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LABORATORY INVESTIGATION

Protection of cerebral microcirculation, mitochondrial function, and electrocortical activity by small-volume resuscitation with terlipressin in a model of haemorrhagic shock

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Abstract

Background: During early treatment of haemorrhagic shock, cerebral perfusion pressure can be restored by small-volume resuscitation with vasopressors. Whether this therapy is improved with additional fluid remains unknown. We assessed the value of terlipressin and lactated Ringer's solution (LR) on the early recovery of the microcirculation, tissue oxygenation, and mitochondrial and electrophysiological function in the rat cerebral cortex.

Methods: Animals treated with LR replacing three times (3LR) the volume bled (n=26), terlipressin (n=27), terlipressin plus 1LR (n=26), 2LR (n=16), or 3LR (n=15) were compared with untreated (n=36) and sham-operated rats (n=17) rats. *In vivo* confocal microscopy was used to assess cortical capillary perfusion, changes in tissue oxygen concentration, and mitochondrial membrane potential and redox state. Electrophysiological function was assessed by cortical somatosensory evoked potentials, spinal cord dorsum potential, and peripheral electromyography.

Results: Compared with sham, haemorrhagic shock reduced the mean (standard deviation) area of perfused vessels [82% (SD 10%) *vs* 38% (12%); P<0.001] and impaired oxygen concentration, mitochondrial redox state [99% (4%) *vs* 59% (15%) of baseline; P<0.001], and somatosensory evoked potentials [97% (13%) *vs* 27% (19%) of baseline]. Administration of terlipressin plus 1LR or 2LR was able to recover these measures, but terlipressin plus 3LR or 3LR alone were not as effective. Spinal cord dorsum potential was preserved in all groups, but no therapy protected electromyographic function.

Conclusions: Resuscitation from haemorrhagic shock using terlipressin with small-volume LR was superior to high-volume LR, with regard to cerebral microcirculation, and mitochondrial and electrophysiological function.

Keywords: brain ischaemia; confocal microscopy; electrophysiology

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Editor's key points

- Haemorrhage is the cause of up to 40% of deaths after trauma.
- Early small volume resuscitation with terlipressin can restore cerebral perfusion after haemorrhagic shock.
- The effect of additional fluid is unclear.
- In an experimental haemorrhage model in rats, resuscitation with low but not high volume fluids plus terlipressin restored cerebral microcirculation and mitochondrial and electrophysiological function.
- Optimum restoration of perfusion after haemorrhage is likely to reduce morbidity and mortality.

Haemorrhage remains a major cause of early death, accounting for 30–40% of trauma mortality, with 33–56% of deaths occurring before arrival at hospital.[1] Life-threatening loss of blood volume causes circulatory collapse.[2] The consequent impairment in oxygen supply to the brain[3] may cause neurological sequelae, most notably altered mentation (including loss of consciousness), seizures, and ischaemic stroke.[2,4,5] The major mechanism is considered to be a cellular energy crisis arising from tissue hypoxia.[3,6,7] In addition to a decrease in the cerebral macrocirculation, studies using animal models of haemorrhagic shock suggest impaired microcirculation[8] and mitochondrial insufficiency.[9] As cell damage potentially starts at the onset of the haemodynamic decompensation,[6,7,10,11] blood supply to the brain must be restored rapidly. However, the optimal method for resuscitation is not established. Standard teaching is to restore adequate volaemia before commencing vasopressor agents. However, despite early fluid resuscitation to restore oxygen delivery to the tissues, cerebral perfusion pressure and oxygenation may fail to recover, especially if there is a persisting loss of vascular tone.[3,12]

Vasopressors can reduce the volume of crystalloid required to recover blood pressure after haemorrhagic shock and can rapidly recover cerebral perfusion pressure during prehospital care.[3,13] Terlipressin, a synthetic analogue of vasopressin, has been proposed for the treatment of haemorrhagic shock,[14,15] and compared with vasopressin, it is longer acting and has higher selectivity for the vasopressin V_1 receptor.[15,16] Although studies in models of haemorrhage have demonstrated that terlipressin can improve cerebral perfusion pressure and tissue oxygenation,[12,17] the efficacy in protecting brain microcirculatory, mitochondrial, and electrophysiological function is unknown. We therefore used confocal imaging to study the circulation and metabolic state of the brain during shock *in vivo*, and in real time. We postulated that small-volume resuscitation with terlipressin would be superior to more aggressive fluid replacement therapy in protecting mitochondrial and electrophysiological function, and perfused vessel density, in a rodent model of haemorrhagic shock.

