J Appl Physiol 118: 20–28, 2015.
First published November 6, 2014; doi:10.1152/japplphysiol.00260.2014.

Effect of nitric oxide on spinal evoked potentials and survival rate in rats with decompression sickness

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Submitted 25 March 2014; accepted in final form 3 November 2014

Randsoe T, Meehan CF, Broholm H, Hyldegaard O. Effect of nitric oxide on spinal evoked potentials and survival rate in rats with decompression sickness. *J Appl Physiol* 118: 20–28, 2015. First published November 6, 2014; doi:10.1152/japplphysiol.00260.2014.—Nitric oxide (NO) releasing agents have, in experimental settings, been shown to decrease intravascular nitrogen bubble formation and to increase the survival rate during decompression sickness (DCS) from diving. The effect has been ascribed to a possible removal of preexisting micronuclei or an increased nitrogen washout on decompression through augmented blood flow rate. The present experiments were conducted to investigate whether a short- or long-acting NO donor [glycerol trinitrate (GTN) or isosorbide-5-mononitrate (ISMN), respectively] would offer the same protection against spinal cord DCS evaluated by means of spinal evoked potentials (SEPs). Anesthetized rats were decompressed from a 1-h hyperbaric air dive at 506.6 kPa (40 m of seawater) for 3 min and 17 s, and spinal cord conduction was studied by measurements of SEPs. Histological samples of the spinal cord were analyzed for lesions of DCS. In total, 58 rats were divided into 6 different treatment groups. The first three received either saline (*group 1*), 300 mg/kg iv ISMN (*group 2*), or 10 mg/kg ip GTN (*group 3*) before compression. The last three received either 300 mg/kg iv ISMN (*group 4*), 1 mg/kg iv GTN (*group 5*), or 75 μg/kg iv GTN (*group 6*) during the dive, before decompression. In all groups, decompression caused considerable intravascular bubble formation. The ISMN groups showed no difference compared with the control group, whereas the GTN groups showed a tendency toward faster SEP disappearance and shorter survival times. In conclusion, neither a short- nor long-acting NO donor had any protective effect against fatal DCS by intravenous bubble formation. This effect is most likely due to a fast ascent rate overriding the protective effects of NO, rather than the total inert tissue gas load.

spinal evoked potential; SEP; tissue bubbles; autochthonous bubbles; isosorbid-5-mononitrate; glycerol trinitrate; nitroglycerine

DURING DIVING, HYPERBARIC air breathing will cause the inert gas of nitrogen (N_2) to dissolve in blood and tissue according to Henry's law. In situations of inadequate decompression, N_2 may supersaturate and form harmful N_2 bubbles within blood and tissue, causing decompression sickness (DCS). It is believed that these bubbles cannot evolve in tissue ex nihilo, but must grow from small preexisting gas entities or micronuclei adhering to the endothelium wall (10, 34, 36), although extravascular bubble formation after decompression has been demonstrated (13, 19). The predominant clinical features in DCS relate to neurological injuries in the central nervous system (CNS) (21). The white matter of the spinal cord is the most commonly affected site (14) due to bubbles exerting a mechanical pressure on the nervous tissue or obstructing blood vessels causing ischemia. The general pathophysiological mechanisms responsible for DCS in the spinal cord are still under debate, and several hypotheses exist (22). These include the formation of intravascular gas bubbles as "arterial emboli" (11) and "venous infarction" theories (17), as well as the extravascular formation of "autochthonous bubbles" in tissue (12, 18, 33) followed by an inflammatory prothrombotic state with complement, platelet, leucocytes, and heat shock protein activation (9, 30, 31, 37).

In experimental settings, the nitric oxide (NO) releasing agents, glycerol trinitrate [nitroglycerine (GNT)] and isosorbide-5-mononitrate (ISMN), have been shown to significantly reduce intravascular bubble formation and to increase the survival rate during DCS from diving (8, 28, 38); however, the exact mechanism by which NO prevents DCS has not been clarified. The most widely accepted theory suggests that NO induces alterations in the hydrophobicity of the endothelial wall, which reduces the stability and density of the micronuclei precursors adhering to the surface (8, 28, 38, 39). It has also been suggested that NO enhances the blood flow rate and thereby promotes bubble shrinkage through an increased N_2 washout from tissues (28). Whether NO donors equally promote protection against DCS in the CNS, i.e., in the spinal cord specifically, has not been reported.

Accordingly, in keeping with the results above, we hypothesized the following: if NO reduces the intravascular bubble formation through elimination of micronuclei precursors, NO donors administered before a dive should reduce intravascular bubble formation, thereby increasing the survival rate and have a protective effect on the spinal cord conduction as evaluated by measurements of spinal evoked potentials (SEPs). Furthermore, it may be argued that, since NO donors cause immediate hemodynamic changes (26, 35), administration of a NO donor before decompression should, everything else being equal, also result in an increased survival rate and a protective effect on spinal cord conduction, due to an enhanced tissue blood flow rate increasing N_2 elimination.

Therefore, in the present experiments, we tested the effects of a short-acting NO donor, GNT (nitroglycerine), and a long-acting NO donor, ISMN, on survival rate and spinal cord conduction during DCS in rats by measurements of SEPs. Accordingly, rats were exposed to a hyperbaric air-breathing dive at 506.6 kPa [40 m seawater (msw)] for 60 min with a decompression phase of 3 min and 17 s. The diving protocol and decompression profile were conducted to cause intravas-

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cular bubble formation with spinal DCS and a lethal outcome in most nontreated animals' 30–60 min post-decompression. NO donors were administered either at "sea level" before the hyperbaric air dive commenced, or at depth before the decompression from 506.6 to 101.3 kPa (sea level pressure).

METHODS

General

The experiments were performed at the Laboratory of Hyperbaric Medicine at Rigshospitalet, The University Hospital of Copenhagen. The experimental protocol was approved by the Danish National Animal Experiment Committee and was in accordance with European Union Regulations and the American Physiological Society's "Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training." All surgery and experiments were performed under anesthesia and were acute, ensuring no suffering.

Female Wistar rats weighing 270–340 g were anesthetized with thiomebumal sodium intraperitoneally (0.1 g/kg), and analgesia was provided by administration of buphrenorphine subcutaneously (0.01–0.05 mg/kg). The anesthetized rat was placed in a supine position and fixed in a stereotactic frame on an operating and heating platform on top of an insulating layer. A cannula was inserted in the trachea (polyethylene tubing, inner diameter 1.5 mm), and a catheter was placed in the left carotid artery for registration of mean arterial blood pressure (MAP). It was kept patent by a continuous infusion of nonheparinized saline by means of a syringe pump (SAGE Instruments model 341) at a rate of 1 ml/h. MAP was measured throughout the experiment by means of a pressure transducer from Edwards Life Sciences placed inside the chamber. A thermometer placed in the vagina was connected to a thermostat to maintain a body temperature of 37°C by suitable heating of the chamber during the experimental procedures. Continuous real-time recordings of temperature and MAP were obtained on a PC using the Picolog data collection software. A catheter was placed in the right vena jugularis externa for administration of saline, ISMN, and GTN. The electrocardiogram (ECG) was monitored continuously, and ascending spinal cord conduction was examined by SEP recordings at the cervical level during bilateral stimulation of the peroneal nerves before compression and after decompression. Rats breathed air spontaneously before and during the compression phase. After decompression, rats were connected to a rat respirator under continuous air breathing, and SEPs were monitored during an observation period of 120 min, or until fatal DCS. For the precise sequence of events, see Fig. 1, experimental protocol.

