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Effect of Nitric Oxide on Spinal Evoked Potentials and Survival Rate in Rats with Decompression Sickness

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ABSTRACT

Introduction: 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 pre-existing micronuclei or an increased nitrogen washout upon decompression through augmented blood flow rate. The present experiments were conducted in order to investigate whether a short- or long acting NO donor [glycerol trinitrate (GTN); isosorbide-5-mononitrate (ISMN), respectively] would offer the same protection against spinal cord DCS evaluated by means of spinal evoked potentials (SEPs). **Methods:** Anesthetized rats were decompressed from a 1-h hyperbaric air dive at 506.6 kPa (40 meter of seawater) for 3 minutes and 17 seconds 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 3 received either saline (group 1), ISMN 300 mg/kg i.v. (group 2) or GTN 10 mg/kg i.p. (group 3) before compression. The last 3 received either ISMN 300 mg/kg i.v. (group 4), GTN 1 mg/kg i.v. (group 5) or GTN 75 µg/kg i.v. (group 6) during the dive, prior to decompression. **Results:** In all groups decompression caused considerable intravascular bubble formation. The ISMN groups showed no difference when compared to the control group, while the GTN groups showed a tendency towards faster SEP disappearance and shorter survival times. **Conclusion:** Neither a short or 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

Glossary

CNS	Central nervous system
CO_2	Carbon dioxide
DCS	Decompression sickness
ECG	Electrocardiogram
GTN	Glycerol trinitrate - nitroglycerine
h	Hour
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
ISMN	Isosorbide-5- mononitrate
MAP	Mean arterial blood pressure
msw	Meter of seawater
N	Number of rats
n	Number of nerves
N_2	Nitrogen
NO	Nitric oxide
O_2	Oxygen
s.c.	Subcutaneous
SEPs	Spinal evoked potentials

INTRODUCTION

During diving, hyperbaric air breathing will cause the inert gas of nitrogen (N_2) to dissolve in blood and tissue according to Henrys 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 pre-existing 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 pro-thrombotic 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 micro-nuclei 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 donor's cause immediate hemodynamic changes (26, 35), administration of an NO donor before decompression should – everything else 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, glycerol trinitrate [nitroglycerine (GNT)] and a long acting NO donor, isosorbid-5-monitrate (ISMN) on survival rate and spinal cord conduction during DCS in rats by measurements of spinal evoked potentials (SEPs). Accordingly, rats were exposed to a hyperbaric air breathing dive at 506.6 kPa (40 meter of seawater, msw) for 60 minutes with a decompression phase of 3 minutes and 17 seconds. The diving protocol and decompression profile were conducted in order to cause intravascular bubble formation with spinal DCS and a lethal outcome in most non-treated animals' 30-60 minutes 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 kPa 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 EU 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 270-340g were anaesthetized with sodium thiomebumal i.p. (0.1 g/kg) and analgesia was provided by administration of buphrenorphine s.c. (0.01-0.05 mg/kg). The anaesthetized rat was placed 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-ID 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 non-heparinized 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 minutes, or until fatal DCS. For the precise sequence of events; see Figure 1 – Experimental Protocol.

Figure 1 here

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 ID 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 i.m. 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 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 kPa to 101.3 kPa (sea level) in three stages with two stops: 1) decompression from 506.5 kPa to 202.6 kPa (10 msw) in 1 minutes (30 m/minute) with a stop at 202.6 kPa for 1 minute; 2) decompression from 202.6 kPa to 152 kPa (5 msw) in 17 seconds (18 m/minute) with a stop at 152 kPa for 43 seconds; 3) decompression from 152 kPa to 101.3 kPa in 17 seconds (18 m/minute). Total decompression time was 3 minutes and 17 seconds; see Figure 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 experiment A-C*, and a group perfusion fixed for histological examinations of the spinal cord; see description of *SEP-control experiment A-C* and *histological examination* below.

For the experimental groups 1-6, rats (N = 58) were divided into groups receiving isosorbide-5-mononitrate [ISMN (Dottikon, Switzerland)] and glycerol trinitrate [GTN (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/minute 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/minute for group 2, 4 and 6, 0.2 ml/minute for group 5 (total infusion time of 5

minutes), 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:

Group 1, (N = 12). Control group; saline i.v. injection initiated 10 minutes before compression.
Group 2, (N = 12). ISMN 300 mg/kg i.v. injection initiated 5-10 minutes before compression.
Group 3, (N = 8). GTN 10 mg/kg i.p. injection 30 minutes before compression.
Group 4, (N = 10). ISMN 300 mg/kg i.v. injection initiated 6 minutes before decompression.
Group 5, (N = 8). GTN 1 mg/kg i.v. injection initiated 8 minutes before decompression.
Group 6, (N = 8). GTN 75 µg/kg i.v. injection initiated 4 minutes before decompression.