Methods

Experiments adhered to the Home Office (UK) 1986 Scientific Procedures Act and European Directive 2010/63/EU and results are reported according to relevant aspects of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, with University College London animal Ethics Committee approval.

Rats (male, in-house, Sprague Dawley, ~150 g) were housed in groups of five in pathogen free cages with a 12 h light/dark cycle at 22°C with standard rat pellets available *ad libitum*. Rats were anaesthetised without recovery throughout the experiments using isoflurane delivered via a vaporizer (induction 5% in an induction cage, maintenance 1.5–2% via nose cone; Iso-Flo, Abbott Labs, Maidenhead, UK) while spontaneously breathing room air. Adequacy of anaesthesia was assessed by ensuring the absence of withdrawal reflex after paw and ear pinch, and by monitoring the values of heart rate, mean arterial pressure (MAP), and respiratory rate to noxious stimulation. Rectal temperature (36–37°C; underblanket, Harvard Apparatus, Cambridge, UK), direct MAP (left femoral artery connected to a pressure transducer; World Precision Instruments, Hitchin, UK), respiratory rate, and end-tidal carbon dioxide ($ETCO_2$; via orotracheal intubation, Microcap, Oridion, Needham, MA, USA) were continuously monitored. The femoral vein was cannulated for fluid and drug administration. A craniotomy ~8 mm in diameter (centred at bregma −2 mm, lateral 2.5 mm) was performed over the left somatosensory cortex and the animals either imaged using *in vivo* confocal microscopy, or assessed electrophysiologically, for the rest of the experiment.

In vivo confocal microscopy

The skull was fixed to a custom-made titanium bar using dental cement (Contemporary Ortho-Jet Powder, Lang Dental Manufacturing Co., Wheeling, IL, USA) mixed with cyanoacrylate glue. The dura was removed, and platinum(II)-5,10,15,20-tetrakis(2,3,4,5,6-pentafluorophenyl)porphyrin (PtPFPP)-based phosphorescent oxygen-sensitive microbeads (Luxcel Biosciences, Cork, Ireland) applied to the cortex. The craniotomy was then sealed with a glass coverslip and petroleum jelly. Time-lapse fluorescence images were acquired with a laser-scanning confocal microscope (512 by 512 pixels, optical slice 37.1 μm; LSM 5 Pascal, Zeiss, Jena, Germany) to assess mitochondrial redox state by imaging endogenous flavoprotein fluorescence (excitation: 488 nm; emission: 505–570 nm), and changes in local oxygen concentration (ex: 543 nm; em: 650 nm). At termination, i.v. fluorescein isothiocyanate-dextran 70 kDA (FITC-dextran; 0.5 mg i.v.; ex: 488 nm; em: 505–570 nm; Sigma-Aldrich, Poole, UK) and topical tetramethylrhodamine methyl ester (TMRM; 1 μM; ex: 543 nm; em: 585 nm; T-668, Molecular Probes, Invitrogen, Paisley, UK) were imaged to establish perfused vessel density and mitochondrial membrane potential, respectively. Images were processed using Fiji/Image J 1.48v (NIH, Bethesda, MD, USA).

Electrophysiology

The right tibial nerve was stimulated (DS2, Digitimer, Welwyn Garden City, UK) percutaneously at the ankle (10 Hz, twice supramaximal), with recording electrodes at the vertebral level T10/T11, and on the cortical dura (−2 mm from bregma, 2.5 mm from midline), with reference electrodes on nearby inactive tissue. Another recording electrode was placed over the ipsilateral metatarsal musculature, with a reference electrode in the third digit. The ground electrode was inserted under the lumbar skin. Recordings of the somatosensory evoked potentials, cord dorsum potentials, and electromyographic signals were amplified (Neurolog System, Digitimer), observed on an oscilloscope (Sigma 60, Nicolet, Madison, WI, USA), and stored as averaged (n=20) compound action potentials. They were monitored as measures of cortical, spinal, and muscular function, respectively.

