Evidence of cell damages caused by circulating bubbles: high level of free mitochondrial DNA in plasma of rats

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Vallée N, Gaillard S, Peinnequin A, Risso J-J, Blatteau J-E. Evidence of cell damages caused by circulating bubbles: high level of free mitochondrial DNA in plasma of rats. *J Appl Physiol* 115: 1526–1532, 2013. First published September 26, 2013; doi:10.1152/japplphysiol.00025.2013.— Bubble formation can occur in the vascular system after diving, leading to decompression sickness (DCS). DCS signs and symptoms range from minor to death. Too often, patients are admitted to a hyperbaric center with atypical symptoms, as bubbles cannot be detected anymore. In the absence of a relevant biomarker for humans, the therapeutic management remains difficult. As circulating DNA was found in the blood of healthy humans and animals, our study was made to correlate the extracellular mitochondrial DNA (mDNA) concentration with the occurrence of clinical DCS symptoms resulting from initial bubble-induced damages. Therefore, 109 rats were subjected to decompression from a simulated 90-m sea water dive, after which, 78 rats survived (71.6%). Among the survivors, 15.6% exhibited typical DCS symptoms (DCS group), whereas the remaining 56% showed no detectable symptoms (noDCS group). Here, we report that the symptomatic rats displayed both a circulating mDNA level ($DNA_{DCS} \rightarrow 2.99 \pm 2.62$) and a bubble grade (median Spencer score = 3) higher than rats from the noDCS group ($DNA_{noDCS} \rightarrow 1.49 \pm 1.27$; Spencer score = 1). These higher levels could be correlated with the platelet and leukocyte consumption induced by the pathogenic decompression. Rats with no detectable bubble had lower circulating mDNA than those with higher bubble scores. We determined that in rats, a level of circulating mDNA >1.91 was highly predictive of DCS with a positive-predictive value of 87.3% and an odds ratio of 4.57. Thus circulating mDNA could become a relevant biomarker to diagnose DCS and should be investigated further to confirm its potential application in humans.

biomarker; bubble; diving; gas; nitrogen

NITROGEN SUPERSATURATION AND bubble formation can occur in the vascular system after diving, leading to decompression sickness (DCS) (9). DCS is generally a mild injury as it occurs today and is rarely fatal (46). Whatever the specific location and mechanism of their formation, bubbles are the pivotal pathologic event in DCS (2). Their number and their size appear proportional to the decompression stress (34). The absence of detectable bubbles is a good indicator of decompression safety. The opposite is sometimes false, and the presence of bubbles is not necessarily proportional to the stress induced, even when high grades of bubbles are detected (36). In a clinical setting, bubbles can be detected within minutes of diving, and bubble activity usually peaks between 30 and 90 min after the end of the dive (20). In practice, serious neurologic decompression illness usually manifests itself within 10 min of decompression, but 90% of cases are symptomatic within 3 h (7). DCS accidents are commonly thought to result in a combination of symptoms and signs, when, in fact, these can also occur later, singly or in an overlapping manner. This contributes to delay the evacuation of potential victims to a hyperbaric center (9).

To define the most adequate therapeutic care of patients caught belatedly with atypical symptoms remains difficult without robust decompression stress markers. A great number of DCS indexes were highlighted in rodent models of DCS, such as platelet count or complement activation, but their relevance to milder human cases is still uncertain. Determination of a biological hallmark, representative to the per se effect of bubbles, should then be considered. This per se effect remains unclear, and two hypotheses are commonly suggested. The first one proposes that the shear stress induced by bubbles may damage both the luminal surfactant layer (12) and endothelial cells (10, 22, 24, 47), which expose collagen and therefore induce platelet activation. The second one suggests that bubbles interact directly with a formed element of blood (21) and may, among other things, stimulate platelet aggregation (24a, 24b, 25, 43) or leukocyte activity (19, 25). In both cases, these actions result in proinflammatory events and prothrombotic phenomena that can degenerate in plurifactorial ischemia, where cytotoxic effects add to the whole panel of the DCS symptomatology (21). All of these deleterious events may explain the failure of recompression treatment in some cases (3).

DNA is now recognized as a reliable biomarker for stroke diagnosis and prognosis (18), and we aimed to develop it in DCS.

Its kinetic in blood is well documented. Approximately 90% of the DNA is accumulated in the liver within 3 min after the injection in a mouse, whereas kidney and spleen would catch only 4-12% (8). Nonetheless, a two-step removal of DNA from circulation was observed (37). At the first step, the oligonucleotides' concentration decreases rapidly, and the half-life of oligonucleotides in circulation is <30 min. At the second step, an oligomer circulation, for a long time at a low concentration, and its half-life are 20–40 h. A similar two-step removal process has been described for mice with an 8-min half-life at the first step and a 409-min half-life at the second step (45).