Recording of SEPs

The cervical vertebral column was exposed by a dorsal midline incision and bore holes were drilled into the 2nd and 5th 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 2d and 5th 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 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 Limited). Signals were then digitized using a CED 1401 analogue to digital converter (Cambridge Electronic Design, Cambridge, UK) and sampled on a computer using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK), 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 in order 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 minute 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 minutes for 120 minutes or until cardiac arrest as measured by the ECG.

Validation of the SEP measurements

In order 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 was recorded for a mean period of 4 h and 37 minutes (SD ±11 minutes) 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 3 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 the SEP-control experiment C continued breathing air spontaneously through the chamber breathing system instead of getting paralyzed and connected to the respirator. In the SEP-control experiment C (N = 5 rats, n = 9 nerves), mean weight of 321.2 g (SD ±10), 4 rats died within a period of 2-62 minutes post decompression while 1 rat survived the observation period. Mean survival time for the whole group was 58 minutes (SD ± 37.8) mean SEP disappearance time was 45 minutes (SD ± 47.7). All of the 4 rats dying during the observation period had ample intravascular gas formation, while 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 in order 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 respectively; 1 rat each from the treatment group 3 and 5 respectively; 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 minutes after decompression except for one rat in group 3 that was fixed after 20 minutes 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 minutes 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 minutes. 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 minutes. 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 minutes. The mean MAP before the pressure exposure and the mean MAP at the time of the SEP disappearance was recorded in mmHg. 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 ±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 (KS) tests were performed followed by nonparametric analysis of variance ANOVAs (Kruskal-Wallis Test). The differences between the mean values of the individual treatment groups were then analyzed using Dunns 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 Fishers 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 post-mortem 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 9 animals surviving the observation period, 5 rats had intravascular gas formation, while no bubbles were visible in the veins of 4 rats.

Table 1 here

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 minutes (SD ± 35.4) with a median range of 44 minutes (17-120). 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 2 nerves and the mean SEP disappearance time was 43.7 minutes (SD ± 39.9) with a median range of 26 minutes (0-120). 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, while no intravascular gas was found in the two rats surviving.

Group 2. The group administered ISMN 300 mg/kg i.v. 5-10 minutes 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 rats died within a period of 23-94 minutes post decompression while 2 rats survived the observation period. The mean survival time for the whole group was 62 minutes (SD ± 32) with a median range of 50 minutes (23-120). 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 minutes and then remained stable during the compression phase. After decompression from the hyperbaric exposure, the conduction was immediately lost in 1 nerve and the mean SEP disappearance time was 42.3 minutes (SD ± 35.2) with a median range of 33 minutes (0-120). 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, while no intravascular gas was observed in 1 of the 2 rats surviving. See Figure 2 for SEP example.

Figure 2 here

Group 3. The group administered GTN 10 mg/kg i.p. 30 minutes 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 minutes post-decompression with a median survival time of 27 minutes and a mean survival time of 40.5 minutes (SD ± 27.2). Before administration of GTN, MAP was stable with a mean of 156.2 mmHg (SD ±24.3). The GTN injection resulted in an abrupt drop in MAP of 30-60%, with a subsequent tendency to fluctuate around the reduced baseline for up to 1½ 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 1 nerve and the mean SEP disappearance time was 18.9 minutes (SD ± 13.6) with a median range of 12.5 minutes (0-53). The mean MAP at SEP disappearance was 90.6 mmHg (SD ± 12.8). All of the 8 rats had ample intravascular gas formation.

Group 4. The group administered ISMN 300 mg/kg i.v. 6 minutes before decompression (N = 10 rats, n = 20 nerves) had a mean weight of 305.8 g (SD ± 17.5); see Table 1. In total 7 rats died within a period of 20-103 minutes post decompression while 3 rats survived the observation period. The mean survival time for the whole group was 65.3 minutes (SD ± 44.9) with a median range of 49 minutes (20-120). 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 minutes. Once decompressed, the MAP was unstable but restored to the initial baseline in most cases. Following the decompression, conduction was immediately lost in 1 nerve and the mean SEP disappearance time was 42.1 minutes (SD ± 41.2) with a median range of 23 minutes (0-120). The mean MAP at SEP disappearance was 82.7 mmHg (SD ± 17.9). All of the 7 rats dying and 1 of the 3 rats that survived the observation period had ample intravascular gas formation, while no intravascular gas was found in remaining 2 rats that survived.