Table 1 Mean bodyweight, mean estimated blood volume withdrawn, and mean time until death after shock. EBV, estimated blood volume

	Bodyweight (g)	Blood withdrawn (%EBV)	Time of death after shock (min)
Sham	162.2 (34.7)	–	–
Shock	164.0 (40.4)	39.3 (7.1)	30 (22)
3LR	163.6 (24.8)	38.2 (8.6)	68 (25)
Terlipressin	162.9 (24.8)	37.8 (8.2)	52 (21)
Terlipressin + 1LR	148.5 (15.7)	43.6 (6.9)	72 (27)*
Terlipressin + 2LR	160.7 (36.9)	42.8 (6.7)	8
Terlipressin + 3LR	160.6 (26.3)	43.2 (11.4)	44 (32)

* vs untreated Shock group $P < 0.05$

Study design

After instrumentation, repeated administrations of 1.5 ml i.v. fluid challenges were given over 10 s every 5 min to ensure normovolaemia at baseline, until MAP failed to increase >10%.[18] Animals were allowed to stabilize for 20 min before randomization envelopes were opened with allocation into one of the seven following groups: [i] not subjected to haemorrhagic shock (Sham; n=17); [ii] subjected to haemorrhagic shock, but untreated (Shock; n=36); [iii] lactated Ringer's solution (LR) given at three times the volume of blood withdrawn (3LR; aggressive fluid resuscitation: n=26); [iv] bolus of 10 μg 100 g^{-1} of terlipressin alone (n=26), or combined with LR in low volumes of [v] one (Terli+1LR; n=26) or [vi] two times (Terli+2LR; n=26) the volume of blood withdrawn; or [vii] combined with LR in a high volume of three times (Terli+3LR; aggressive fluid resuscitation; n=26) the volume of blood withdrawn. The dose of terlipressin was titrated in a pilot study, starting from a dose previously described.[14]

Haemorrhagic shock was achieved by removing blood from the arterial line, targeting a MAP of 40 mm Hg, maintained for 30 min by withdrawing or re-infusing blood when necessary, before treatment.

Data were recorded at baseline, after 30 min with MAP of 40 mm Hg (shock), and at 5, 60, and 120 min (T5, T60, and T120) after treatment. After 150 min all surviving rats were culled under 5% isoflurane anaesthesia.

Statistical analysis

The sample size was calculated in preliminary experiments using a power analysis that indicated a minimum of 26 rats/group was required for a 95% chance (with 5% risk) to detect a difference between groups, of 60%, 40%, and 46% in the cortical somatosensory evoked potentials (n=6/group), mitochondrial redox state (n=10 rats/group), and changes in tissue oxygen concentration (n=10 rats/group), respectively, considering a