Breathing System

The rat was placed in the stereotactic frame inside a small experimental pressure chamber. In the bottom of the chamber, penetrations were made for a chamber heating system consisting of an electrical heater and a small fan mixing the chamber atmosphere. The rat tracheal cannula was connected to a T-shaped tube in the chamber breathing system, and air was supplied continuously at a pressure slightly above chamber pressure. The air provided flowed inside the chamber through an 8-mm-inner diameter silicone tube with a small latex rubber breathing bag reflecting the rats' respiratory motion. The T-shaped tube was further connected to an exhaust outlet via a specially designed overboard dump valve. During the compression and decompression phase, rats breathed air spontaneously while connected to the chamber breathing system. Once decompressed, all rats in the experimental *groups 1–6* were disconnected from the chamber breathing system and the tracheal cannula connected to a custom made rat respirator. Subsequently, rats were paralyzed with pancuronium bromide (Pavulon, 2 mg/kg) by intramuscular injection and ventilated artificially by the respirator, maintaining a normal arterial CO_2 tension measured with a Radiometer ABL 30 blood-gas analyzer. Rats continued air breathing throughout the entire observation period.

Pressurizing Protocol

Once the breathing system, arterial and venous catheters, and temperature, ECG, SEP, and stimulation electrodes were connected to their respective chamber penetrations, the top steel lid of the pressure chamber was mounted, and all of the experimental groups of animals were then exposed to a 1-h hyperbaric air dive at 506.6-kPa absolute pressure (40 msw). Rats were then decompressed from 506.6 to 101.3 kPa (sea level) in three stages with two stops: *1*) decompression from 506.5 to 202.6 kPa (10 msw) in 1 min (30 m/min) with a stop at 202.6 kPa for 1 min; *2*) decompression from 202.6 to 152 kPa (5 msw) in 17 s (18 m/min) with a stop at 152 kPa for 43 s; and *3*) decompression

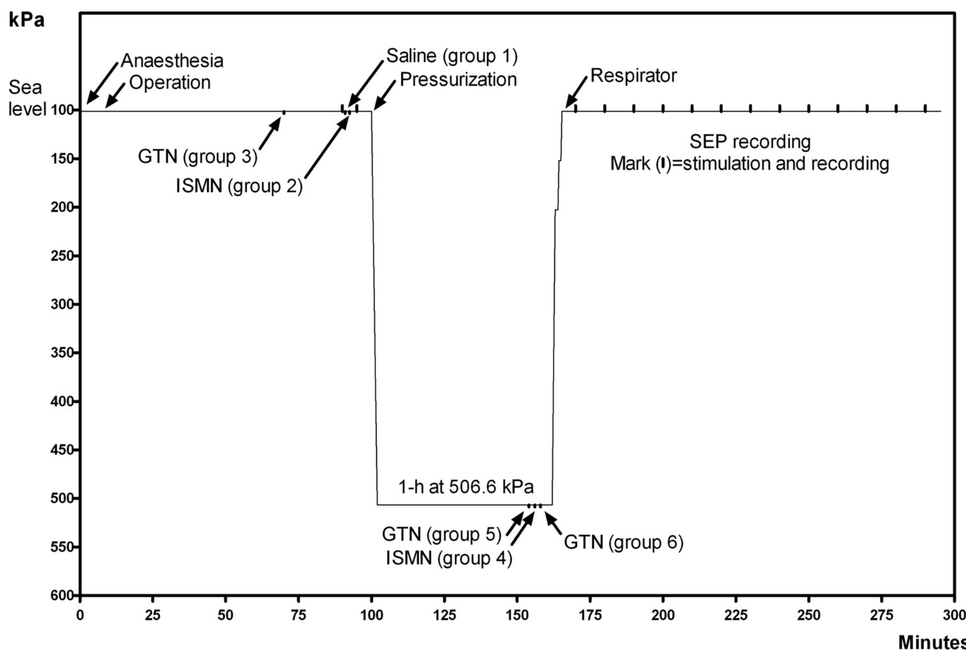

Fig. 1. Experimental protocol. Anesthetized rats were exposed to a 1-h hyperbaric air dive to 506.6 kPa (40 m seawater) and decompressed to 101.3 kPa (sea level) in 3 min and 17 s during spontaneous air breathing. Following the decompression phase, rats were paralyzed and were subsequently mechanically ventilated with air using a respirator. Spinal evoked potentials (SEPs) were measured both immediately before the air dive and post-decompression during an observation period at sea level of up to 2 h or until death by cardiac arrest. Rats were administered either glycerol trinitrate (GTN) or isosorbide-5-mononitrate (ISMN) at sea level before the dive (*groups 2* and *3*) or during the compression phase (*groups 4–6*).

from 152 to 101.3 kPa in 17 s (18 m/min). Total decompression time was 3 min and 17 s; see Fig. 1.

Experimental Groups

In total, 77 rats were used during the experiments of which 58 rats were assigned to 6 experimental groups. The remaining 19 rats were assigned to the validation of the SEP measurements and divided into SEP-control *experiments A–C*, and a group perfusion fixed for histological examinations of the spinal cord; see description of SEP-control *experiments A–C* and histological examination below.

For the experimental *groups 1–6*, rats (N = 58) were divided into groups receiving ISMN (Dottikon) and GNT (nitroglycerine) at sea level before compression and at high pressure before decompression. The experimental control *group 1* received 1 ml of saline with an injection rate of 1 ml/min before compression. In the treatment groups, ISMN and GTN were, for all groups, dissolved in saline, giving a total volume of 1 ml. The injection rate was 1 ml/min for *groups 2*, *4*, and *6*, 0.2 ml/min for *group 5* (total infusion time of 5 min), and a bolus injection for *group 3*. The doses of the NO donors (except *group 3*) were chosen according to the hemodynamic effect reported in previous studies (26, 35). The 58 rats from the 6 experimental groups were divided into the following: *1*) *group 1* (N = 12), control group, saline intravenous injection initiated 10 min before compression; *2*) *group 2* (N = 12), 300 mg/kg iv ISMN injection initiated 5–10 min before compression; *3*) *group 3* (N = 8), 10 mg/kg ip GTN injection 30 min before compression; *4*) *group 4* (N = 10), 300 mg/kg iv ISMN injection initiated 6 min before decompression; *5*) *group 5* (N = 8), 1 mg/kg iv GTN injection initiated 8 min before decompression; and *6*) *group 6* (N = 8), 75 μg/kg iv GTN injection initiated 4 min before decompression.

