Remote ischaemic conditioning in a rat model subjected to decompression stress

Nikolaj Hjort Schmidt, Kasper Hansen, Henrik Lauridsen, Annie Vesterby, Jens Randel Nyengaard, Alf Brubakk and Michael Pedersen

Abstract

Schmidt NH, Hansen K, Lauridsen H, Vesterby A, Nyengaard JR, Brubakk A, Pederson M. Remote ischaemic conditioning in a rat model subjected to decompression stress. *Diving and Hyperbaric Medicine*. 2016 September;46(3):170-175.)

Introduction: Vascular bubble formation after decompression has been associated with inflammation, necrosis, and platelet activation. This study evaluates remote ischaemic conditioning (RIC), performed before or after decompression, on bubble formation, platelet activation and ischaemic brain lesions.

Methods: Forty-two female Wistar rats were pressurised to 600 kPa air pressure for 45 min followed by linear decompression (50 kPa·min^{-1}). Rats received RIC (5 min ischaemia followed by 5 min reperfusion, repeated four times) one day before (pre-RIC, n = 10) or immediately after an air-dive (post-RIC, n = 10). The other animals served as air-dived sham rats (n = 11) or non-dived controls (n = 11). Bubbles were evaluated by ultrasonography of the pulmonary artery for 140 min after the dive. Blood was collected before and after the dive to evaluate platelet and metabolic changes (i.e., pH, lactate, glucose, free calcium ions, oxygen and carbon dioxide tensions, haemoglobin and haemoglobin oxygen saturation). Rats were euthanized two days after the dive to investigate potential brain infarctions evaluated by 2,3,5-triphenyltetrazolium chloride (TTC) staining.

Results: Pre-RIC and sham rats exhibited a similar bubble response. In contrast to this, post-RIC animals had significantly higher bubble grades that lasted for ~40 min longer than the sham group. Additionally, a 50% mortality was noted for post-RIC animals. No significant platelet or metabolic changes were observed and the dive profile did not produce TTC-verifiable cerebral ischaemic changes in any groups.

Conclusion: Pre-conditioning does not alter the response to decompression contrary to post-conditioning which seems to aggravate the bubble response.

Key words

Decompression sickness; venous gas embolism; platelets; metabolism; central nervous system; ischaemic preconditioning

Introduction

During decompression to the surface following a dive, if the tissue supersaturation of inert gas exceeds a certain threshold, bubbles may form to restore gas equilibrium within the body.[1,2] Gas bubbles have served as a biomarker for development of decompression profiles because no bubbles or very low bubble grades after decompression are correlated with a low probability of decompression sickness (DCS).[3]

Intravascular bubbles have the potential to obstruct blood vessels, which results in inflammation, platelet activation and depletion and development of ischaemia/necrosis in the adjacent tissue.[4–9] Prevention of DCS or attenuation of the pathophysiological responses to decompression ('decompression stress') through pre-dive procedures has been sought for decades through different conditioning procedures.[10] A novel idea is to evaluate the effects of remote ischaemic conditioning (RIC), which implies that short non-lethal periods of ischaemia in a remote bodily structure (e.g., a leg) protect against longer lasting harmful periods of ischaemia in another bodily structure/organ (e.g., the brain). RIC has provided impressive pre-clinical as well clinical results, e.g., pre-hospital salvage of myocardium in patients with suspected acute myocardial infarction;[11] improved glomerular filtration rate and renal perfusion after kidney transplantation in pigs;[12] and reduced cerebral infarction after transient middle cerebral artery occlusion in rats.[13]

The nomenclature pre-, per-, and post-RIC, denotes whether RIC is performed before, during, or after the insult. RIC can be performed with a blood pressure cuff non-invasively.[14] Therefore, if RIC demonstrates a protective effect against 'decompression stress' this could be a promising future adjuvant pre-hospital DCS treatment in a practical diving context. Accordingly, the objective of this study was to investigate whether RIC performed prior to (pre-RIC) or immediately after (post-RIC) simulated dives reduces bubble formation, platelet activation, and/or diminishes ischaemic insults in the brain in a decompression-stressed rat model.