Levels of plasma DNA increase in response to a variety of conditions (41), including necrosis (32) or apoptosis of nuclear cell elements of the blood or endothelium (14), erythrocyte and platelet maturation, and active secretion of nucleic acids into the extracellular space (41). Although the putative sources of

circulating DNA are diverse, its endogenous origin is commonly accepted for a major part as extracellular DNA. There is an estimated concentration of 60 ng/ml DNA in the blood of a healthy patient (39, 40).

Necrosis is normally far less widespread than apoptosis; nonetheless, this death mechanism seems to be similar to the one observed consecutively to a bubble flow. Necrosis is known to be induced by severe, irreversible injuries, caused by prolonged ischemia, exposure to a high dose of ionizing radiation, high temperatures, or cell membrane-damaging agents. Since necrotic cells are usually absent from healthy patients (17), this type of cell death could hardly be the source of the considerable basal amount of DNA in the plasma of these patients. Necrosis can substantially contribute to the circulating level of DNA, in some cases, for instance, in injuries. Indeed, a significant correlation was found between the circulating DNA concentration and the severity of an injury (16). Plasma concentration of DNA is elevated within 3 h of ischemic and hemorrhagic stroke onset, and patients who experience a poor outcome or die as a result of their stroke display higher levels than those who do not (31, 33).

The purpose of this study was to investigate mitochondrial DNA (mDNA) as a potential biomarker of cell damage, resulting from decompression, and as a predictor of DCS in the rat.

MATERIALS AND METHODS

Study population. Male Sprague-Dawley rats (Harlan Laboratories, Venray, The Netherlands), weighing ~350 g, were used. Rats were kept at $22 \pm 1°C$ under a 12-h light/12-h dark cycle (lights on at 7 AM) with food (A03; Usine Alimentation Rationnelle, Villemoisson sur Orge, Essone, France) and water available ad libitum. Before experiments, rats were housed in an accredited animal care facility. Procedures were in accordance with the European Communities Council rules (Brussels, Belgium), directive of November 24, 1986 (86/609/EEC), as enshrined in French law (decree 87/848). The Ethics Committee of the Institut de Recherche Biomédicale des Armées approved this study (No. 01.2012). Our investigator (N. Vallée) is associated with agreement number 83.6, delivered by the Health and Safety Directorate of our department, as stated in French rules R.214-93, R.214-99, and R.214-102.

Rats were randomly assigned to their batch.

Hyperbaric procedure. Hyperbaric protocol was performed within a period of 2 wk. Each numbered rat was weighed 30 min before the dive. Batches of eight rats (four/cage) were subjected to the hyperbaric protocol in a 200-liter tank fitted with three ports for observation. The rats were free to move inside of the cage.

Rats underwent the compression procedure at a rate of 100 kPa/min to a pressure of 1,000 kPa (90 m sea water), maintained for 45 min while breathing air. At the end of the exposure period, rats were decompressed down to 200 kPa at a rate of 100 kPa/min with a 5-min stop at 200 kPa, 5-min stop at 160 kPa, and 10-min stop at 130 kPa. Decompression between 200 kPa and atmospheric pressure was performed at a rate of 10 kPa/min. The decompression was controlled automatically by a computer linked to an analog/digital converter (NI USB-6211; National Instruments, Austin, TX), itself plugged to a solenoid valve (LR24A-SR; Belimo, Hinwil, Switzerland) and a pressure transmitter (Pressure Transmitter 8314; Burkert Fluid Control Systems, Ingelfingen, Germany). The program was designed on DasyLab (DASYLab; National Instruments).

Compressed air was generated using a diving compressor (Mini Verticus III; Bauer, Munich, Germany), coupled with a 100-liter tank at 300 bar. The oxygen analyzer was based on a Micro-Fuel electrochemical cell (G18007; Teledyne Analytical Instruments, City of Industry, CA). Water vapor and carbon dioxide, produced by the animals, were captured with soda lime (<300 parts/million captured by the soda lime) and Secca Gel (relative humidity: 40–60%). Gases were mixed by an electric fan. The day-night cycle was respected throughout. The temperature inside of the tank was measured using a platinum-resistance temperature probe (Pt100; Invensys Eurotherm, Dardilly Cedex, France).

Clinical observation. After decompression, rats were transferred into individual cages and observed for DCS for 30 min. During this period, respiratory distress, convulsions, walking difficulties (including limping, failure to maintain balance, sideways gait, falling, difficulty righting after a fall), and forelimb and/or hindlimb paralysis were considered as DCS signs that onset was recorded to the nearest 30 s of the first sign. The presence of isolated subclinical manifestation i.e., prostration (without moving difficulties after stimulation), was not considered as a specific sign of DCS.