Recording of SEPs

The cervical vertebral column was exposed by a dorsal midline incision, and bore holes were drilled into the second and fifth cervical vertebra using a dental drill (Bravo Micromotor, Danish Nordenta A/S, hard metal round burs RA 1/008+1/009), leaving the dura intact. Two silver electrodes were placed in the holes on the dura and fixated with dental cement. Vents were left open to the vertebral canal by cutting openings in the ligamentum flavum adjacent to the second and fifth cervical vertebra to allow the escape of gas accidentally introduced. A polyethylene tube was placed in the operation field as a drain, and the incision was closed. Both peroneal nerves were dissected free and placed on stimulating electrodes (custom-made "tunnel electrodes") and the incision closed. The front extremities were perforated with needles and connected to electrodes for ECG registration. SEPs were registered from the cervical electrodes (i.e., over the surface of the dorsal funiculus) during alternate bilateral stimulation of the peroneal nerves using a NL 800A Linear Constant Current Stimulus Isolator and amplifier from NeuroLog Systems (Digitimer). Signals were then digitized using a CED 1401 analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and sampled on a computer using the Spike 2 software (Cambridge Electronic Design), which also used custom scripts to trigger the stimulator. To reduce the electrical interference from the heart, the stimulator was triggered by the R-peak of the ECG with a delay to place the stimulus and SEP within the isoelectric phase of the ECG. During SEP recordings, the chamber heating system was briefly disconnected to eliminate electrical interference. Stimulation intensity was chosen to give the maximal amplitude of the evoked potential, and the averaging was performed over a 2-min period of consecutive stimulation of each nerve. Before the hyperbaric exposure, the stimulation was also performed, and mean values were obtained for statistical comparison. In the post-decompression observation period at sea level, SEPs were recorded at intervals of 10 min for 120 min or until cardiac arrest, as measured by the ECG.

Validation of the SEP measurements

To evaluate the reliability of the SEP measurements, a number of experiments were performed to control for the fibers stimulated (the afferents directly projecting to the dorsal nuclei through the dorsal column), the possible relationship to blood pressure, and the effects of immobilization and artificial respiration.

SEP-control experiment A. In two rats, stimulation and recordings were performed in both directions (i.e., peripheral stimulation with central recording and central stimulation with peripheral recording) to characterize the neuronal path involved. It was found that action potentials traveled in both directions with the same latency, i.e., delay from stimulation to initiation of the first wave (and expressed in ms). In total, for the 130 nerves that were stimulated in 68 rats (i.e., 58 rats from experimental *group 1–6*, 5 rats from SEP-control *experiment B*, and 5 rats from SEP-control *experiment C*, almost all with stimulation of both peroneal nerves), the mean latency time before the pressure exposure was 3.36 ms (SD ±0.22). This is similar to the nerve conduction velocity measured in a previous report, in which it had been concluded did not allow for a synaptic delay (20).

SEP-control experiment B. The aim was to evaluate a possible relationship between SEPs, MAP, and the effect of immobilization without the hyperbaric exposure. Rats (N = 5 rats; n = 9 nerves) with a mean weight of 296.4 g (SD ±39.1) underwent the same experimental procedures as the experimental control *group 1*, without the air dive, and SEP and MAP were recorded for a mean period of 4 h and 37 min (SD ±11) at sea level. At the beginning, mean MAP was 177.5 mmHg (SD ±16.6) and remained stabile with a tendency for a slow decrease throughout the entire observation period, ending up with a mean MAP of 151 mmHg (SD ±16). The initial mean latency was 3.32 ms (SD ±0.15), ending up with a mean latency time of 3.34 ms (SD ±0.09) at the end of the observation period. There were no changes in the amplitudes of the recorded SEPs. At the end of the observation period, three rats were perfusion fixed, as described below.

SEP-control experiment C. To more accurately visualize the SEPs without interference from electromyography activity from moving muscles, rats in the experimental *groups 1–6* were paralyzed and artificially ventilated. This, therefore, reduced the stimulation time necessary to obtain a stable and clear average of the SEP recording, especially when the rat was dying of DCS and displaying myoclonic twitches. To clarify whether the respirator could influence survival rate or conductivity of the spinal cord during DCS, an additional group (SEP-control *experiment C*) was monitored and exposed to a 1-h hyperbaric air dive, similar to experimental *group 1*. Once decompressed to sea level, rats in SEP-control *experiment C* group continued breathing air spontaneously through the chamber breathing system instead of getting paralyzed and being connected to the respirator. In SEP-control *experiment C* [N = 5 rats, n = 9 nerves; mean weight of 321.2 g (SD ±10)], four rats died within a period of 2–62 min post-decompression, while 1 rat survived the observation period. Mean survival time for the whole group was 58 min (SD ±37.8) with a median range of 60 min (2–120); mean SEP disappearance time was 45 min (SD ±47.7). All of the four rats dying during the observation period had ample intravascular gas formation, whereas no intravascular gas was found in the rat surviving.

Comparing experimental control *group 1* with the SEP-control *experiment C*, there was no significant difference regarding weight, MAP, survival rate, survival time, or spinal cord conductivity. Accordingly, we found no justifiable reason against using a respirator.

Histological Examination

Perfusion fixation of the spinal cord was performed in 12 separate rat experiments to obtain histological examples of possible decompression induced lesions: 3 rats from the SEP-control *experiment B*; 2 rats each from the experimental *groups 1*, *4* and *6*; 1 rat each from the experimental *groups 3* and *5*; and 1 rat was fixed immediately after

anesthesia without operation or hyperbaric exposure. Due to difficulties transporting the respirator, none of the perfusion-fixed rats were paralyzed or connected to the respirator but breathed spontaneously.

All of the decompressed rats were fixed 30 min after decompression, except for one rat in *group 3* that was fixed after 20 min because of terminal respiration and one rat in *group 4* that died upon arrival to sea level and was fixed immediately after. The rats were fixed by vascular perfusion through the left ventricle of the heart with venous opening in the right atrium. Preceding the fixative, the vascular system was flushed for 2 min with 200 ml phosphate-buffered saline to which heparin had been added (15,000 IU/l). Subsequent to this, the rats were perfused with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6) for 15 min. After perfusion fixation, the spinal cords were removed and postfixed for 12 h in the same fixative. The spinal cords were then divided into three parts (cervical, thoracic, and lumbar) and after dehydration embedded in Epon. The cervical and lumbar parts were then cut into one block each while the thoracic part was divided into two blocks. From each Epon block, 7–12 1-μm-thick sections were cut and stained with Toluidin blue.

Data Analysis and Statistics

Time from the beginning of the observation period until the loss of spinal cord conductivity was for each nerve defined as SEP disappearance time and expressed in minutes. When the conductivity was compromised immediately post-decompression, the SEP disappearance time was registered as zero. When conductivity was maintained during the entire observation period, the SEP disappearance time was registered as 120 min. Rats were also measured with respect to survival time, defined as the time from beginning of the observation period until death by cardiac arrest and expressed in minutes. If a rat survived the observation period, the survival time was registered as 120 min. The mean MAP before the pressure exposure and the mean MAP at the time of the SEP disappearance were recorded in millimeters of mercury. If a SEP was preserved throughout the observation period, the MAP at the SEP disappearance was defined as the last recorded MAP at the end of the observation period. The mean values of weight, MAP, SEP disappearance time, and survival time are given as means ± SD. For all comparisons, $P < 0.05$ is regarded as the criteria for significance.

To examine whether the differences between mean values of weight, MAP, SEP disappearance time, and survival time were significantly different from zero, tests for normality by means of Kolmogorov and Smirnov tests were performed, followed by nonparametric ANOVAs (Kruskal-Wallis Test). The differences between the mean values of the individual treatment groups were then analyzed using Dunn's multiple-comparison tests (3, 4, 15). Rats were also compared with respect to death or survival between the different treatment groups by means of a contingency table using Fisher's exact test (3, 4, 15).