Methods

Forty-two female Wistar rats (Taconic, Ry, Denmark), body mass 0.252 ± 0.001 (mean ± SE) kg, were randomly assigned to the following groups: control, not dived (n = 11); sham, a 'dived control' group, i.e., not receiving RIC (n = 11); pre-dive RIC (n = 10) or post-dive RIC (n = 10). Two pre-RIC rats were subsequently excluded from the study due to pressure chamber malfunction. Rats were housed at 24.1 ± 0.04^{O}C, relative humidity of 49.6 ± 0.8% and a 12/12 h day/night cycle with free access to food and

water. All experiments were approved by the national animal experimental inspectorate (license: 2014-15-0201-00101) and conducted in accordance with the Danish Ministry of Environment and Food animal research act.

STUDY DESIGN

The study was designed as a randomised block experiment and each rat was handled for four consecutive days (Figure 1). Primary endpoints were bubble grade, platelet fluctuations, and ischaemic brain lesions. Secondary endpoints were mortality, cardiac output (CO) and stroke volume (SV), and blood pH, lactate, glucose, free calcium ions, oxygen (O_2) and carbon dioxide (CO_2) tensions, haemoglobin and haemoglobin oxygen saturation.

Day 1: 23 h prior to simulated diving, all groups were anaesthetised for 1 h. Anaesthesia was induced with a subcutaneous injection (3 mL·kg^{-1}) of hypnorm (fentanyl 0.315 mg·mL^{-1} and fluanisone 10 mg·mL^{-1}) mixed with midazolam (5 mg·mL^{-1}) and sterile water (ratio 1:1:2), with a maintenance dose (1.5 mL·kg^{-1}) injected after 20 min.

Pre-RIC was initiated 15 min following injection of the anaesthetic induction dose and was performed with a custom-made tourniquet fitted around the upper part of the right hind leg, a method of temporary ischaemia previously used and validated.[13,15] One cycle consisted of 5 min of ischaemia followed by 5 min of reperfusion, repeated four times (Figure 1). Sufficient restriction of the blood supply during ischaemia was validated in pilot studies by ultrasonography of leg vessels distal to the tourniquet, and by paw pulse oximetry (data not shown). Furthermore, the paw turned blue during the ischaemic period, which subsequently changed to a deep red from hyperaemia after release of the tourniquet.

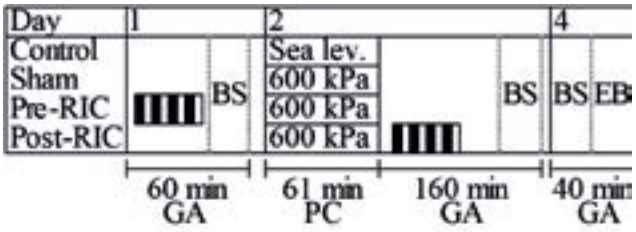
Heart rate (HR), respiratory frequency (RF) and temperature (T) were measured and displayed in real-time using the Vevo 2100 (FujiFilm VisualSonics, Toronto, ON, Canada) imaging system's physiologic monitoring equipment (Vevo Imaging Station) consisting of four electrodes mounted to the rat's paws, and a rectal probe. One hour after the anaesthetic induction, HR, RF, and T were recorded. Subsequently, venous (~0.5 mL) and arterial (~0.5 mL) blood samples were withdrawn from the tongue and tail, respectively. The venous blood samples were collected in EDTA-coated tubes (Microvette 500 K3E, Sarstedt, Nümbrecht, Germany) and analysed for platelets and haemoglobin. Arterial blood samples were obtained in heparin-coated syringes (Pico 50, Radiometer, Brønshøj, Denmark) and analysed for pH, lactate, glucose, free calcium ions, O_2- and CO_2 tension, and haemoglobin oxygen saturation using an ABL 700 (Radiometer, Brønshøj, Denmark).

Day 2: Sham, pre- and post-RIC animals were subjected to a simulated air-dive in an automated pressure chamber

Figure 1

Study design; animals were handled under general anaesthesia (GA), but were awake in the pressure chamber (PC); blood samples (BS) were taken at the end of each anaesthetic period; brains were removed after euthanasia (EB); Sea lev. - sea level pressure

▮▮▮▮ – repetitive remote ischemic conditioning



system (volume ≈ 3 L). A continuous airflow through the chamber of 1.5 L·min^{-1} was maintained while a rat was in the chamber. Awake rats were compressed over 6 min with atmospheric air to 600 kPa, which was maintained for 45 min, and followed by linear decompression of 50 kPa·min^{-1} to ambient pressure (Figure 1), representing moderate to severe exposure with a previously described 33% mortality following such an air-dive.[16] Animals in the control group stayed in the chamber at sea level pressure for 61 min.