Group 5. The group administered GTN 1 mg/kg i.v. 8-3 minutes 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 minutes post-decompression with a median of 19 minutes and mean survival time of 27.9 minutes (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 minutes. 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 4 nerves and the mean SEP disappearance time was 13.7 minutes (SD ± 12.5) with a median range of 12 minutes (0-43) and the mean MAP at SEP disappearance was 86.6 mmHg (SD ± 21.8). All of the 8 rats had ample intravascular gas formation.

Group 6. The group administered GTN 75 µg/kg i.v. 4 minutes before decompression (N = 8 rats, n = 14 nerves) had a mean weight of 306 g (SD ±24.5); see Table 1. In total, 6 rats died within a period of 20-43 minutes post-decompression while 2 rats survived the observation period. The mean survival time for the whole group was 51 minutes (SD ± 43.3) with a median range of 31 minutes (20-120). 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 minutes 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 1 nerve and the mean SEP disappearance time was 32.6 minutes (SD ± 39.2) with a median range of 12 minutes (0-120) and the mean MAP at SEP disappearance was 104.4 mmHg (SD ± 29.5). All of the 6 rats that died and the 2 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 (Dunns) 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 (Dunns) among the groups showed that SEPs disappeared significantly faster in group 5 when compared to both group 1 ($P < 0.05$) and group 2 ($P < 0.01$), while SEPs in group 3 disappeared significantly faster than 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, while there was no difference in survival time among the rest of the groups.

Comparison of death and survival

A Fishers 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 Figure 3a. 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 Figure 3b. There was no clearly visible vessel disruption or bleeding in the area.

414 **Figure 3a and 3b here**

415

416 ## DISCUSSION

417

418 In the present study we found, that a short or long acting NO donor administrated before a
419 hyperbaric air dive or during the dive before decompression to surface showed no protective effect on
420 spinal cord conduction or the survival rate in rats with DCS. This result stands in contrast to previous
421 reports, in which NO releasing agents in experimental settings in different mammalian species have
422 been shown to significantly reduce intravascular bubble formation and increase survival rate during
423 DCS from diving. These reports include a short acting NO donor, glycerol trinitrate [nitroglycerine
424 (GTN)], decreasing the intravascular gas formation in pigs decompressed from a saturation dive (28)
425 and humans decompressed from both open-water and simulated hyperbaric air dive (8) as well as a
426 long acting NO donor, isosorbide-5-mononitrate (ISMN) increasing the survival rate and reducing
427 intravascular bubble formation in rats exposed to an otherwise fatal bout of decompression (38). The
428 beneficial effect of NO has been opposed by administration of a nonselective inhibitor of NO synthase
429 (NOS), increasing intravascular bubble formation and turning a dive from safe to unsafe (6, 39).

430 Nitrates are known to cause venous dilation with sequestration of blood from the central arterial
431 circulation to the capacitance bed, thereby reducing cardiac preload (1, 2, 16, 23) and increasing the
432 peripheral blood flow rate in the extremities (24, 25, 27). Whereas the venous vessels are maximally
433 dilated with relatively small doses of nitrates, the arterioles or resistance vessels dilate with high
434 amounts of nitrates (1, 2), with GTN influencing the systemic vascular resistance far more potently
435 than ISMN (26). So far, the optimal treatment drug (short vs. long acting NO donor), the optimal
436 dosage, the optimal time of delivery prior to 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. Further, 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 Wisløff 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 micro nuclei 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 group 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.

459 This has been discussed in a recent report by Blatteau et al. (5) who found, that a potent vasodilator,
460 sildenafil (Viagra™), administered before a dive, significantly increased the manifestations of
461 neurological DCS in a rat model, an effect the authors ascribe to enhanced cerebral blood flow and
462 subsequent augmented inert gas load during the hyperbaric exposure. Therefore, in order to promote
463 the beneficial effect, NO donors should be dispensed just prior to decompression. In a previous report
464 by Møllerløkken et al. (28) of DCS in a swine saturation model, it was found, that GTN significantly
465 decreased the intravascular bubble formation when given at a dosage twice that recommended in
466 humans during a dive to 500 kPa (40 msw) subsequent to a linear decompression phase of 2 h.
467 Accordingly, in group 4-6 the NO donors were administered 3-8 min before initiation of
468 decompression and at a high dose that would inflict a hemodynamic impact during the decompression
469 procedure (26, 35). However, when compared to the control group, NO donors in group 4-6 showed no
470 protective effect on survival rate or spinal cord conduction when administered immediately before
471 decompression. On the contrary, administration of a high dose of GTN in group 5 resulted in
472 significantly faster SEP disappearance and recipients showed a tendency towards shorter survival times
473 than the control group while a lower dose of GTN in group 6 also caused a faster SEP disappearance,
474 although this was not significantly different. Further, administration of ISMN in group 4 had no
475 detrimental effect on SEP disappearance or survival time when compared to the control group, despite
476 the fact that that the dose and method of administration for ISMN is causing a peak pulse pressure
477 effect after 10.5 min ± 4.7 (35).
478 In the saturation swine model by Møllerløkken et al. (28), the GTN infusion significantly
479 elevated the heart rate and reduced MAP, although, it could not be determined, whether the protective
480 effect of GTN was attributed to hemodynamic changes or to removal of micronuclei precursors.
481 Similarly in the present experiment, it cannot be excluded, that NO donors administered prior to