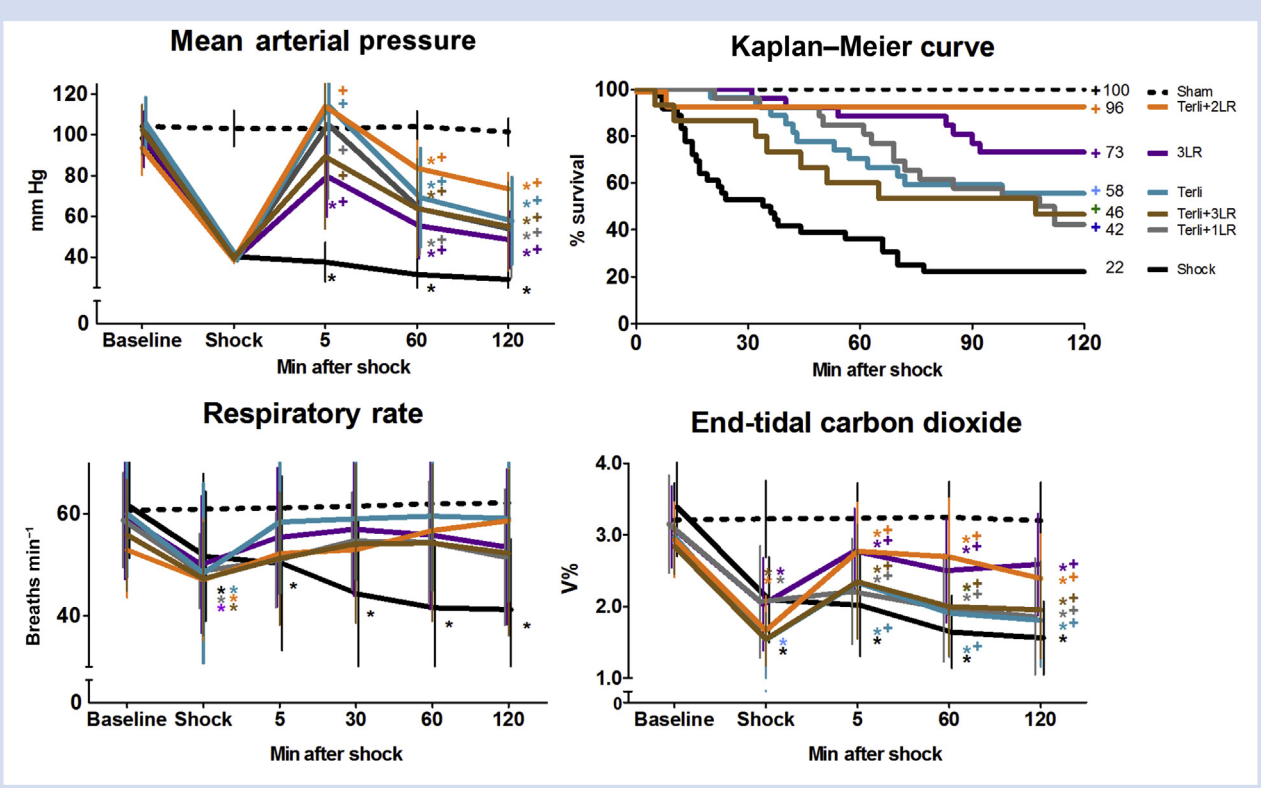


Fig 1. Kaplan–Meier curve and changes in mean arterial pressure, respiratory rate, and end-tidal carbon dioxide induced by haemorrhagic shock, and treatment with lactated Ringer's solution (LR), terlipressin, and combined treatments of LR plus terlipressin. *vs Sham (at least $P<0.05$); + vs untreated Shock group (at least $P<0.05$).

standard deviation (SD) of 8%, 15%, and 5%, respectively. Endogenous flavoprotein fluorescence was analysed by determining the ratio between the mean intensity of areas adjacent to veins and arteries (perivenular:periarterial ratio).[19,20] The mean phosphorescence of the oxygen-sensitive beads was analysed by selecting up to six beads representing proximity to different vascular regions (the beads distribute randomly). The emission signals of flavoproteins and oxygen-sensitive beads analysed at each time-point were compared with their corresponding emission signal at baseline. The TMRM images were analysed 150 min after shock, as described for flavoproteins. The FITC-dextran fluorescence was analysed at 120 min after shock by determining the number of vessels crossing three equidistant horizontal and vertical lines, divided by the total length of the lines, and the area occupied by fluorescent vessels above a threshold brightness. In the electrophysiological recordings, the measurements at each time-point were compared with the measurement at baseline. Data were assessed for normality using the Kolmogorov–Smirnov test and were compared within and between groups using repeated measure two-way analysis of variance followed by Tukey's *post hoc* testing (GraphPad Prism 5.03, GraphPad Software Inc., La Jolla, CA, USA). The 'last observation carried forward' method was used when animals died before the end of the study. Pearson's coefficient was calculated to assess correlation between variables. A 0–40 scoring system was calculated for each variable according to the percentage difference at T120/T150 compared with baseline/sham: (0) for 0–20%, (1) for 21–40%, (2) for 41–60%, (3) for 61–80%, and (4) for 81–100%. The sum of the scores (0=worst, 40=best) was calculated to compare the effectiveness of each treatment. Data are presented as mean and SD. Statistical significance was $P<0.05$.

Results

Bleeding and survival

Ten rats from the Shock group were not included in the statistical analysis because they were used to generate the data upon which to base the power calculation. Only a single bolus of 1.5 ml fluid challenge was necessary in all rats to ensure normovolaemia at baseline. Removal of approximately 40% (approximately 4 ml) of the estimated blood volume (EBV) of each rat [EBV (ml)=0.06 × body weight (g)+0.77][21] (approximately 10 ml) was necessary to induce haemorrhagic shock as defined by a MAP <40 mm Hg (Table 1). Most animals died under anaesthesia when not treated after shock ($P<0.001$ Shock *vs* Sham; Fig. 1). Survival was higher in all treated groups; the most effective treatment was Terli+2LR (one death; $P<0.001$ *vs* Shock).