Using the same anaesthetic agent and doses as described for day 1, each rat was anesthetised immediately following return to sea level and received maintenance doses 40 and 80 minutes later. The post-RIC group received RIC at 11.5 min after anaesthesia. Ultrasonography of the pulmonary artery was performed 20 min after return to sea level and continued for 130 min, and was acquired using the previously described Vevo 2100 imaging system fitted with a 21 MHz linear array transducer (frame rate 210 min^{-1}). The pulmonary artery was visualised through the parasternal longitudinal axis view. In B-mode, 420 frames were obtained every 20 min using a trigger mechanism; hence, the images were acquired independently of bubbles observed by the operator. Vascular bubbles in the pulmonary artery were graded according to the Eftedal-Brubakk (EB) scale.[17] Three consecutive cycles of four beats were evaluated, and the average bubble grade reported.

Similarly to Day 1, HR, RF and T were measured. After the last bubble scan, CO was calculated by multiplying HR with SV; the latter was estimated in the expiratory phase through three consecutive heart cycles by measuring the pulmonary arterial diameter distal to the pulmonary valves along with the velocity time integral (VTI) derived from pulse-waved Doppler measurements. All ultrasound data were analysed with the observer blinded to the type of exposure.

Following ultrasonic evaluation, both venous and arterial blood samples were obtained as previously described. For pain relief, buprenorphine (0.05 mg·kg^{-1}) was injected subcutaneously 2 h after return to sea level and further

administered through the drinking water (7.5 mg·L^{-1}) for the remainder of the experiment.

Day 4: After 43 h of recovery, surviving rats were anaesthetised, had blood withdrawn for analysis, and were euthanized by an intra-cardiac injection of pentobarbital (50 mg·mL^{-1}). The brain was immediately removed from the skull and cut according to the systematic uniform random sampling technique;[18] i.e., first coronal cut was randomly positioned between 0 to 2 mm from end of the frontal lobes. Hereafter, a new cut was positioned every 2 mm caudally. The slices where then placed in a pre-heated (37^{O}C) 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 3 h under constant stirring in an incubator (37^{O}C). The brain slices were subsequently transferred to 4% formaldehyde and stored in the refrigerator for later histological processing. The olfactory bulb and cerebellum were removed prior to histological examination. Each brain slice (~7 per brain) was placed on the caudal side in a vibratome (Vibratome Series 3000 Plus) and 390 µm tissue was initially removed from the rostral side. Next, a 65 µm section was prepared and mounted for later analysis with bright-field microscopy.

STATISTICS

All data are reported as median (interquartile range). The number of animals allocated to each group was based on previous conditioning studies.[19,20] Because most data were not normally distributed, non-parametric Wilcoxon rank sum tests were used for analysis with the control, pre-RIC and post-RIC groups compared to the sham group individually. The Kruskal-Wallis test was used to evaluate baseline platelet levels across groups. Mortality was evaluated using 2x2 tables with Fisher's exact test. $P < 0.05$ was considered statistically significant. All data analyses were performed in Stata 13 (StataCorp LP, TX, USA).

Results

PHYSIOLOGY

Pre-RIC animals had a significantly higher RF on Day 1 compared to the sham group ($RF_{pre\text{-}RIC}$ = 72 (67.5–92) min^{-1}; RF_{sham} = 53 (47.5–61) min^{-1}; $P = 0.03$), and the control group had a slightly higher T 20 min after return to sea level on Day 2 compared to the sham group ($T_{control}$ = 37.0 (36.5-37.1)^{O}C; T_{sham} = 36.0 (36.0-36.6)^{O}C; $P = 0.02$). For the remaining observation points at Day 1 and 2, pre-RIC and control animals exhibited no differences with respect to HR, RF, T, CO, and SV when compared individually to the sham group.

HR in the post-RIC group was significant higher on Day 1 compared to the sham group ($HR_{post\text{-}RIC}$ = 476 (444.5–492.5) min^{-1}; HR_{sham} = 420 (408.5–440) min^{-1}; $P = 0.02$), which was also observed on Day 2 at 20 min ($HR_{post\text{-}RIC}$ = 483 (475–505) min^{-1}; HR_{sham} = 440 (426–465.5) min^{-1}; $P = 0.003$), 60 min ($HR_{post\text{-}RIC}$ = 505 (493–512) min^{-1}; HR_{sham} = 453 (438.5–471.5) min^{-1}; $P = 0.007$) and 100 min ($HR_{post\text{-}RIC}$ = 507.5 (501.8-514.8) min^{-1}; HR_{sham} = 465 (444–488.5) min^{-1}; $P = 0.03$) after chamber exit. At 40, 80, 120 and 140 min after chamber exit on Day 2, similar but insignificant differences were observed. Comparable T were observed between post-RIC and sham both Day 1 and 2.