decompression could initiate a demise of pre-existing 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 group 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 prior to 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. Further, in a previous report by Wisløff et al. (38), rats were administered ISMN 65 mg/kg by gastric intubation 20 h or 30 min prior to 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 pre-existing gas nuclei. In the present experiment, we replicated these intervals from the administration of NO donors to the decompression induced impact, hence group 2 and 3 were administered ISMN and GTN respectively 5-10 and 30 min before compression. Nonetheless, just like group 4-6, administration of GTN showed a tendency towards 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 $T_{½}$ for ISMN of 268 ± 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

505 (28). However, in the previous report by Wisløff et al. (38), rats of similar weight in control group VI
506 and VII (310 ± 7 g and 308 ± 6 g; data from (38)) were decompressed in 12 min resulting in a median
507 survival range of 27 (2-39) and 19 (8-60) minutes (group VI and VII in (38)). Since rats in the control
508 group 1 and *SEP-control experiment C* (rats breathing spontaneously without being connected to a
509 respirator) of the present experiment survived with a median range of respectively 44 (17-120) and 60
510 (2-120) minutes post decompression, it appears that the present decompression profile used is less
511 harsh than the profile used by Wisløff et al. (38).

512 The specific physiological mechanism responsible for the discrepancy of survival found
513 in (38) and the present set of experiments is speculative and it seems premature to provide a clear
514 explanation. However, considering the slow ascent rate of 5 m/minute in (38) as compared to the much
515 faster ascent rate used in the present experiment of 30 and18 m/minute, the ascent rate per se during
516 decompression may be crucial for the effect of NO donors on DCS prevention. In keeping with the
517 tendency to reduce survival time and enhance neurologic deterioration upon infusion of a high dose of
518 GTN in group 5 (GTN 1 mg/kg i.v; infusion started at depth 8 minutes and terminated 3 minutes before
519 decompression) it cannot be excluded, that enhanced blood flow by NO donors may augment the inert
520 gas uptake prior to decompression thereby increasing the risk of injury as it seems to have been
521 demonstrated by Blatteau et al. (5). Accordingly, since NO donors showed no therapeutic effect in any
522 of the experimental groups and even caused a poorer outcome in group 5, administration of NO donors
523 prior to emergency decompression procedures such as during submarine escapes seems
524 contraindicated.

525 Histological evidence of ischemia in the spinal cord takes 30-60 min to develop from the onset
526 of the ischemic insult (11). Therefore the present experiments do not allow for a histological evaluation
527 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 to 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 prior to 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.

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LEGENDS:

Table 1: The effect of ISMN and GTN administration before and during a hyperbaric exposure on spinal cord conductivity and survival in rats with DCS

[1]) SEP disappearance time in group 3 different from SEP disappearance time in group 2 ($P < 0.05$). [2]) SEP disappearance time in group 5 different from SEP disappearance time in group 1 ($P < 0.05$) and group 2 ($P < 0.01$). [3]) Survival time in group 5 different from survival time in group 2.

Figure 1: Experimental protocol

Anaesthetized rats were exposed to a 1-h hyperbaric air dive to 506.6 kPa (40 meter of seawater) and decompressed to 101.3 kPa (sea level) in 3 minutes and 17 seconds 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) ore isosorbide-5-mononitrate (ISMN) at sea level before the dive (group 2 and 3) or during the compression phase (group 4-6).

Figure 2: Examples of SEPs

Illustrated are spinal evoked potentials for one rat from group 2, with ISMN administered at sea level prior to the hyperbaric exposure. Spinal evoked potentials are demonstrated before, and at various time intervals (as indicated) after the dive. Stimulus artifacts have been truncated.