Control rats were placed in the same chamber and maintained under atmospheric pressure for an equivalent period of time.

At the end of the experiment, blood samples were collected, and rats were killed by injecting pentobarbital (200 mg/kg ip; Sanofi, Paris, France).

Platelet, leukocyte, and erythrocyte counts. Counts were carried out in an automatic analyzer (Scil Vet ABC; SCIL Animal Care, Altorf Bas-Rhin, France) on blood samples collected before the dive and 30 min after. Leukocytes, erythrocytes, and platelets were counted in 20 μl samples taken from the tip of the tail and diluted in an equivalent volume of 2 mM EDTA (Sigma, Saint-Quentin-Fallavier, France).

Bubble detection. Bubble detection and collection of blood samples for mDNA analysis were performed after the end of the clinical observation. Rats were anesthetized 30 min after surfacing by intraperitoneal injection of a mixture of 16 mg/kg xylazine (Rompun 2%; Bayer HealthCare Pharmaceuticals, Leverkusen, Germany) and 100 mg/kg ketamine (Imalgene 1000; Laboratoire Rhône-Mérieux, Ancenis, France). Circulating bubbles were detected in the heart—with a portable ultrasound echocardiography system (MicroMaxx Ultrasound System; SonoSite, Bothell, WA), equipped with a 4- to 8-MHz probe—by an experienced operator, using first, a two-dimensional mode for the location of the heart and then, the pulsed Doppler shoot mode on the right cavity. The quantity of bubbles was graded using the Spencer scale (38). Basically, *grade 0* corresponds to no bubble detected, *grade 1* is a few bubbles, *grade 2* is "some bubbles at each heartbeat," *grade 3* is "many bubbles per heartbeat," and *grade 4* is "continuous bubbles." A 5-min recording was given to each animal.

mDNA extraction and amplification. Circulating mDNA was quantified from blood samples collected 30 min after the end of the dive. Quantification of mDNA was chosen, since its levels are not limiting for PCR analysis (5).

Blood samples (900 μl) were collected in the vena cava with a disposable syringe containing 100 μl of 2 mM EDTA. Samples were centrifuged immediately for 10 min at 1,500 g at 4°C. Supernatants were centrifuged subsequently for 10 min at 20,000 g at 4°C, and the plasma samples collected were stored at −80°C until analysis.

Enhanced green fluorescent protein (EGFP) plasmid (1 μl; 10^{-5} M), for internal normalization, was added to plasma samples before DNA extraction. DNA was extracted using the QIAmp UltraSens Virus Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Quantitative PCR was carried out with a LightCycler 2.0 (soft LC Run 5.32; Roche Diagnostics, Basel, Switzerland) on 5 μl DNA extract added to 16 μl reaction mixture. For the negative controls, water was substituted for the extract. The reaction mixture contained 9.8 μl water, 0.4 μl primers F and R (20 mM; Eurofins MWG Operon, Ebersberg bei Munchen, Germany), 0.4 μl TaqMan probe P1 (10 μM; Eurofins MWG Operon), and 4 μl of an amplification mixture (MasterPlus Plus; Roche Diagnostics). Mitochondrial and EGFP primers and probes {16s mitochondrial primer forward (F) [5′ CAC GAA CCC CGC CTG TTT AC 3′]; 16s mitochondrial primer reverse (R) [5′ CGC GGC CGT GGA ACT TTA 3′]; 16s mitochondrial probe F

[5′ 6-carboxyfluorescein (FAM) TGG CAT CGC CTG CCC AGT GAC 3′ *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine (TAM)]; EGFP primer F [5′ ACG ACG GCA ACT ACA AGA CC 3′]; EGFP primer R [5′ GTC CTC CTT GAA GTC GAT GC 3′]; EGFP probe F [5′ FAM CGACACCCTGGTGAACC 3′ TAM]} were used with the following thermocycler settings: initialization step 95°C/10 min (denaturation step 95°C/10 s; annealing and elongation step 60°C/20 s) × 60 cycles; cooling 40°C/30 s. Settings were the same for both probes, whereas PCR was carried out independently. All assays were carried out in duplicate. Specificity was checked by melting-curve analysis, as described previously (23).

Statistical analyses. Blood cell counts were expressed as the percentage change vis-à-vis the control reading. The reference value (100%) was based on measurements before hyperbaric exposure. Data were then compiled. Numerical data points were expressed as mean and SD. Spearman rank correlation test was used for the measure of statistical dependence among variables. Different groups were compared using Kruskal-Wallis or Mann-Whitney (MW) tests, and matched comparisons within groups were analyzed using a Wilcoxon (W) test.