Generally, RF values were comparable between the post-RIC and the sham group over the entire observation period except 20 min after chamber exit on Day 2 ($RF_{post\text{-}RIC}$ = 79 (70–90) min^{-1}; RF_{sham} = 52 (36.5–67.5) min^{-1}; $P = 0.03$). CO was identical between the post-RIC and sham groups; however, SV was all but significantly lower in post-RIC animals compared to the sham group on Day 2 ($SV_{post\text{-}RIC}$ = 137.2 (112.1–202.4) mm^{3}; SV_{sham} = 234.0 (196.8–304.8) mm^{3}; $P = 0.05$).

BUBBLE GRADES AND MORTALITY

Comparing control with sham, the bubble grades were significantly elevated in the entire observation period, except for the last scan 140 min after return to sea level (data not shown, since no bubbles were observed in control rats). Generally, pre-RIC animals had lower bubble grades (n.s.) and the variation among rats (i.e., the bubble range) was smaller compared to the sham group. Post-RIC animals exhibited a significantly elevated bubble response at 100 min ($P = 0.04$), with a similar trend at 120 min ($P = 0.07$), after return to sea level when compared to the sham group. At all other time points the medians were statistically identical (Figure 2).

The maximum bubble grade observed in each rat across the observation period is presented in Table 1 for the various groups, i.e., if a rat exhibited grade 2 bubbling at most time points during the observation period and then at one time point exhibited grade 4, then grade 4 is reported in the table, thus each rat only appears once in the table.

There were no deaths in the control group. One of 11 rats died in the sham dive group, two of eight in the pre-RIC group and five of 10 in the post-RIC group. These differences in mortality were not statistically significant, although there may have been a trend of higher mortality in the post-RIC group ($P = 0.06$). When pooled for survival, rats that died during follow-up had a median maximum bubble grade of 4, compared to a median maximum grade of 1 in the sham, pre- and post-animals that survived the recovery period and were euthanized on Day 4.

BLOOD ANALYSES

No differences were observed between the control, pre-RIC, and post-RIC groups compared to the sham group,

Figure 2
Bubble grades (Eftedal-Brubakk (EB) scale[17]), median + interquartile ranges; A) Sham (black squares) vs. pre-RIC (grey circles). B) Sham (black squares) vs. post-RIC (grey triangles). * $P < 0.05$

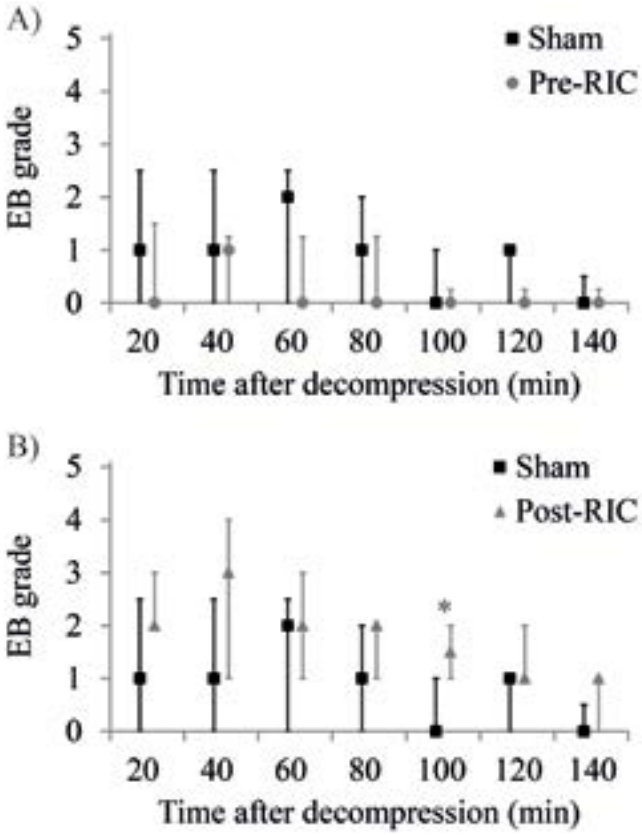


Table 1
Maximum bubble grade (Eftedal-Brubakk scale[17]) observed at some point for (*n*) rats in the individual groups across the 140-min observation period