Figure 3: Toludine blue staining of lesions

A. Toludine blue staining of a 1-µm-thick transverse cervical section of the spinal cord of one rat from group 6 who received NTG (75µg/kg) 4 minutes before decompression (magnification x 25). The arrow indicates a lesion.

B. Same section as above but at higher magnification (x 40). The arrow indicates an example of unmyelinated axons.

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Table 1:

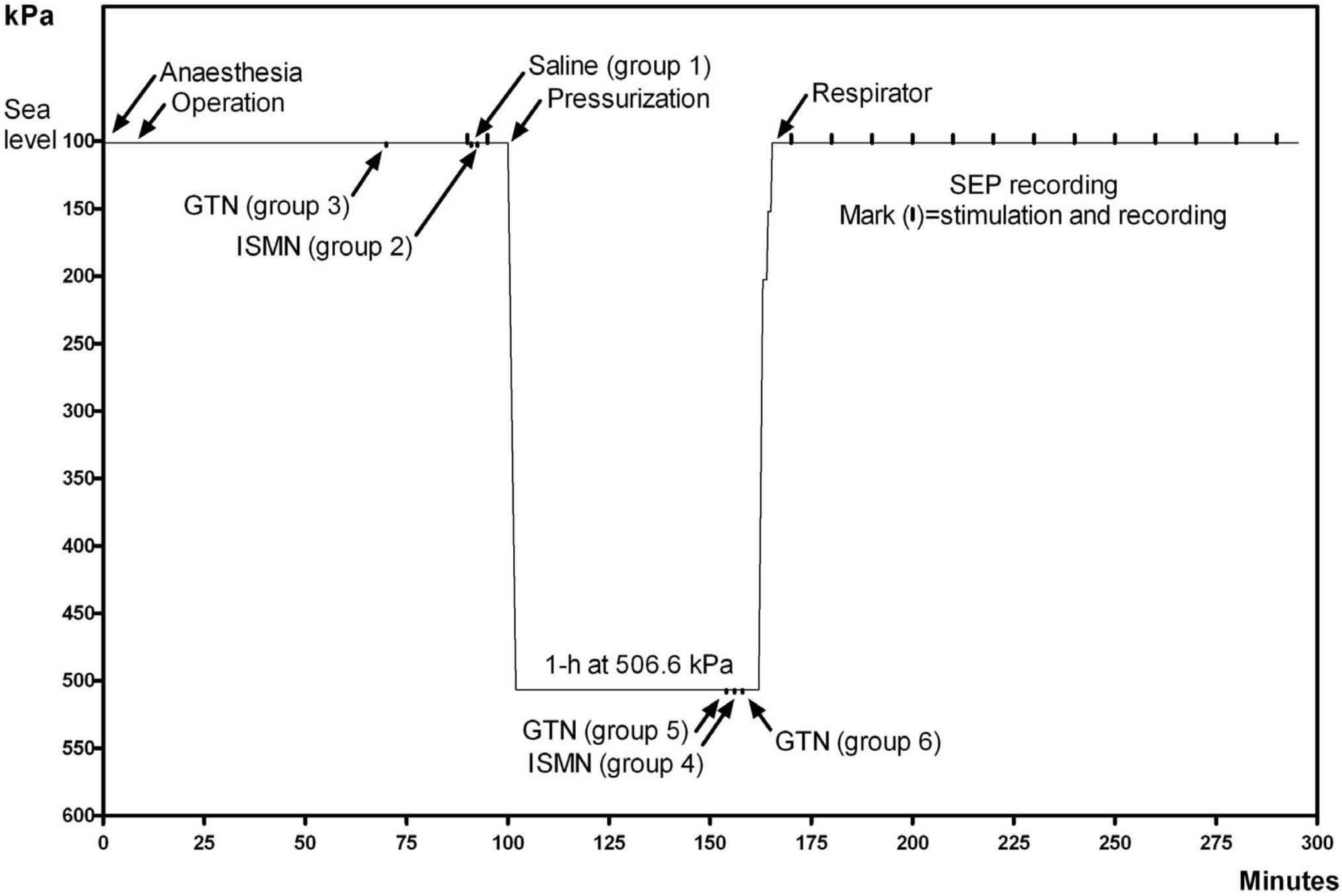
The effect of ISMN and GTN administration before and during a hyperbaric exposure on spinal cord conductivity and survival in rats with DCS