Data for bubble score were noted using median value and the 25th–75th percentiles.

The optimal cutoff level for mDNA that can discriminate between symptomatic or asymptomatic animals was determined using the receiver operating characteristic (ROC) curve. The diagnostic value of this test was estimated through the calculation of sensitivity, specificity, negative-predictive value, and positive-predictive value. The two-tailed Fisher exact test was used to detect differences in the frequencies between symptomatic or asymptomatic animals from mDNA threshold; odds ratio (OR) with 95% confidence intervals (CI) was also calculated.

The significance threshold was 95% with an α-risk of 5%. A statistical trend was considered when $5\% < \alpha < 10\%$.

RESULTS

One hundred twenty-one Sprague-Dawley rats, weighing 367.3 ± 18.8 g, were used in this study. Twelve of them, which were not subjected to the decompression protocol, were used as control for plasma mDNA basal-level determination in a preliminary study.

Clinical observations. In the experimental group ($n = 109$), death occurred inside of the hyperbaric chamber during the decompression phase ($n = 22$) or rapidly after surfacing ($n = 9$), within 10.8 ± 7.16 min (mean ± SD) in 31 rats. Although they displayed the whole spectrum of DCS signs, these rodents were excluded from the blood analysis and the bubble-detection protocol because of a too-short death latency time for the sampling techniques (Fig. 1).

Platelet, leukocyte, and erythrocyte counts, as well as mDNA levels, were determined in blood samples from the remaining 78 rats that survived the hyperbaric protocol. Pulmonary and neurological DCS symptoms with abnormal breathing, limb paralysis, and walking difficulties (paraparesis) occurred in 17 rats (forming the "DCS" group), within the 30-min observation period (5.8 ± 7.3 min on average). Bubble detection was performed on eight rats from this group.

Finally, 61 rats (forming the "noDCS" group) survived during the 30-min observation period following surfacing without apparent DCS symptoms. The bubble-detection protocol was applied randomly on 36 rats of the noDCS group.

Bubble counts. In the rats of the DCS group ($n = 8$), one rat showed no bubble, two displayed a Spencer grade at 2, three had *grade 3*, and two rats had a *grade 4*. In the group of rats
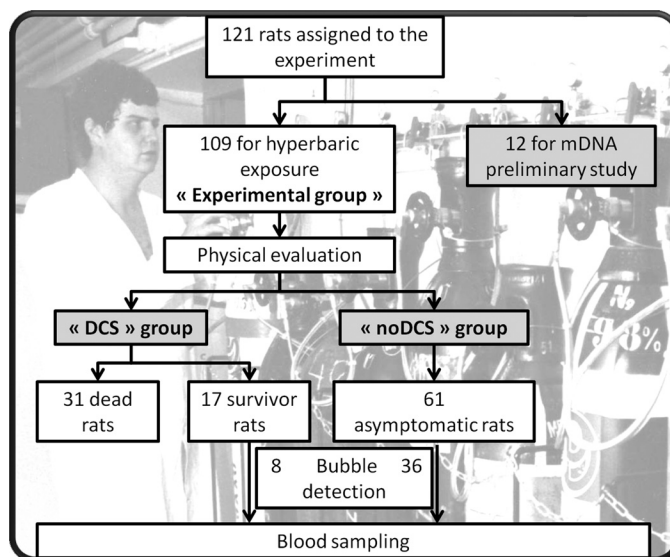

Fig. 1. Flow chart describing the experimental design. mDNA, mitochondrial DNA; DCS group, group showing decompression sickness (DCS) symptoms; noDCS group, group showing no DCS symptoms.

without symptoms ($n = 36$), 16 rats showed no bubble, three had a *grade 1*, five a *grade 2*, 11 had a *grade 3* in their blood sample, and one rat had a *grade 4*.

Rats displaying DCS symptoms had a significant bubble score higher than asymptomatic rats [Spencer$_{DCS}$ = 2.0/**3.0**/4.0, Spencer$_{noDCS}$ = 0.0/**1.0**/3.0 (first quartile/**median**/third quartile); MW$_{DCS/noDCS}$: $n = 8/36$, $\alpha = 0.05$, $P = 0.044$]. When converted into binary data (DCS → 1; noDCS → 0), the Spearman test showed a correlation between the clinical status (DCS or noDCS) and the Spencer bubble grade (Spearman$_{clinic/bubble}$: $n = 44$, $\alpha = 0.05$, $P = 0.028$).

mDNA levels. To evaluate basal levels of circulating mDNA in plasma and the feasibility of this study, preliminary assays were conducted on a group of 12 rats, 3 mo before hyperbaric experiments. The recorded level of circulating mDNA averaged 1.50 ± 0.93 in plasma.