RESULTS

General Conditions of Rats

In total, 58 rats from the 6 experimental groups were used with stimulation of 112 nerves (4 nerves omitted for technical reasons) before the hyperbaric exposure. Once decompressed to sea level, the decompression-induced insult immediately compromised the SEP in 10 nerves. Of the 58 rats, 49 died during the observation period with a pronounced drop in blood pressure preceding death, while 9 rats survived with a tendency for a slow decrease in MAP. An autopsy was performed immediately postmortem or at the end of the observation period. When the abdominal and thoracic cavities of the 49 rats dying during the observation period were opened for microscopic scan, ample intravascular gas formation was clearly visible in the veins and the right atrium. In the nine animals surviving the observation period, five rats had intravascular gas formation, while no bubbles were visible in the veins of four rats.

Effect of ISMN and GTN on MAP, SEP, and Survival Time

Group 1. The control group administered saline ($N = 12$ rats, $n = 24$ nerves) had a mean weight of 305.6 g (SD ±19), 10 rats died within a period of 17–93 min post-decompression, while 2 rats survived the observation period; see Table 1. The mean survival time for the whole group was 55.7 min (SD ±35.4) with a median range of 44 min (17–120 min). Before the pressure exposure, MAP was stable with a mean of 176.2 mmHg (SD ±17.8). Following decompression from the hyperbaric exposure, conductivity was immediately lost in two nerves, and the mean SEP disappearance time was 43.7 min (SD ±39.9) with a median range of 26 min (0–120 min). The mean MAP at SEP disappearance was 114.1 mmHg (SD ±51.9). All of the 10 rats dying during the observation period had ample intravascular gas formation, whereas no intravascular gas was found in the two rats surviving.

Group 2. The group administered 300 mg/kg iv ISMN 5–10 min before compression ($N = 12$ rats, $n = 22$ nerves) had a mean weight of 307.2 g (SD ±17.2); see Table 1. In total, 10

Table 1. *The effect of ISMN and GTN administration before and during a hyperbaric exposure on spinal cord conductivity and survival in rats with decompression sickness*

Treatment Group	*Group 1*, Saline iv 10 min Before Compression	*Group 2*, 300 mg/kg iv ISMN 5–10 min Before Compression	*Group 3*, 10 mg/kg ip GTN 30 min Before Compression	*Group 4*, 300 mg/kg iv ISMN 6 min Before Decompression	*Group 5*, 1 mg/kg iv GTN 3–8 min Before Decompression	*Group 6*, 75 μg/kg iv GTN 4 min Before Decompression
Rats, *N*	12	12	8	10	8	8
Nerves, *n*	24	22	16	20	16	14
Weight, g	305.6 ± 19	307.2 ± 17.2	301.1 ± 19.2	305.8 ± 17.5	304.2 ± 20	306 ± 24.5
Survival, no.	2 of 12	2 of 12	0 of 8	3 of 10	0 of 8	2 of 8
SEP disappearance time, min						
Mean	43.7 ± 39.9	42.3 ± 35.2	18.9 ± 13.6*	42.1 ± 41.2	13.7 ± 12.5†	32.6 ± 39.2
Median (range)	26 (0–120)	33 (0–120)	12.5 (0–53)	23 (0–120)	12 (0–43)	12 (0–120)
Survival time, min						
Mean	55.7 ± 35.4	62 ± 32	40.5 ± 27.2	65.3 ± 44.9	27.9 ± 19.1‡	51 ± 43.3
Median (range)	44 (17–120)	50 (23–120)	27 (17–93)	49 (20–120)	19 (9–67)	31 (20–120)

ISMN, isosorbide-5-mononitrate; GTN, glycerol trinitrate; SEP, spinal evoked potential. *SEP disappearance time in *group 3* different from SEP disappearance time in *group 2* ($P < 0.05$). †SEP disappearance time in *group 5* different from SEP disappearance time in *group* 1 ($P < 0.05$) and *group 2* ($P < 0.01$). ‡Survival time in *group 5* different from survival time in *group 2*.

rats died within a period of 23–94 min post-decompression, whereas 2 rats survived the observation period. The mean survival time for the whole group was 62 min (SD ±32) with a median range of 50 min (23–120 min). Before the pressure exposure, MAP was stable with a mean of 167.9 mmHg (SD ±20.9). The ISMN injection resulted in an abrupt drop in MAP of 50–60% followed by a tendency to increase. The MAP was restored to the initial baseline after 8–12 min and then remained stable during the compression phase. After decompression from the hyperbaric exposure, the conduction was immediately lost in one nerve, and the mean SEP disappearance time was 42.3 min (SD ±35.2) with a median range of 33 min (0–120 min). The mean MAP at SEP disappearance was 73.7 mmHg (SD ±19.5). All of the 10 rats dying and the 1 rat surviving the observation period had ample intravascular gas formation, whereas no intravascular gas was observed in 1 of the 2 rats surviving. See Fig. 2 for SEP example.

Group 3. The group administered 10 mg/kg ip GTN 30 min before compression (N = 8 rats, n = 16 nerves) had a mean weight of 301.1 g (SD ±19.2); see Table 1. All rats died within a period of 17–93 min post-decompression with a median survival time of 27 min and a mean survival time of 40.5 min (SD ±27.2). Before administration of GTN, MAP was stable with a mean of 156.2 mmHg (SD ±24.3). The GTN injection