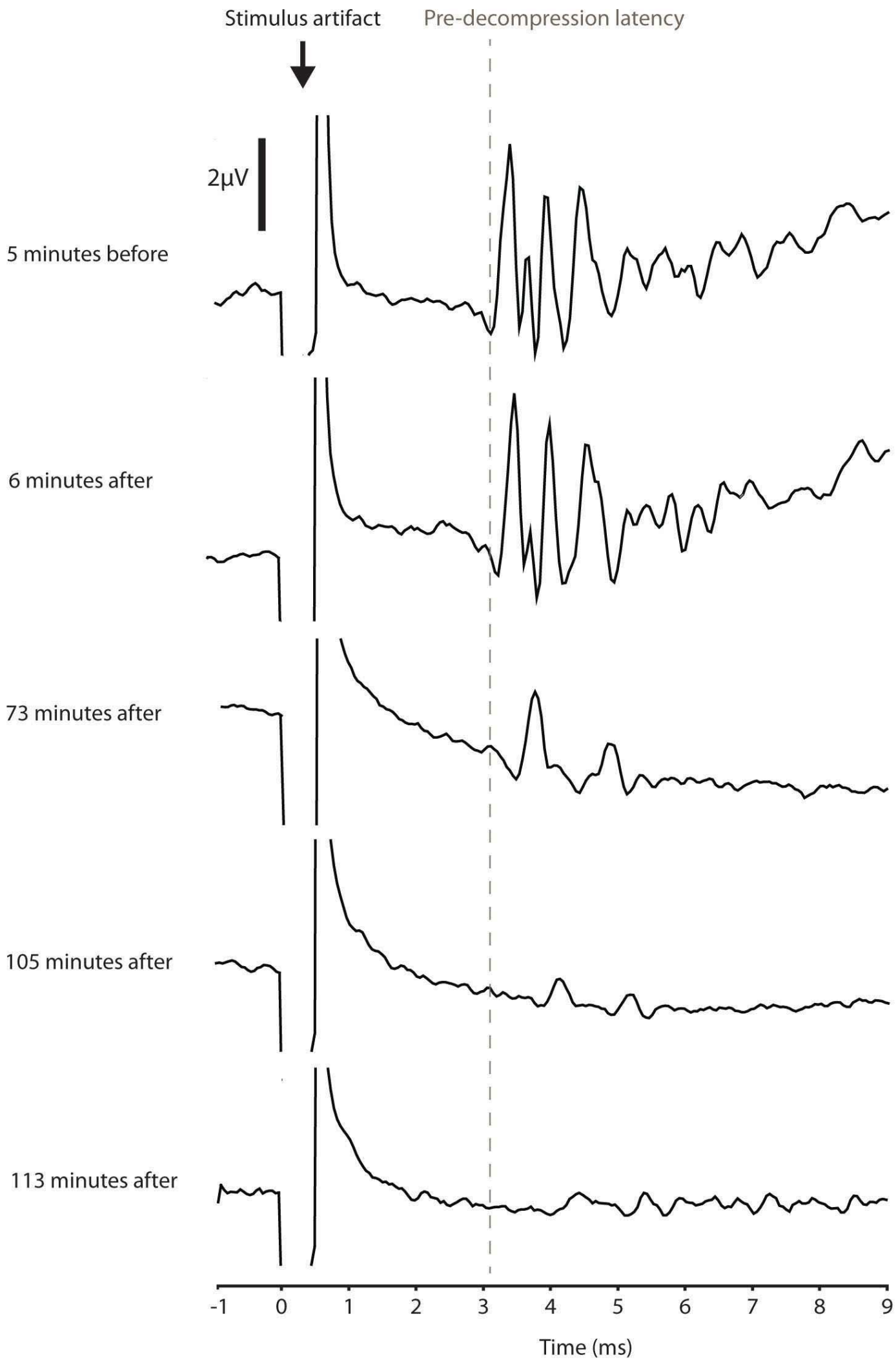
Treatment group	Group 1 Saline iv. 10 min before compression	Group 2 ISMN 300 mg/kg iv. 5-10 min before compression	Group 3 GTN 10 mg/kg ip. 30 min before compression	Group 4 ISMN 300 mg/kg iv. 6 min before decompression	Group 5 GTN 1 mg/kg iv. 3-8 min before decompression	Group 6 GTN 75 µg/kg i.v. 4 min before decompression
N = rats	12	12	8	10	8	8
n = nerves	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	2 of 12	2 of 12	0 of 8	3 of 10	0 of 8	2 of 8
SEP disappearance time minutes Mean Median range	 43.7 ± 39.9 26 (0-120)	 42.3 ± 35.2 33 (0-120)	 18.9 ± 13.6 [1] 12.5 (0-53)	 42.1 ± 41.2 23 (0-120)	 13.7 ± 12.5 [2] 12 (0-43)	 32.6 ± 39.2 12 (0-120)
Survival time minutes Mean Median range	 55.7 ± 35.4 44 (17-120)	 62 ± 32 50 (23-120)	 40.5 ± 27.2 27 (17-93)	 65.3 ± 44.9 49 (20-120)	 27.9 ± 19.1 [3] 19 (9-67)	 51 ± 43.3 31 (20-120)