Levels of plasma mDNA were significantly higher in the DCS (DNA$_{DCS}$ = 2.99 ± 2.62) than in the noDCS groups (DNA$_{noDCS}$ = 1.49 ± 1.27; MW$_{noDCS/DCS}$: $n = 61/17$, $\alpha = 0.05$, $P = 0.005$; Fig. 2).

Blood elements. Intragroup comparisons revealed significant differences in platelet counts before and after the dive in both DCS and noDCS groups (W: $\alpha = 0.05$, noDCS $n = 61$, $P < 0.001$; DCS $n = 17$, $P = 0.002$). Percent-falls in platelets were $9.4 \pm 11.4\%$ and $18.5 \pm 16.6\%$ in the noDCS and DCS groups, respectively, with a statistical trend in platelet consumption between DCS and noDCS groups (MW$_{noDCS/DCS}$: $n = 61/17$, $\alpha = 0.05$, $P = 0.052$; Fig. 3).

Concerning leukocyte counts, intragroup comparisons revealed significant differences before and after the dive in the noDCS and the DCS group (W: $\alpha = 0.05$, noDCS $n = 61$, $P < 0.001$; DCS $n = 17$, $P = 0.006$). Percent-fall in leukocytes averaged $16.1 \pm 25.4\%$ and $19.1 \pm 20.2\%$ in noDCS and DCS groups, respectively. No statistical difference was observed between DCS and noDCS groups (MW$_{noDCS/DCS}$: $n = 61/17$, $\alpha = 0.05$, $P = 0.706$; Fig. 3).

Finally, intragroup comparisons revealed a significant difference in erythrocyte counts before and after the dive in the
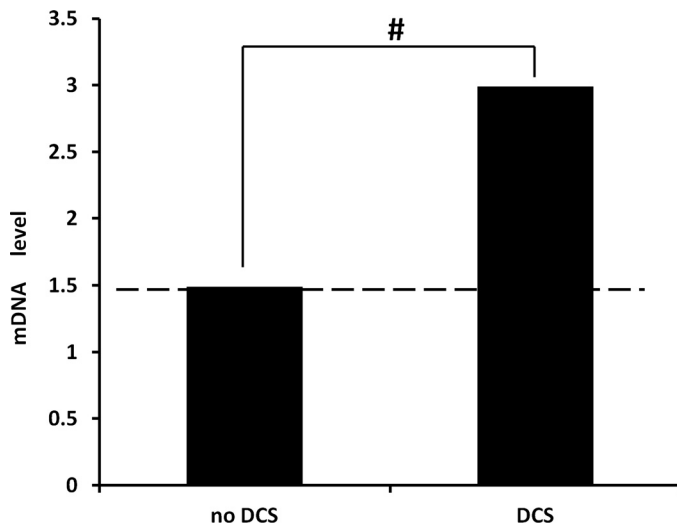
Fig. 2. Circulating levels of mDNA. mDNA detection was carried out on the DCS group (rats that suffered from decompression protocol) and on the noDCS group (rats that did not express physical troubles after the decompression protocol). #Significant difference [$\alpha = 0.05$, Mann-Whitney (MW) test] between clinical groups (noDCS, DCS). Preliminary assays were conducted on a control group of 12 rats, 3 mo before hyperbaric experiments (dotted line).

noDCS group only (W: $\alpha = 0.05$, noDCS $n = 61$, $P < 0.001$; DCS $n = 17$, $P = 0.635$). On average, percent-variation in erythrocytes was $-6.0 \pm 12.2\%$ and $4.6 \pm 14.1\%$ in noDCS and DCS groups, respectively. A significant difference was observed between the DCS and the noDCS groups (MW$_{\text{noDCS/DCS}}$: $n = 61/17$, $\alpha = 0.05$, $P = 0.018$).

Correlation among bubbles, mDNA levels, and blood elements. Platelet, leukocyte, and erythrocyte counts were compared with mDNA values on rats subjected to the decompression protocol, without consideration of the DCS status (DCS, noDCS). Spearman test showed a correlation between mDNA levels-increase and percent-fall in platelet counts (Spearman$_{\text{mDNA/platelet}}$: $n = 78$, $\alpha = 0.05$, $P < 0.0001$), but there was no significant correlation for mDNA levels-increase and leukocyte-falls (Spearman$_{\text{mDNA/leukocytes}}$: $n = 78$, $\alpha = 0.05$, $P = 0.423$) or erythrocyte-counts variation (Spearman$_{\text{mDNA/erythrocytes}}$: $n = 78$, $\alpha = 0.05$, $P = 0.504$). Nonetheless, the platelet consumption was significantly correlated with both the leukocyte decrease (Spearman$_{\text{platelet/leukocytes}}$: $n = 78$, $\alpha = 0.05$, $P = 0.010$) and the erythrocyte decrease (-3.9 ± 13.1; Spearman$_{\text{platelet/erythrocytes}}$: $n = 78$, $\alpha = 0.05$, $P = 0.020$).