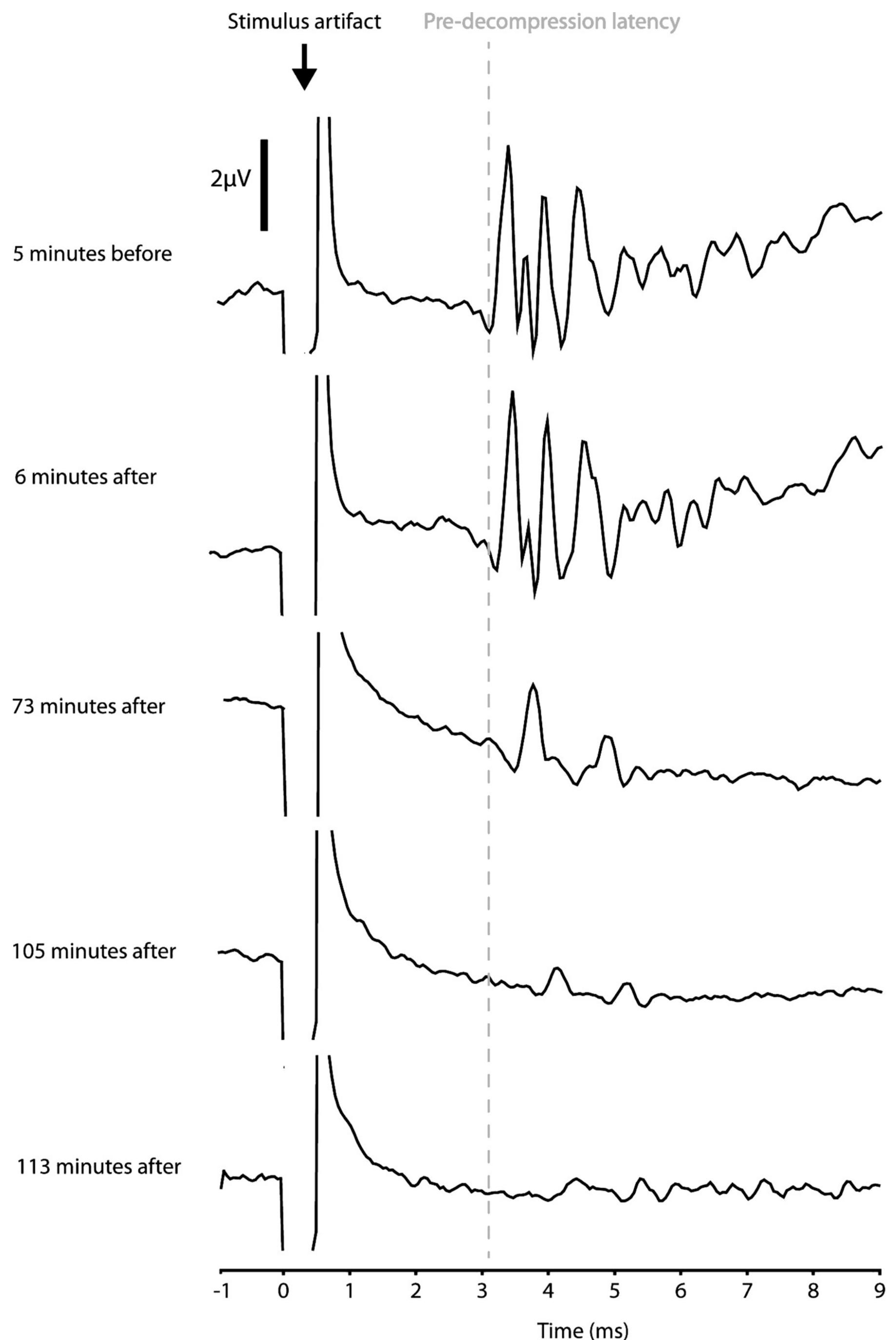

Fig. 2. Examples of SEPs. Illustrated are SEPs for one rat from *group 2*, with ISMN administered at sea level before the hyperbaric exposure. SEPs are demonstrated before and at various time intervals (as indicated) after the dive. Stimulus artifacts have been truncated.

resulted in an abrupt drop in MAP of 30–60%, with a subsequent tendency to fluctuate around the reduced baseline for up to 1.5 h, followed by a tendency for a slow increase during the decompression phase. After decompression from the hyperbaric exposure, the conduction was immediately lost in one nerve, and the mean SEP disappearance time was 18.9 min (SD ±13.6) with a median range of 12.5 min (0–53 min). The mean MAP at SEP disappearance was 90.6 mmHg (SD ±12.8). All of the eight rats had ample intravascular gas formation.

Group 4. The group administered 300 mg/kg iv ISMN 6 min before decompression ($N = 10$ rats, $n = 20$ nerves) had a mean weight of 305.8 g (SD ±17.5); see Table 1. In total, seven rats died within a period of 20–103 min post-decompression, whereas threee rats survived the observation period. The mean survival time for the whole group was 65.3 min (SD ±44.9) with a median range of 49 min (20–120 min). Before the pressure exposure, the MAP was stable with a mean of 165.5 mmHg (SD ±16.9). Subsequent to the ISMN injection, the MAP had an abrupt drop of 40–60% in 8–10 min. Once decompressed, the MAP was unstable but restored to the initial baseline in most cases. Following the decompression, conduction was immediately lost in one nerve, and the mean SEP disappearance time was 42.1 min (SD ±41.2) with a median range of 23 min (0–120 min). The mean MAP at SEP disappearance was 82.7 mmHg (SD ±17.9). All of the seven rats dying and one of the three rats that survived the observation period had ample intravascular gas formation, while no intravascular gas was found in remaining two rats that survived.

Group 5. The group administered 1 mg/kg iv GTN 8-3 min before decompression ($N = 8$ rats, $n = 16$ nerves) had a mean weight of 304.2 g (SD ±20); see Table 1. All rats died within a period of 9–67 min post-decompression with a median of 19 min and mean survival time of 27.9 min (SD ±19.1). Before the pressure exposure, the MAP was stable with a mean of 151.9 mmHg (SD ±28.2). The GTN injection caused an abrupt fall in the MAP of 20–50% for 8–10 min. In most rats, the MAP was restored to the initial baseline upon decompression but with a fluctuating tendency to be unstable. Following the decompression, conduction was immediately lost in four nerves, and the mean SEP disappearance time was 13.7 min (SD ±12.5) with a median range of 12 min (0–43 min), and the mean MAP at SEP disappearance was 86.6 mmHg (SD ±21.8). All of the eight rats had ample intravascular gas formation.

Group 6. The group administered 75 μg/kg iv GTN 4 min before decompression ($N = 8$ rats, $n = 14$ nerves) had a mean weight of 306 g (SD ±24.5); see Table 1. In total, six rats died within a period of 20–43 min post-decompression, whereas two rats survived the observation period. The mean survival time for the whole group was 51 min (SD ±43.3) with a median range of 31 min (20–120 min). Before the pressure exposure, the MAP was stable with a mean of 151.9 mmHg (SD ±26.2). The GTN injection resulted in a transient drop in the MAP of 40–50% for 3–5 min, after which the MAP was restored to the initial baseline but with a tendency for a fluctuating instability. Following the decompression, conduction was immediately lost in one nerve, and the mean SEP disappearance time was 32.6 min (SD ±39.2) with a median range of 12 min (0–120 min), and the mean MAP at SEP disappearance was 104.4 mmHg (SD ±29.5). All of the six rats that died and the two rats that survived the observation period had ample intravascular gas formation.

Comparability of the Experimental Groups

A Kruskal-Wallis test followed by multiple-comparisons tests (Dunn's) showed no significant difference between the groups with respect to either weight or MAP before the hyperbaric exposure.

Comparison of SEP Disappearance Time and Survival Time

A Kruskal-Wallis test followed by a multiple-comparisons test (Dunn's) among the groups showed that SEPs disappeared significantly faster in *group 5* compared with both *group 1* ($P < 0.05$) and *group 2* ($P < 0.01$), whereas SEPs in *group 3* disappeared significantly faster than those in *group 2* ($P < 0.05$). There was no difference in SEP disappearance times between the remaining groups. The survival time was significantly shorter in *group 5* than *group 2*, whereas there was no difference in survival time among the rest of the groups.

Comparison of Death and Survival

A Fisher's exact test showed that the number of rats surviving was not significant different between groups.

Histological Examination of the Spinal Cord

Occasional perfusion/fixation artifacts were visible throughout the spinal cord as clearly demarcated light areas. At one level in the cervical columna spinalis in the ventral and partly lateral funiculi, some white matter lesions were seen; see Fig. 3*A*. The illustrated lesion was poorly stained and had ill-defined margins. There appeared to be edema of the tissue disrupting the normal architecture. The myelin membranes were thinned and split up, and axons were lying unmyelinated; see Fig. 3*B*. There was no clearly visible vessel disruption or bleeding in the area.