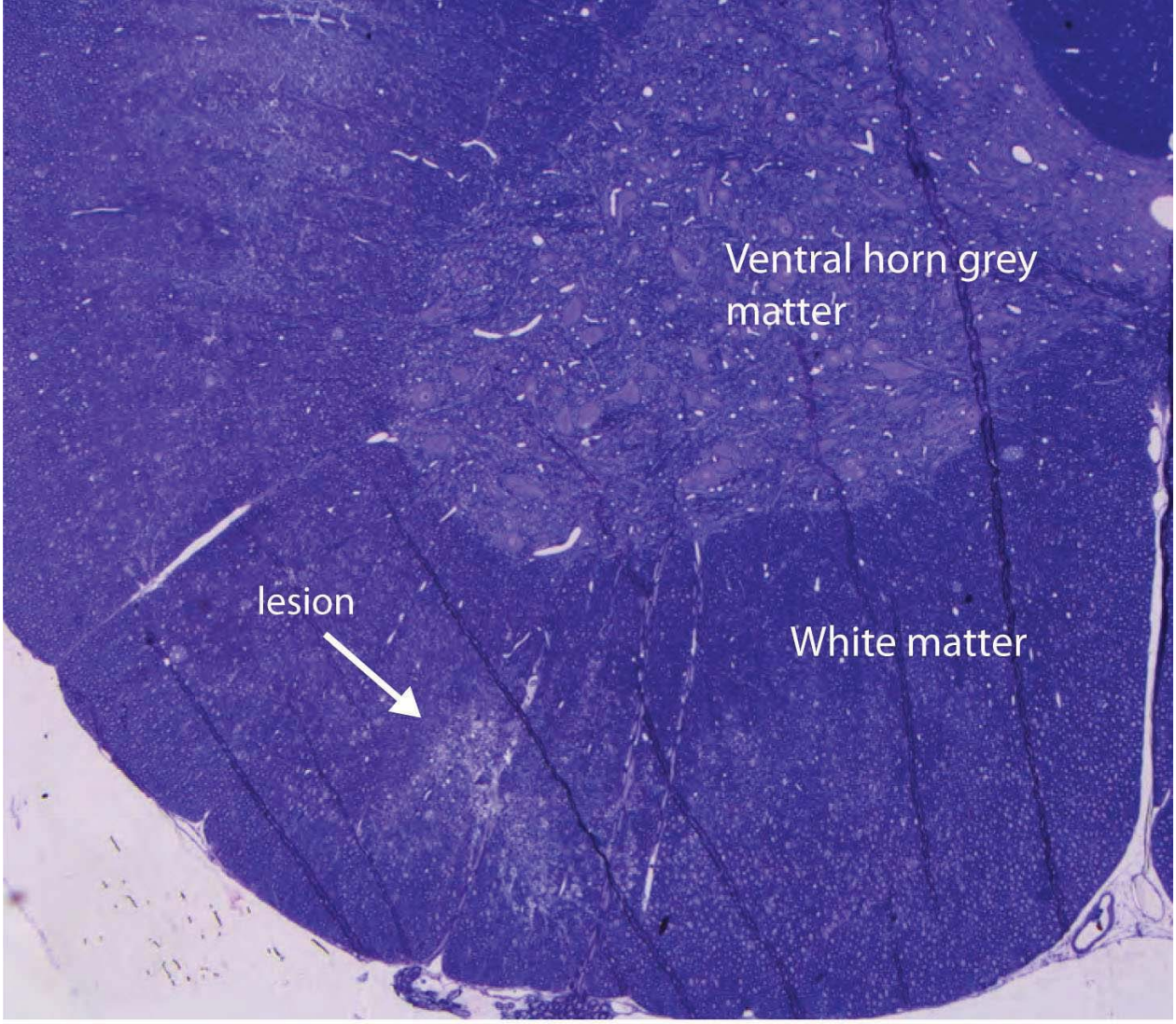
[1]) SEP disappearance time in group 3 different from SEP disappearance time in group 2 ($P < 0.05$).