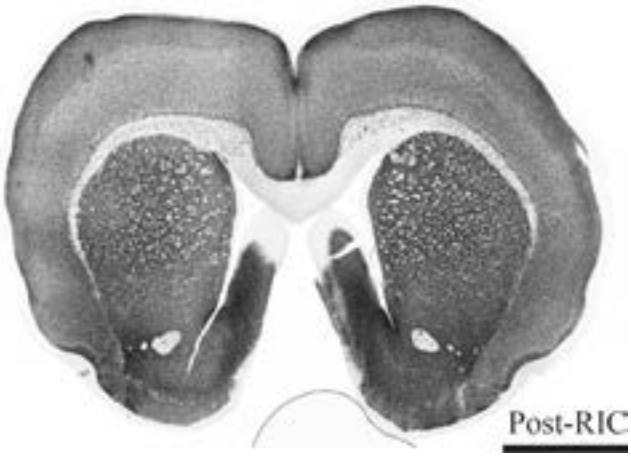
EB grade	Control	Sham	Pre-RIC	Post-RIC
0	11	4	4	3
1	0	0	2	0
2	0	3	0	1
3	0	1	1	1
4	0	1	1	4
5	0	2	0	1

respectively, with regard to platelets, pH, lactate, glucose, free calcium-ions, O_2- and CO_2-tension, haemoglobin and haemoglobin oxygen saturation on Days 1, 2 or 4.

HISTOLOGY

Five control and five sham animals, the latter selectively chosen as the animals displaying the highest bubble grades, and all pre- and post-RIC animals were evaluated microscopically, but no signs of ischaemic lesions were found in any of the samples. A representative example of a post-RIC 65 µm section (grey-scale) is shown in Figure 3.

Figure 3
Representative post-RIC 2,3,5-triphenyltetrazolium chloride (TTC) stained coronal brain section. Scale bar = 3 mm. Optical magnification x 40 (grey-scale image)



Discussion

The present study evaluated the effects of both pre- and post-RIC in a rat model subjected to 'decompression stress'. No differences in the physiological, metabolic or haematological parameters measured were observed, nor were ischaemic brain lesions seen. In the post-RIC group, maximum bubble counts were significantly higher than in the sham dive group, but the lack of a significant difference in bubble grades between the sham and pre-RIC animals suggests that the study probably had insufficient strength (i.e., a Type II error) to detect any difference that might have been present.

Unlike in previous studies, the present dive profile did not produce changes in platelet numbers.[7] This may be explained by the lesser decompression stress of the 600 kPa exposure in the present study compared to the 1,000 kPa exposure used previously.[7] Thus, we are unable to say if RIC attenuates decompression-induced changes in platelets.

Administration of a NO-donor prior to pressurisation has been reported to lower the median EB bubble grade from 5 to 0, when compared with dived controls.[20] Thus, it is surprising that we were not able to demonstrate a similar effect based on the observation that early-phase nitric oxide (NO) and nitrite release has been associated with remote ischaemic conditioning.[21,22] However, RIC has been described to be highly time-dependent comprising of both an early (minutes) and long-term (days) phase of protection, depending on the assessed organ.[14] Thus, as it has been seen with exercise prior to decompression, we cannot preclude the possibility that different timing of the RIC-procedure would mediate a different response. Furthermore, differences in

number and duration of RIC cycles may mediate different protective responses against global cardiac ischaemia.[15] Thus, it seems possible that an alternative RIC-protocol to the one used in this study would mediate an altered decompression stress response.

