area for rats from group PD and that for the dead rats from group AD (Table 1, Figure 6). In the obese rats, the %Area for rats from group PD, the dead rats from group AD, and the control rats was 77.3%, 58.9%, and 45.4%, respectively, with significant differences between the 3 groups (Table 1, Figure 6).

Microscopic examination of fat emboli

No fat emboli were observed in the lungs, kidneys, or cerebrum in the non-obese rats from any group. In contrast, fat emboli were observed in the alveolar capillaries of obese rats not only from group AD but also in obese rats from groups PD and controls (Figure 7). In these obese rats from all 3 groups, fat emboli were also observed in the renal capillaries but not in the cerebrum.

DISCUSSION

Depth, duration, the ascent rate for diving, and obesity are the important risk factors affecting DCI [4, 11-14]. High pressure, long exposure time, and obesity increase the amount of dissolved nitrogen gas in the body, and rapid decompression makes it hard to discharge the bubbles that are thus formed. In this study, the mortality rate and the degree of bubbles were greater for durations of 2 and 3 h than for 1 h (Table 1). Further, in obese rats, these parameters tended to be greater than those in non-obese rats (Table 1); we thus confirmed that long durations of diving and obesity are indeed risk factors of DCI, as demonstrated by previous studies [4, 12-14]. In the comparison between the surviving rats and dead rats in this study, a greater number of intravascular bubbles were observed in the dead rats than in the surviving rats (Table 1). This result suggests the following 2 possibilities: (1) the presence of numerous bubbles caused death, and (2) the survival time of the dead rats following decompression was too short to discharge the dissolved nitrogen gas through breathing, leading to the presence of numerous bubbles.

In the course of decompression of the rats from 0.2 MPa to atmospheric pressure (0.1 MPa), marked arrhythmia, heart rate changes, and abnormal respiration were observed (Figure 4). This is well explained by Boyle's law: at a given temperature, the volume and absolute pressure of a gas vary inversely. Accordingly, the greater change in volume would occur in shallow water than in deep water, which appeared to be reflected in the physiological changes observed in this study. Past studies have indicated that intravascular bubbles and DCI can occur even in shallow diving to depths less than 10 m [15-17]. Diving in deep water is a well-known risk factor of DCI; however, diving in shallow water is not always safe from the point of view of avoiding DCI. This little-known factor should be more widely publicized to people participating in diving activities.

In medico-legal autopsies of diving-related deaths, the differentiation between postmortem decompression and antemortem decompression is important. Distinguishing divers who have died before surfacing (postmortem decompression) from divers who have died after surfacing (antemortem decompression) would aid in clarifying the exact cause and mechanism of death in divers. With increasing numbers of elderly people engaging in recreational diving, deaths from endogenous diseases under water are increasing [3]. Therefore, differentiating between postmortem and antemortem decompression becomes more important in such situations. We therefore focused attention on the degree of pulmonary emphysema as a barometer for

differentiation between postmortem and antemortem decompression. The severe pulmonary emphysema observed in group PD rats and the dead rats from group AD could adequately distinguish these animals from the surviving rats from group AD, who showed lesser pulmonary emphysema (Figure 5). In particular, we quantitatively evaluated pulmonary emphysema using ImageJ software; among obese rats, the values thus obtained were significantly greater in group PD rats than in the dead rats from group AD (Table 1, Figure 6). Previous studies have indicated that intravascular bubbles and pulmonary emphysema are formed as a result of postmortem decompression [5, 18]. However, the present results indicate the potential utility of this quantitative technique in distinguishing between divers who have died before surfacing and divers who have died shortly after surfacing. In the future, if the cutoff values for such quantitative evaluation are determined, we would be able to diagnose DCI more accurately, with possible practical applications to autopsy diagnosis in human beings.

It is known that fat emboli are often observed in the lungs of DCI subjects [19-21]. We considered that fat emboli would be a useful finding for the diagnosis of antemortem decompression. However, in the obese rats, fat emboli were observed not only in groups AD and PD but also in controls (Figure 7). Chamber temperature, severe obesity, fatty liver, hyperlipidemia, and intraperitoneal anesthesia might have induced fat emboli [21-24]. We consider that appropriate modifications of study protocols are needed for the additional examination of fat emboli.

CONCLUSION

We have clarified certain useful findings for the autopsy diagnosis of DCI using 2 types of DCI rat models (non-obese rats and obese rats) along with the histopathological findings, physiological data, and risk factors of DCI. The results of our study indicate that not only obesity and a long duration of diving but also decompression in shallow water are important risk factors of DCI. The diagnosis of DCI should not be based on the presence of intravascular bubbles alone. The quantitative evaluation of pulmonary emphysema, which we have presented in this paper, would help to distinguish between divers who died before surfacing, who died shortly after surfacing, and who died long after surfacing. Moreover, these results could be useful for the autopsy diagnosis of scuba diving-related deaths, including DCI.

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