Cardiorespiratory variables

Haemorrhagic shock caused a decrease in respiratory rate, $ETCO_2$, and MAP in all groups compared with Sham ($P<0.001$; Fig. 1). This cardiorespiratory impairment did not recover at any time in the Shock group. MAP was significantly higher at T120 in all treated groups ($P<0.001$ *vs* Shock), although it remained lower compared with Sham ($P<0.001$). The respiratory rate was restored by all treatments at T120. However, the

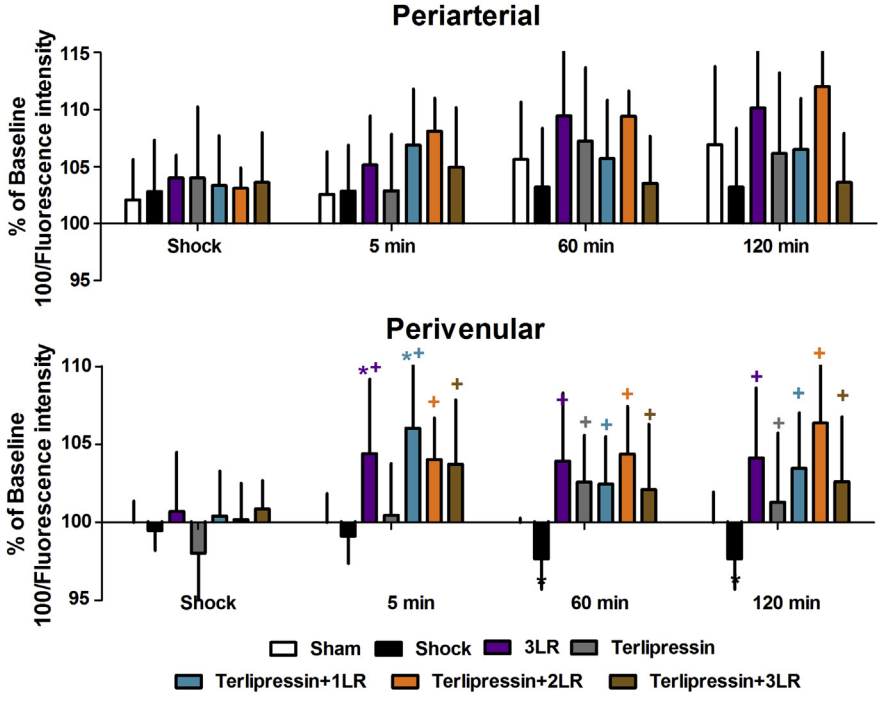


Fig 2. Changes in phosphorescence of oxygen-sensitive microbeads according to their location relative to the veins and arteries throughout the study. * *vs* Sham of the same time-point (at least $P<0.05$); + *vs* untreated Shock group of the same time-point (at least $P<0.05$). LR, lactated Ringer's solution.

improvement in $ETCO_2$ was lower than in Sham, although higher than in Shock ($P<0.001$; Fig. 1).

Cortical tissue oxygenation

Haemorrhagic shock caused a significant decrease in tissue oxygenation near veins at T60 and T120 in the untreated Shock group compared with Sham ($P<0.05$; Fig. 2). At these same time-points, the perivenular tissue oxygenation was significantly higher after all treatments ($P<0.05$ *vs* Shock). Oxygenation near arteries was maintained throughout.

Cerebral vascular density

The induction of haemorrhagic shock resulted in a significant decrease in both the density [0.05 (SD 0.01) *vs* 0.17 (0.01) n μm^{-2} for Sham, $P<0.001$] and percentage area of perfused vessels [38% (12%) *vs* 82% (10%) for Sham, $P<0.001$] at T120 (Fig. 3). Treatment with terlipressin and Terli+2LR improved both density and the percentage area of perfused vessels [0.14 (0.02) and 0.14 (0.02) n μm^{-2}, $P<0.001$ *vs* Shock; 66% (14%) and 73% (9%), $P<0.001$ *vs* Shock, respectively] achieving results that were similar to Sham (Fig. 3). The other treatments were either inferior to Sham (3LR and Terli+1LR), or no better than untreated animals (Terli+3LR).

Cerebral mitochondrial redox potential

In all groups, haemorrhagic shock caused a significant decrease in flavoprotein fluorescence (i.e. increased reduced state) adjacent to veins, but fluorescence persisted in a 'halo' around arteries (Fig. 4A), reflected by a reduction in the perivenular:periarterial fluorescence ratio ($P<0.05$; Fig. 4B). In the Shock group, the ratio was lower at T120 compared with Sham [59% (16%) *vs* 99% (4%) of baseline; $P<0.001$; Fig. 4B]. All treatments were effective in increasing the fluorescence around veins at T5. At study end, administration of 3LR [73% (20%) of baseline; $P<0.001$] and Terli+3LR [74% (18%) of baseline; $P<0.001$] resulted in a lower perivenular:periarterial flavoprotein ratio compared with Sham, although higher than in Shock ($P<0.05$). At T120, animals treated with Terli, Terli+1LR and Terli+2LR showed ratios not significantly different to Sham [87% (20%), 82% (20%), and 92% (15%) of baseline, respectively], but higher than Shock ($P<0.001$) (Fig. 4).