DISCUSSION

In the present study, we found that a short- or long-acting NO donor administrated before a hyperbaric air dive or during the dive before decompression to surface showed no protective effect on spinal cord conduction or the survival rate in rats with DCS. This result stands in contrast to previous reports, in which NO releasing agents in experimental settings in different mammalian species have been shown to significantly reduce intravascular bubble formation and increase survival rate during DCS from diving. These reports include a short-acting NO donor, GNT (nitroglycerine), decreasing the intravascular gas formation in pigs decompressed from a saturation dive (28) and humans decompressed from both open-water and simulated hyperbaric air dive (8), as well as a long-acting NO donor, ISMN, increasing the survival rate and reducing intravascular bubble formation in rats exposed to an otherwise fatal bout of decompression (38). The beneficial effect of NO has been opposed by administration of a nonselective inhibitor of NO synthase, increasing intravascular bubble formation and turning a dive from safe to unsafe (6, 39).

Nitrates are known to cause venous dilation with sequestration of blood from the central arterial circulation to the capacitance bed, thereby reducing cardiac preload (1, 2, 16, 23) and

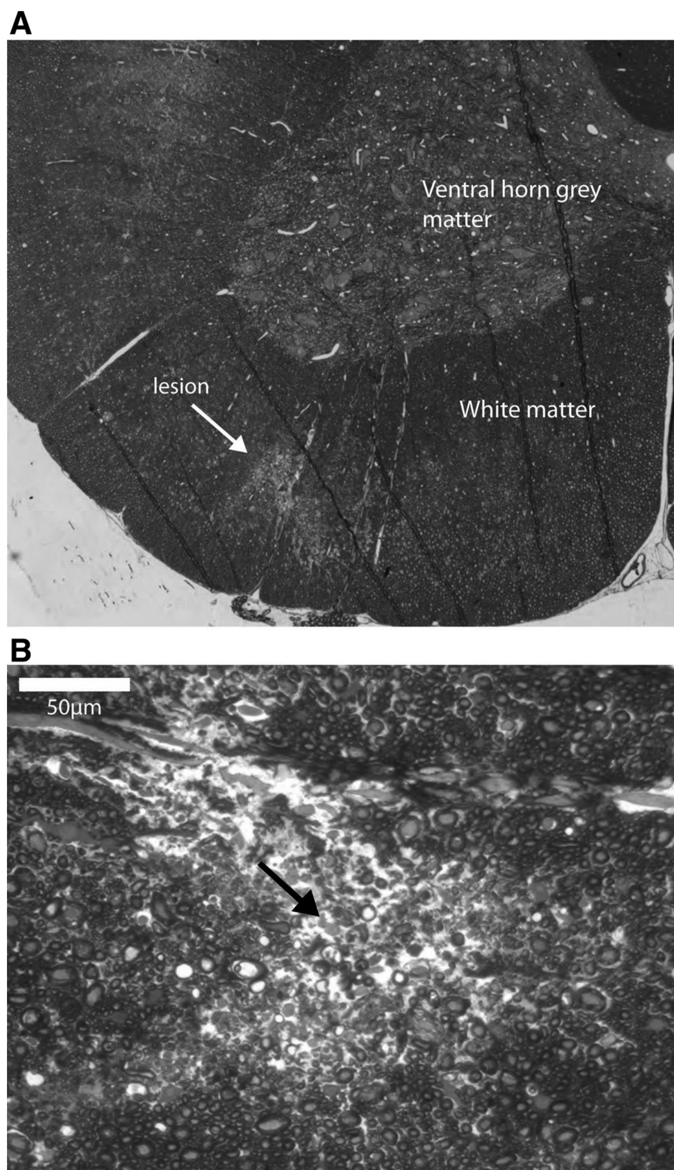

Fig. 3. *A*: Toludine blue staining of lesions. Toludine blue staining of a 1-µm-thick transverse cervical section of the spinal cord of one rat from *group 6* who received GTN (75 µg/kg) 4 min before decompression (magnification ×25) is shown. The arrow indicates a lesion. *B*: same section as above but at higher magnification (×40). The arrow indicates an example of unmyelinated axons.

increasing the peripheral blood flow rate in the extremities (24, 25, 27). Whereas the venous vessels are maximally dilated with relatively small doses of nitrates, the arterioles or resistance vessels dilate with high amounts of nitrates (1, 2), with GTN influencing the systemic vascular resistance far more potently than ISMN (26). So far, the optimal treatment drug (short- vs. long-acting NO donor), the optimal dosage, the optimal time of delivery before the decompression induced insult, and the exact physiological mechanism responsible for the therapeutic effect during DCS are unknown and remain to be established. It has been suggested that NO induces alterations in the hydrophobicity of the endothelial wall, which reduces the stability and density of the nuclei precursors adhering to the surface (8, 28, 38, 39), causing less gas to evolve as bubbles. Furthermore, it has also been suggested that NO, through augmented blood flow, may increase the N_2 washout and thereby promote bubble shrinkage (28); however, as discussed by Moon (29) and Wisloff et al. (39), GTN has a very short half-time, and, since reduced flow during decompression appears of minor importance, the first hypothesis seems more attractive (29, 39).

Whether NO donors offer protection against DCS in the spinal cord has not been reported. We hypothesize that NO donors show a protective effect on spinal cord conduction in cases where NO donors reduce the intravascular bubble formation through the elimination of micronuclei precursors and in cases where the arterial emboli (11), venous infarction (17), or complement activation (9, 37) are the primary mechanism responsible for DCS in CNS. However, if autochthonous bubbles (12, 18, 33) are the primary cause for DCS in CNS, then removal of intravascular nuclei precursors may increase the survival rate, whereas the neurological injuries may persist. On the other hand, if NO increases the N_2 washout through augmented blood flow rate, the time of administration may be a decisive factor for survival and neurological detriment, due to the short interval of hemodynamic changes caused by nitrates (35).

In the present experiments, a hemodynamic alteration as a possible mechanism for preventing DCS in CNS was tested by administration of both ISMN in *group 4* and GTN in *groups 5* and *6* during the dive. If increased blood flow enhances the N_2 elimination during the decompression phase, it seems reasonable to assume that a similar uptake of N_2 ought to commensurate during the preceding dive. This has been discussed in a recent report by Blatteau et al. (5), who found that a potent vasodilator, sildenafil (Viagra), administered before a dive, significantly increased the manifestations of neurological DCS in a rat model, an effect the authors ascribe to enhanced cerebral blood flow and subsequent augmented inert-gas load during the hyperbaric exposure. Therefore, to promote the beneficial effect, NO donors should be dispensed just before decompression. In a previous report by Mollerlokken et al. (28) of DCS in a swine saturation model, it was found that GTN significantly decreased the intravascular bubble formation when given at a dosage twice that recommended in humans during a dive to 500 kPa (40 msw), subsequent to a linear decompression phase of 2 h. Accordingly, in *groups 4–6*, the NO donors were administered 3–8 min before initiation of decompression and at a high dose that would inflict a hemodynamic impact during the decompression procedure (26, 35). However, compared with the control group, NO donors in *groups 4–6* showed no protective effect on survival rate or spinal cord conduction when administered immediately before decompression. On the contrary, administration of a high dose of GTN in *group 5* resulted in significantly faster SEP disappearance, and recipients showed a tendency toward shorter survival times than the control group, while a lower dose of GTN in *group 6* also caused a faster SEP disappearance, although this was not significantly different. Furthermore, administration of ISMN in *group 4* had no detrimental effect on SEP disappearance or survival time compared with the control group, despite the fact that the dose and method of administration for ISMN is causing a peak pulse pressure effect after 10.5 ± 4.7 min (35).