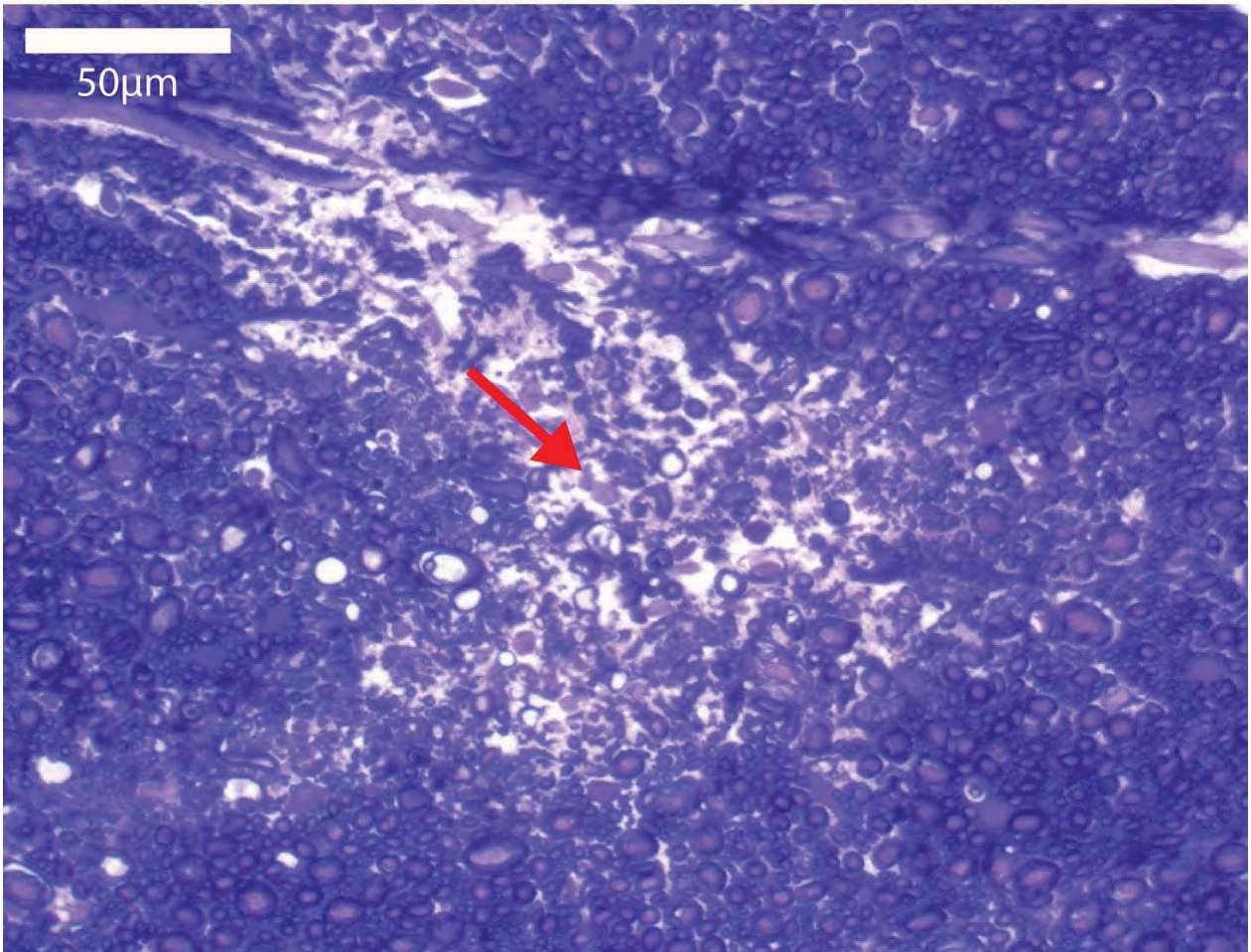
[2]) SEP disappearance time in group 5 different from SEP disappearance time in group 1 ($P < 0.05$) and group 2 ($P < 0.01$).

[3]) Survival time in group 5 different from survival time in group 2


kPa
Anaesthesia
Operation
Saline (group 1)
Pressurization
Respirator
Sea level
100
GTN (group 3)
ISMN (group 2)
SEP recording
Mark (I)=stimulation and recording
150
200
250
300
350
400
450
1-h at 506.6 kPa
500
GTN (group 5)
ISMN (group 4)
GTN (group 6)
550
600
0
25
50
75
100
125
150
175
200
225
250
275
300
Minutes


Stimulus artifact
Pre-decompression latency
2μV
5 minutes before
6 minutes after
73 minutes after
105 minutes after
113 minutes after
-1
0
1
2
3
4
5
6
7
8
9
Time (ms)


Ventral horn grey matter
lesion
White matter


50µm