In rats that were considered for bubble detection, Spearman tests showed no correlation between mDNA levels and the Spencer bubble grade (Spearman$_{\text{mDNA/bubble}}$: $n = 44$, $\alpha = 0.05$, $P = 0.235$). Nonetheless, rats without bubble (Spencer bubble grade $= 0$; $n = 17$) displayed significantly lower mDNA levels than those with circulating bubbles ($n = 29$; mean $\pm$ SD: mDNA$_{\text{noBubble}} = 1.22 \pm 0.77$ vs. mDNA$_{\text{Bubbles}} = 2.06 \pm 1.34$; MW$_{\text{noBubble/Bubble}}$: $n = 16/28$, $\alpha = 0.05$, $P = 0.038$).

Platelet, leukocyte, and erythrocyte counts were also compared with mDNA values in rats displaying clinical symptoms (DCS group). Spearman test showed a correlation between mDNA levels-increase and percent-fall in platelet counts (DCS group, Spearman$_{\text{mDNA/platelet}}$: $n = 17$, $\alpha = 0.05$, $P = 0.0002$), but there was no significant correlation for mDNA levels-increase and leukocyte-falls (DCS group, Spearman$_{\text{mDNA/leukocytes}}$: $n = 17$,

$\alpha = 0.05$, $P = 0.566$) or erythrocyte-counts variations (DCS group, Spearman$_{\text{mDNA/erythrocytes}}$: $n = 17$, $\alpha = 0.05$, $P = 0.630$).

Additionally, platelet, leukocyte, and erythrocyte counts were compared with mDNA values in rats of the noDCS group. Spearman test revealed no correlation between mDNA levels and other variables in this group (noDCS group, Spearman$_{\text{mDNA/platelet}}$: $n = 61$, $\alpha = 0.05$, $P = 0.267$; Spearman$_{\text{mDNA/leukocytes}}$: $n = 61$, $\alpha = 0.05$, $P = 0.516$; Spearman$_{\text{mDNA/erythrocytes}}$: $n = 61$, $\alpha = 0.05$, $P = 0.625$), whereas a strong correlation was found between platelet count and leukocyte or erythrocyte counts (noDCS group, Spearman$_{\text{platelet/leukocytes}}$: $n = 61$, $\alpha = 0.05$, $P = 0.002$; Spearman$_{\text{platelet/erythrocyte}}$: $n = 61$, $\alpha = 0.05$, $P < 0.001$).

ROC curve analysis. With the use of ROC curve analysis, the threshold value of mDNA, highly predictive of clinical symptoms in animals, was determined as >1.91, with corresponding values of sensitivity, specificity, negative-predictive value, and positive-predictive value as follows: 59% (95% CI, 33–82%), 76% (95% CI, 64–86%), 40% (95% CI, 21–61%), and 87% (95% CI, 75–94%), respectively. Differences in the frequencies between symptomatic or asymptomatic animals from the mDNA threshold were significant (Fisher exact test, $P = 0.008$), and the proportion of animals presenting clinical symptoms of DCS was higher when levels of mDNA exceeded 1.91 (OR 4.6; 95% CI, 1.5–14.1).

Concerning the ROC curve analysis of platelet counts, the threshold-predictive value of clinical symptoms in animals was determined as -19%. The cutoff value attributed to the bubble score was 2. Their corresponding values of sensitivity, specificity, negative-predictive value, and positive-predictive value were, respectively, as follows: platelet counts 62.0% (95% CI, 32–86%), 81% (95% CI, 68–90%), 90% (95% CI, 79–97%), and 42% (95% CI, 20–67%); bubble score 87% (95% CI, 47–100%), 55% (95% CI, 38–72%), 95% (95% CI, 76–100%), and 30% (95% CI, 13–53%). Differences in the frequencies between symptomatic or asymptomatic animals were significant for platelet counts (Fisher exact test, $P = 0.004$) and bubble counts ($P = 0.048$). In both cases, the proportion of animals without any clinical symptoms of DCS was lower
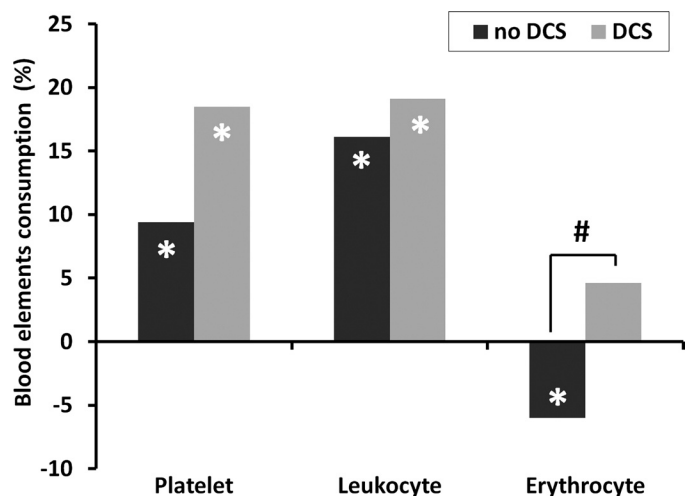