In the saturation swine model by Mollerlokken et al. (28), the GTN infusion significantly elevated the heart rate and reduced MAP, although it could not be determined whether the

protective effect of GTN was attributed to hemodynamic changes or to removal of micronuclei precursors. Similarly, in the present experiment, it cannot be excluded that NO donors administered before decompression could initiate a demise of preexisting gas nuclei. However, if that is the case, it seems evident that removal of nuclei requires a therapeutic window exceeding the present interval from NO donor administration to the decompression-induced insult in *groups 4–6*. Since the hemodynamic effect of NO donors wears off within minutes (26, 35) when administered at a clinical dose and since the regeneration time for a depleted nuclei population is 10–100 h (40), any protective effect of NO donors when administered before a dive advocates for a decrease in nuclei density as the predominant factor rather than conditioning by flow limitations. In a previous report by Dujic et al. (8), divers received 0.4 mg GTN by oral spray 30 min before both a 30-min open water dive to 30 msw and a 80-min hyperbaric air dive to 18 msw, followed by a decompression phase of, respectively, 6 and 9 min. Furthermore, in a previous report by Wisloff et al. (38), rats were administered 65 mg/kg ISMN by gastric intubation 20 h or 30 min before a 45-min hyperbaric air dive to 700 kPa, after which they were decompressed to surface in 12 min. In both divers and rats, NO donors significantly decreased the intravascular bubble formation as well as increased the survival rate in rats, an effect ascribed to a possible reduction of preexisting gas nuclei. In the present experiment, we replicated these intervals from the administration of NO donors to the decompression-induced impact, hence *groups 2* and *3* were administered ISMN and GTN, respectively, 5–10 and 30 min before compression. Nonetheless, just like *groups 4–6*, administration of GTN showed a tendency toward shorter survival and a faster SEP disappearance, although dispensed at a high dose, while administration of ISMN had no detrimental effect on survival time or spinal cord conduction despite a half-time for ISMN of 268 $\pm$ 40 min (35).

In the present experiment, it could be speculated that the absence of a protective effect of NO donors could be ascribed to the fast ascent rate used in the decompression profile causing a DCS impact overriding the therapeutic effect. This could explain why administration of GTN, combined with a protracted decompression, reduced the intravascular bubble formation in the saturation swine model (28). However, in the previous report by Wisloff et al. (38), rats of similar weight in control groups VI and VII (310 $\pm$ 7 and 308 $\pm$ 6 g, respectively; data from Ref. 38) were decompressed in 12 min, resulting in a median survival range of 27 (2–39) and 19 (8–60) min (groups VI and VII in Ref. 38). Since rats in the control *group 1* and SEP-control *experiment C* (rats breathing spontaneously without being connected to a respirator) of the present experiment survived with a median range of, respectively, 44 (17–120) and 60 (2–120) min post-decompression, it appears that the present decompression profile used is less harsh than the profile used by Wisloff et al. (38).

The specific physiological mechanism responsible for the discrepancy of survival found in Ref. 38 and the present set of experiments is speculative, and it seems premature to provide a clear explanation. However, considering the slow ascent rate of 5 m/min in Ref. 38 compared with the much faster ascent rate used in the present experiment of 30 and 18 m/min, the ascent rate per se during decompression may be crucial for the effect of NO donors on DCS prevention. In keeping with the tendency to reduce survival time and enhance neurological deterioration upon infusion of a high dose of GTN in *group 5* (1 mg/kg iv GTN; infusion started at depth 8 min and terminated 3 min before decompression) it cannot be excluded that enhanced blood flow by NO donors may augment the inert gas uptake before decompression, thereby increasing the risk of injury as it seems to have been demonstrated by Blatteau et al. (5). Accordingly, since NO donors showed no therapeutic effect in any of the experimental groups and even caused a poorer outcome in *group 5*, administration of NO donors before emergency decompression procedures, such as during submarine escapes, seems contraindicated.

Histological evidence of ischemia in the spinal cord takes 30–60 min to develop from the onset of the ischemic insult (11). Therefore, the present experiments do not allow for a histological evaluation of the mechanisms underlying spinal cord DCS, although lesions were observed in one animal. However, as previous reports have demonstrated, there may not be any correlation between the extent of observable lesions in the spinal cord compared with the functionality, as evaluated by SEPs during DCS (7, 20, 32).

In conclusion, we found no protective effect of a short- or long-acting NO donor during DCS upon a provocative dive with a fast ascent rate, regardless of dose and the interval from administration to the decompression-induced insult. Accordingly, the results do not indicate that NO donors constitute beneficial properties as a consequence of nuclei demise or hemodynamic alterations, an observation we assume to be caused by the fast ascent rate during decompression, causing a DCS impact overriding the therapeutic effect of NO donors. On the contrary, we found that a high dose of GTN administered at depth before decompression significantly increased the manifestations of spinal DCS, presumably caused by enhanced blood flow and thereby increased inert-gas uptake. Further investigations are necessary to establish the optimal dose and time of delivery of NO donors for survival rate and spinal cord conductivity during different decompression profiles, as well as providing a deeper insight into possible adverse effects of NO donors during DCS.

ACKNOWLEDGMENTS

Thanks are given to Professor Hans Hultborn for setting up the SEP experiments, without which this work would have been impossible. The assistance of senior Hyperbaric Supervisor Michael Bering Sifakis with chamber support and maintenance is greatly appreciated.

GRANTS

The Lundbeck Foundation, The Laerdal Foundation for Acute Medicine, and Rigshospitalets Forskningsudvalg supported the present work.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.R. performed experiments; T.R., C.F.M., H.B., and O.H. analyzed data; T.R., H.B., and O.H. interpreted results of experiments; T.R., C.F.M., and H.B. prepared figures; T.R. and O.H. drafted manuscript; T.R., C.F.M., and O.H. edited and revised manuscript; T.R., C.F.M., H.B., and O.H. approved final version of manuscript; O.H. conception and design of research.

REFERENCES

1. **Abrams J.** Hemodynamic effects of nitroglycerin and long-acting nitrates. *Am Heart J* 110: 216–224, 1985.