Post-RIC rats exhibited an elevated bubble response, and the elevated RF, HR and reduced SV post-dive on Day 2 may reflect compromised cardio-respiratory function due to bubble filtering in the lungs and circulatory stress from intravascular bubbles, as previously described.[23] However, the post-RIC HR were already elevated on Day 1 when compared to the sham animals HR, although the two groups had been treated identically at this point. We cannot comment on the SV and CO Day 1, but it may be that the post-RIC group, by chance, were more susceptible to 'decompression stress' due to their altered cardiovascular status at enrolment. We also speculate whether muscle trauma, mediated by the tourniquet during the RIC procedure, may have been the cause of the elevated bubble response seen in post-RIC animals as suggested previously.[24]

Mortality may have been greater in the post-RIC group but, again, the study probably had insufficient strength to confirm this despite the size of the study being based on previous similar work. The 25% mortality in the pre-RIC group is similar to that in a previous comparable study.[16] It is possible that the elevated bubble grade observed in post-RIC animals is the cause of the increased mortality in this group, as an increased bubble grade has been associated with early mortality in other rodent studies.[16,20]

No necrotic brain lesions were detected, as was also the case in a previous study.[16] TTC has successfully been used as a marker for ischaemic brain lesions in a rat stroke model, where pre- or per-RIC significantly reduced the infarcted area when compared to controls.[13] Therefore, despite our negative findings, we expect TTC to be useful in identifying cerebral gas embolisms following a more radical dive profile. However, the use of opioid analgesia in our study may have blunted potential cerebral ischaemic damage since opioids have been found to contribute to the neurogenic pathway for RIC protection against brain infarction.[25]

Impressive multi-tissue protection has been documented for RIC in various organs and intervention models.[11–13,26] Thus, if RIC provides a similar protection against the systemic adverse effect of DCS, RIC could be a useful technique for diving purposes, since it can be performed simply and non-invasively with a blood pressure cuff. However, in the light of the present results, further research is needed before RIC could become a future tool in the pre-hospital treatment of divers with DCS.

Conclusions

Remote ischaemic pre-conditioning conducted 23 h prior to a simulated 600 kPa air-dive did not significantly affect pulmonary bubble load in rats. Instead, remote ischaemic post-conditioning performed immediately after the dive worsened the bubble response and possibly increased mortality. No metabolic or platelet changes were observed in RIC-treated animals compared to sham dive animals, nor were cerebral ischaemic changes demonstrated using TTC staining.

References

1 Randsoe T, Hyldegaard O. Threshold altitude for bubble decay and stabilization in rat adipose tissue at hypobaric exposures. *Aviat Space Environ Med*. 2013;84:675-83.

2 Yang M, Milovanova TN, Bogush M, Uzun G, Bhopale VM, Thom SR. Microparticle enlargement and altered surface proteins after air decompression are associated with inflammatory vascular injuries. *J Appl Physiol*. 2012;112:204-11.

3 Eftedal OS, Lydersen S, Brubakk AO. The relationship between venous gas bubbles and adverse effects of decompression after air dives. *Undersea Hyperb Med*. 2007;34:99-105.

4 Mollerlokken A, Gaustad SE, Havnes MB, Gutvik CR, Hjelde A, Wisloff U, et al. Venous gas embolism as a predictive tool for improving CNS decompression safety. *Eur J Appl Physiol*. 2012;112:401-9.

5 Flick MR, Perel A, Staub NC. Leukocytes are required for increased lung microvascular permeability after microembolization in sheep. *Circ Res*. 1981;48:344-51.

6 Nyquist PA, Dick EJ, Jr., Buttolph TB. Detection of leukocyte activation in pigs with neurologic decompression sickness. *Aviat Space Environ Med*. 2004;75:211-4.

7 Pontier JM, Vallee N, Bourdon L. Bubble-induced platelet aggregation in a rat model of decompression sickness. *J Appl Physiol*. 2009;107:1825-9.

8 Palmer AC, Calder IM, Yates PO. Cerebral vasculopathy in divers. *Neuropathol Appl Neurobiol*. 1992;18:113-24.

9 Schneeweis C, Fleck E, Gebker R. Myocardial infarction after scuba diving. *Eur Heart J*. 2012;33:2224.

10 Gempp E, Blatteau JE. Preconditioning methods and mechanisms for preventing the risk of decompression sickness in scuba divers: a review. *Res Sports Med*. 2010;18:205-18.

11 Botker HE, Kharbanda R, Schmidt MR, Bottcher M, Kaltoft AK, Terkelsen CJ, et al. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. *Lancet*. 2010;375:727-34.

12 Soendergaard P, Krogstrup NV, Secher NG, Ravlo K, Keller AK, Toennesen E, et al. Improved GFR and renal plasma perfusion following remote ischaemic conditioning in a porcine kidney transplantation model. *Transpl Int*. 2012;25:1002-12.