Fig. 3. Fall in blood platelet leukocyte and erythrocyte count (%) after decompression in rats. Blood tests were carried out on the DCS group and on the noDCS group. *Significant difference ($\alpha = 0.05$, Wilcoxon test) between pre- and postdecompression count into a group; #significant difference ($\alpha = 0.05$, MW test) between clinical groups.

when a decrease in platelet count was less than −19% (OR 6.7; 95% CI, 1.8–24.0) or when the bubble score was <2 (OR 8.8; 95% CI, 1.3–60.2).

No significant difference was observed in the frequencies of symptomatic and asymptomatic animals for leukocyte (Fisher exact test, $P = 1$; OR 1.2; 95% CI, 0.34–4.0) or red cell counts ($P = 0.082$; OR 3.2; 95% CI, 0.91–11.0) using the ROC curve analysis.

DISCUSSION

The decompression protocol used in this study was comparable with that used in other studies with rats of similar weight (28–30). This protocol generated circulating bubbles and induced DCS with abnormal breathing, motor and locomotor impairments, and convulsions.

In this study, we pointed out an increase in circulating mDNA plasmatic levels in rats subjected to the decompression protocol. Rats displaying DCS symptoms presented mDNA levels twice as high as the others. The mDNA plasmatic levels were correlated with the loss of circulating platelets. We also demonstrated that symptomatic rats had higher bubble grades than those without symptoms. These results are in accordance with literature data (34, 36) that report correlations between bubbles and DCS stress. As a matter of fact, bubbling rats had higher mDNA levels than nonbubblers, although all bubblers were not injured. It is interesting to note that the mDNA average level of 2.06 in the bubble group is in accordance with the DCS-predictive threshold value of 1.91, calculated in this study.

Bubble per se effect theories. We firstly hypothesized that bubble-induced shear stress and therefore, endothelial or blood cells' destruction generate free mDNA release in blood. Although this study does not allow identification of the cell type impacted by bubbles, the high level of mDNA found in the plasma of DCS and bubbling rats corroborated this theory. Circulating mDNA could come from platelet, leukocyte, erythrocyte, or endothelial cells' degradation. Nonetheless, only a part of leukocytes and platelets should be implicated in this cell-destruction process, considering that: *1*) diapedesis is well documented in DCS (6, 11, 48–51), *2*) some antithrombotic substances protect from DCS (30), *3*) endothelial damages induced by bubbles can be observed (2, 22, 24, 47), *4*) erythrocyte counts tend to increase in rats suffering from DCS, and *5*) no correlation was found between the mDNA levels-increase and leukocyte consumption. In all likelihood, free mDNA levels recorded from blood in this study should come from vascular endothelium after the bubble-induced shear stress.

Another suggested mechanism about the per se effect of bubbles involved a direct interaction between blood components and bubbles, leading to proinflammatory and prothrombotic events without damaging cells. Even though a drop in platelet and leukocyte counts corroborates this theory as much as the other one, it cannot explain the increase of mDNA level in blood. Nonetheless, the presence of DNA does not invalidate this theory, because bubbles could activate cells without killing them. In rats without DCS symptoms, no correlation was found between the mDNA level and the blood-element counts, suggesting that according to our hypothesis, no blood cells were destroyed. Nonetheless, in the same group, strong correlations

among blood elements themselves—platelet and leukocyte and erythrocyte—were noticed. Whereas these rats were not symptomatic, it argues in favor of this direct interaction with bubbles and an emergence of an immune-inflammatory cascade. Circulating microparticles could be the missing link. Derived from various cellular types, they may initiate neutrophils' activation and subsequent vascular injuries (42). Hence, we could hypothesize for further experiments that microparticles could be one of the initiating processes inducing cell death. A survey of Juffermans et al. (15) indicated that bubbles could initiate the production of microparticles by the stimulation of a big conductance potassium channel. Otherwise, it tends to demonstrate that mDNA release in blood of DCS rats could not come from blood elements but rather, from endothelial cells, for example.