2. **Abrams J.** Nitroglycerin and long-acting nitrates. *N Engl J Med* 302: 1234–1237, 1980.
3. **Altman DG.** *Practical Statistics for Medical Research*. London: Chapman and Hall, 1991, p. xii, 611.
4. **Armitage P, Berry G, Matthews JNS.** *Statistical Methods in Medical Research*. Malden, MA: Blackwell Science, 2001, p. xi, 817.
5. **Blatteau JE, Brubakk AO, Gempp E, Castagna O, Risso JJ, Vallee N.** Sidenafil pretreatment promotes decompression sickness in rats. *PLos One* 8: e60639, 2013.
6. **Bondi M, Cavaggioni A, Michieli P, Schiavon M, Travain G.** Delayed effect of nitric oxide synthase inhibition on the survival of rats after acute decompression. *Undersea Hyperb Med* 32: 121–128, 2005.
7. **Calder IM.** The pathology of spinal cord decompression sickness. In: *Describing Decompression Sickness*. Hampshire, UK: Institute for Naval Medicine, 1990, p. 29–45.
8. **Dujic Z, Palada I, Valic Z, Duplancic D, Obad A, Wisloff U, Brubakk AO.** Exogenous nitric oxide and bubble formation in divers. *Med Sci Sports Exerc* 38: 1432–1435, 2006.
9. **Ersson A, Linder C, Ohlsson K, Ekholm A.** Cytokine response after acute hyperbaric exposure in the rat. *Undersea Hyperb Med* 25: 217–221, 1998.
10. **Evans A, Walder DN.** Significance of gas micronuclei in the aetiology of decompression sickness. *Nature* 222: 251–252, 1969.
11. **Francis TJ, Pezeshkpour GH, Dutka AJ.** Arterial gas embolism as a pathophysiologic mechanism for spinal cord decompression sickness. *Undersea Biomed Res* 16: 439–451, 1989.
12. **Francis TJ, Pezeshkpour GH, Dutka AJ, Hallenbeck JM, Flynn ET.** Is there a role for the autochthonous bubble in the pathogenesis of spinal cord decompression sickness? *J Neuropathol Exp Neurol* 47: 475–487, 1988.
13. **Francis TJR, Griffin JL, Homer LD, Pezeshkpour GH, Dutka AJ, Flynn ET.** Bubble-induced dysfunction in acute spinal cord decompression sickness. *J Appl Physiol* 68: 1368–1375, 1990.
14. **Gempp E, Blatteau JE.** Risk factors and treatment outcome in scuba divers with spinal cord decompression sickness. *J Crit Care* 25: 236–242, 2010.
15. **Graph-Pad.** *Instat version 3.06 for Windows*. San Diego, CA: Graph-Pad Software, 2003.
16. **Haber HL, Simek CL, Bergin JD, Sadun A, Gimple LW, Powers ER, Feldman MD.** Bolus intravenous nitroglycerin predominantly reduces afterload in patients with excessive arterial elastance. *J Am Coll Cardiol* 22: 251–257, 1993.
17. **Hallenbeck JM.** Cinephotomicrography of dog spinal vessels during cord-damaging decompression sickness. *Neurology* 26: 190–199, 1976.
18. **Hills BA, James PB.** Spinal decompression sickness: mechanical studies and a model. *Undersea Biomed Res* 9: 185–201, 1982.
19. **Hyldegaard O, Madsen J.** Influence of heliox, oxygen, and N_2O-O_2 breathing on N_2 bubbles in adipose tissue. *Undersea Biomed Res* 16: 185–193, 1989.
20. **Hyldegaard O, Moller M, Madsen J.** Protective effect of oxygen and heliox breathing during development of spinal decompression sickness. *Undersea Hyperb Med* 21: 115–128, 1994.
21. **James T, Francis R, Mitchell J.** Manifestations of decompression disorders. In: *Bennett and Elliot's Physiology and Medicine of Diving* (5th ed.), edited by **Brubakk AO and Neumann TS. Edinburgh, UK: Saunders, 2003, p. 578–599.**
22. **James T, Francis R, Mitchell J.** Pathophysiology of decompression sickness. In: *Bennett and Elliot's Physiology and Medicine of Diving* (5th ed.), edited by **Brubakk AO and Neuman TS. Edinburgh, UK: Saunders, 2003, p. 530–556.**
23. **Kirsten R, Nelson K, Kirsten D, Heintz B.** Clinical pharmacokinetics of vasodilators. Part II. *Clin Pharmacokinet* 35: 9–36, 1998.
24. **Leier CB, Magorien RD, Desch CE, Thompson MJ, Unverferth DV.** Hydralazine and isosorbide dinitrate: comparative central and regional hemodynamic effects when administered alone or in combination. *Circulation* 63: 102–109, 1981.
25. **Loos D, Schneider R, Schorner W.** Changes in regional body blood volume caused by nitroglycerin. *Z Kardiol* 72, *Suppl* 3: 29–32, 1983.
26. **Manabe T, Yamamoto A, Satoh K, Ichihara K.** Tolerance to nitroglycerin induced by isosorbide-5-mononitrate infusion in vivo. *Biol Pharm Bull* 24: 1370–1372, 2001.
27. **Mason DT, Braunwald E.** The effects of nitroglycerin and amyl nitrite on arteriolar and venous tone in the human forearm. *Circulation* 32: 755–766, 1965.
28. **Mollerlokken A, Berge VJ, Jorgensen A, Wisloff U, Brubakk AO.** Effect of a short-acting NO donor on bubble formation from a saturation dive in pigs. *J Appl Physiol* 101: 1541–1545, 2006.
29. **Moon RE.** Nitroglycerine: relief from the heartache of decompression sickness? *J Appl Physiol* 101: 1537–1538, 2006.
30. **Pekna M, Ersson A.** Complement system response to decompression. *Undersea Hyperb Med* 23: 31–34, 1996.
31. **Pontier JM, Vallée N, Bourdon L.** Bubble-induced platelet aggregation in a rat model of decompression sickness. *J Appl Physiol* 107: 1825–1829, 2009.
32. **Zhang RJ, Liu K, Kang ZM, Fan DF, Ni XX, Liu Y, Lian QL, Sun XJ, Tao HY, Xu WG.** Combined effects of intravenous perfluorocarbon emulsion and oxygen breathing on decompression-induced spinal cord injury in rats. *Undersea Hyperb Med* 38: 335–343, 2011.
33. **Sykes JJ, Yaffe LJ.** Light and electron microscopic alterations in spinal cord myelin sheaths after decompression sickness. *Undersea Biomed Res* 12: 251–258, 1985.
34. **Tikuisis P, Gerth WA.** Decompression theory. In: *Bennett and Eliott's Physiology and Medicine of Diving* (5th ed.), edited by **Brubakk AO and Neuman TS. Edinburgh, UK: Saunders, 2003, p. 419–454.**
35. **Tzeng TB, Fung HL.** Pharmacokinetic/pharmacodynamic relationship of the duration of vasodilating action of organic mononitrates in rats. *J Pharmacol Exp Ther* 261: 692–700, 1992.
36. **Vann RD, Grimstad J, Nielsen CH.** Evidence for gas nuclei in decompressed rats. *Undersea Biomed Res* 7: 107–112, 1980.
37. **Ward CA, McCullough D, Yee D, Stanga D, Fraser WD.** Complement activation involvement in decompression sickness of rabbits. *Undersea Biomed Res* 17: 51–66, 1990.
38. **Wisloff U, Richardson RS, Brubakk AO.** Exercise and nitric oxide prevent bubble formation: a novel approach to the prevention of decompression sickness? *J Physiol* 555: 825–829, 2004.
39. **Wisloff U, Richardson RS, Brubakk AO.** NOS inhibition increases bubble formation and reduces survival in sedentary but not exercised rats. *J Physiol* 546: 577–582, 2003.
40. **Yount D, Strauss R.** On the evolution, generation and regeneration of gas cavitation nuclei. *J Acoust Soc Am* 65: 1431–1439, 1982.