13 Hahn CD, Manlhiot C, Schmidt MR, Nielsen TT, Redington AN. Remote ischemic per-conditioning: a novel therapy for acute stroke? *Stroke*. 2011;42:2960-2.

14 Kanoria S, Jalan R, Seifalian AM, Williams R, Davidson BR. Protocols and mechanisms for remote ischemic preconditioning: a novel method for reducing ischemia reperfusion injury. *Transplantation*. 2007;84:445-58.

15 Johnsen J, Pryds K, Salman R, Lofgren B, Kristiansen SB, Botker HE. The remote ischemic preconditioning algorithm: effect of number of cycles, cycle duration and effector organ mass on efficacy of protection. *Basic Res Cardiol*. 2016;111:10.

16 Havnes MB, Wideroe M, Thuen M, Torp SH, Brubakk AO,

Möllerlökken A. Simulated dive in rats lead to acute changes in cerebral blood flow on MRI, but no cerebral injuries to grey or white matter. *Eur J Appl Physiol*. 2013;113:1405-14.
17 Eftedal O, Brubakk AO. Agreement between trained and untrained observers in grading intravascular bubble signals in ultrasonic images. *Undersea Hyperb Med*. 1997;24:293-9.
18 Muhlfeld C, Nyengaard JR, Mayhew TM. A review of state-of-the-art stereology for better quantitative 3D morphology in cardiac research. *Cardiovasc Pathol*. 2010;19:65-82.
19 Wisloff U, Richardson RS, Brubakk AO. NOS inhibition increases bubble formation and reduces survival in sedentary but not exercised rats. *J Physiol*. 2003;546:577-82.
20 Wisloff U, Richardson RS, Brubakk AO. Exercise and nitric oxide prevent bubble formation: a novel approach to the prevention of decompression sickness? *J Physiol*. 2004;555:825-9.
21 Pickard JM, Botker HE, Crimi G, Davidson B, Davidson SM, Dutka D, et al. Remote ischemic conditioning: from experimental observation to clinical application: report from the 8th Biennial Hatter Cardiovascular Institute Workshop. *Basic Res Cardiol*. 2015;110:453.
22 Rassaf T, Totzeck M, Hendgen-Cotta UB, Shiva S, Heusch G, Kelm M. Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning. *Circ Res*. 2014;114:1601-10.
23 Butler BD, Hills BA. The lung as a filter for microbubbles. *J Appl Physiol.* 1979;47:537-43.
24 Harvey EN. Decompression sickness and bubble formation in blood and tissues. *Bull N Y Acad Med*. 1945;21:505-36.
25 Zhou Y, Fathali N, Lekic T, Ostrowski RP, Chen C, Martin RD, et al. Remote limb ischemic postconditioning protects against neonatal hypoxic-ischemic brain injury in rat pups by the opioid receptor/Akt pathway. *Stroke*. 2011;42:439-44.
26 Kim YH, Yoon DW, Kim JH, Lee JH, Lim CH. Effect of remote ischemic post-conditioning on systemic inflammatory response and survival rate in lipopolysaccharide-induced systemic inflammation model. *J Inflamm* (London). 2014;11:16.

Acknowledgements

We would like to acknowledge Anette Funder, Helene Andersen and Maj-Britt Lundorf for their indispensable histological assistance.

Funding

The work was supported by The Danish Council for Independent Research (Grant-ID: DFF – 4004-00472B). The Centre for Stochastic Geometry and Advanced Bioimaging is supported by the Villum Foundation.

Conflicts of interest: nil

Submitted: 05 November 2015; revised 24 April and 29 June 2016
Accepted: 01 July 2016

Nikolaj Hjort Schmidt[1], Kasper Hansen[1], Henrik Lauridsen[1], Annie Vesterby[2], Jens Randel Nyengaard[3], Alf Brubakk[1], Michael Pedersen[1]

[1]Department of Clinical Medicine – Comparative Medicine Lab, Aarhus University, Aarhus N, Denmark
[2]Department of Forensic Medicine, Aarhus University
[3]Stereology and Electron Microscopy Laboratory, Centre for Stochastic Geometry and Advanced Bioimaging, Department of Clinical Medicine, Aarhus University, Denmark

Address for correspondence:
Nikolaj H Schmidt
Department of Clinical Medicine – Comparative Medicine Lab
Aarhus University, Palle Juul-Jensens Boulevard 99
DK-8200 Aarhus N
Denmark
schmidt@clin.au.dk