Could mDNA be a relevant biomarker in DCS? With the use of ROC curve analysis, we found that the value of mDNA, determined as >1.91, was predictive of DCS symptoms in animals with a positive-predictive value of 87.3% and an OR of 4.57. However, the negative-predictive value was only 40%. In contrast with mDNA values, the statistical analysis permitted us to conclude that the absence of platelet consumption or absence of bubbles is a good indicator of a safe decompression, but it is not an efficient predictor of DCS. We believe that the combined analysis of mDNA, platelets, and bubbles could be relevant to the assessment of DCS.

In this study, the mDNA plasmatic levels of rats injured by the protocol were correlated with platelet- and leukocyte-fall, which was considered as a relevant index of DCS severity in animal models (24a, 29). High mDNA levels have also been shown in bubbler and DCS rats. We believe that mDNA could be helpful to diagnose patients presenting only minor clinical DCS symptoms. In DCS, experiments have strongly suggested a role for coagulation system activation (13, 25) that is self-sustained after the resolution of bubble. Considering the time of the end of the dive and DNA degradation time (37), this marker could afford a scheme of the impact on the endothelium and therefore, be helpful for diagnosis and prognosis. Origin of mDNA should be determined to better identify cells destructed by bubbles.

A certain concentration of extracellular DNA is normally maintained in the blood of healthy patients (39), as in the group of rats from our preliminary study. In our study, an average level of free mDNA in the preliminary study is similar (free mDNA ~1.50) to that of the noDCS group (free mDNA ~1.49). Assuming that mDNA is a reliable biomarker, we could conclude that the noDCS group did not suffer from the decompression protocol. On one hand, it is partly true, as no phenotypic symptom was detected during physical examinations. On the other hand, we could suggest that the noDCS rats had some circulating bubbles, considering their loss in platelet and leukocyte counts. The bubble effect may not have been strong enough to induce cell destruction and mDNA release. These circulating bubbles could be considered as "silent bubbles" (4).

Physiopathological effect of free DNA in DCS. DNA not only plays the key role in storing genetic information, but it also exerts an immune-stimulating effect (35). DNA is involved in the pathogenesis of some disorders, where an elevated content of immune complexes with DNA induces inflammation (44, 48). As a prothrombotic event of DCS can degen-

erate into a disseminated ischemia, where cytotoxic effects add to the whole panel of the sickness (22), it is possible that ischemia induced by DCS leads to a novel release of circulating DNA in blood, as it was found in a trauma patient (33), itself adding an novel, immune-stimulating effect.

Limitations. Animal experimentation is especially useful in studies that would pose unacceptable risks in human subjects. The rat model was quite severe (28% death, 15% serious manifestations) compared with human DCS (46). Moreover, mDNA samples were obtained 30 min postdive. Therefore, the applicability of the results to humans remains to be determined. Nonetheless, the use of an animal model is relevant in DCS evaluation (1, 30); however, specific problems are encountered. For example, a postdive bubble detection and blood sampling were not possible for all rats, especially in dead rats. Indeed, the average time from surfacing to onset of initial DCS symptoms was very short, i.e., 5.8 min with a high mortality rate. Therefore, we could only suppose that dead rats had very high levels of bubbles and circulating mDNA.

Conclusion. In our rat model of DCS that generates circulating bubbles, the increase of mDNA level in the plasma is significantly predictive of symptoms' occurrence related to DCS. Moreover, a significant correlation is observed between the extracellular mDNA level and the drop in platelet and leukocyte in symptomatic rats.

These results suggest that cell destructions could be induced by circulating bubbles, even though they cannot differentiate between bubble-induced vessel-wall injury and bubble-blood component interactions in DCS. In both cases, these actions result in proinflammatory events and platelet-clotting activity. If endothelial cell destruction is reckoned, further analysis should be conducted to identify mDNA origins better.

In our rat model, the concentration of circulating mDNA, when combined with other markers, seems diagnostically valuable in estimating the DCS severity. Further studies are needed to assess the relevance of mDNA as a DCS biomarker in humans.

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GRANTS

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.V. and J-E.B. conception and design of research; N.V., A.P., and J-E.B. performed experiments; N.V. and J-E.B. analyzed data; N.V., S.G., A.P., J-J.R., and J-E.B. interpreted results of experiments; N.V. and J-E.B. prepared figures; N.V., S.G., J-J.R., and J-E.B. drafted manuscript; N.V., S.G., A.P., J-J.R., and J-E.B. edited and revised manuscript; N.V., S.G., A.P., J-J.R., and J-E.B. approved final version of manuscript.

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