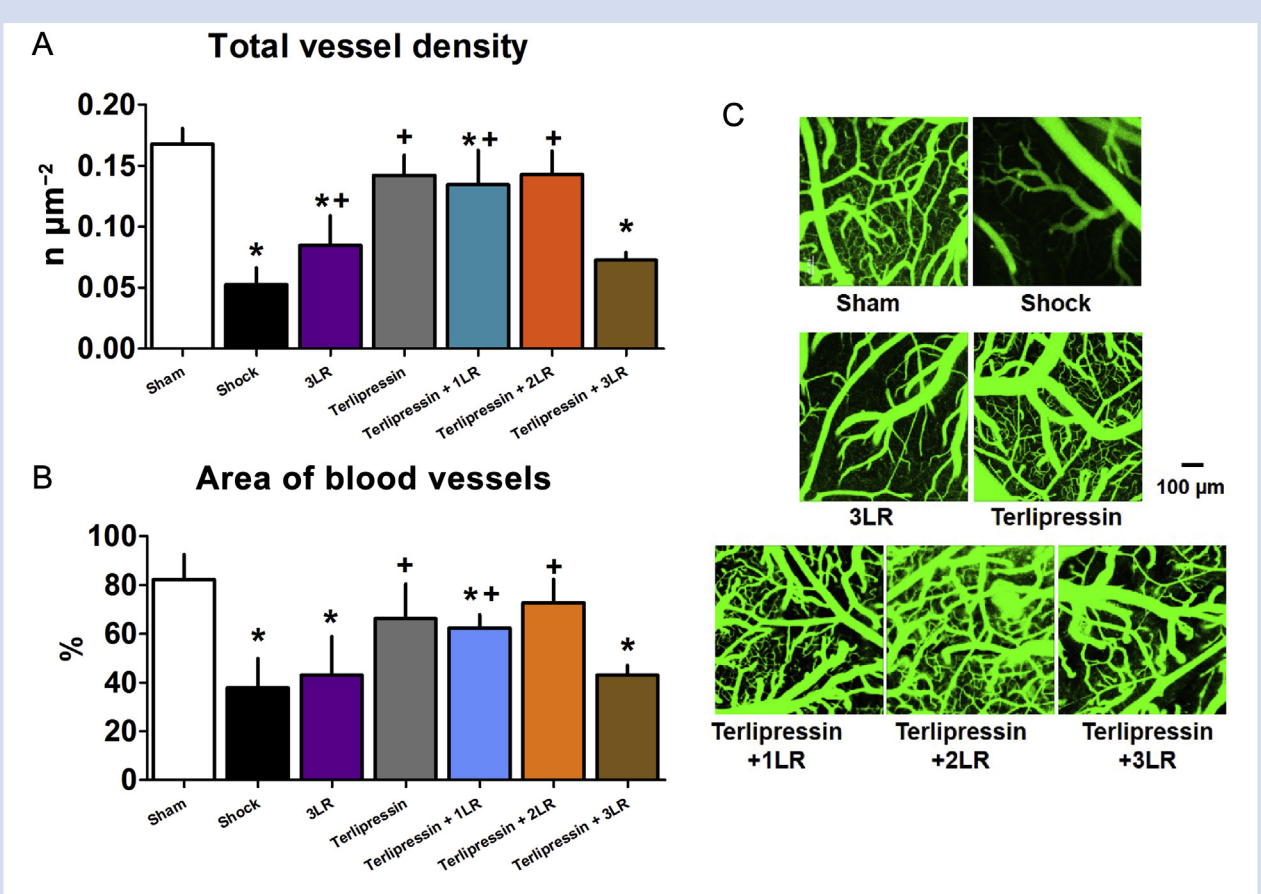


Fig 3. Vasculature of the cerebral cortex assessed by fluorescein isothiocyanate (FITC)-dextran after 120 min of haemorrhagic shock. (A) Total perfused vessel density shows quantity of blood vessels with flow. (B) Representative *in vivo* confocal images of the cortical vasculature. (C) Area fraction of blood vessels representing relative area covered by FITC-dextran fluorescence. Notably fewer vessels in untreated Shock, 3LR and Terli+3LR groups than Sham. Images of rats receiving Terli and Terli+2LR show no difference to Sham. * *vs* Sham (at least $P<0.05$); $^+$ *vs* untreated Shock group (at least $P<0.05$). LR, lactated Ringer's solution.

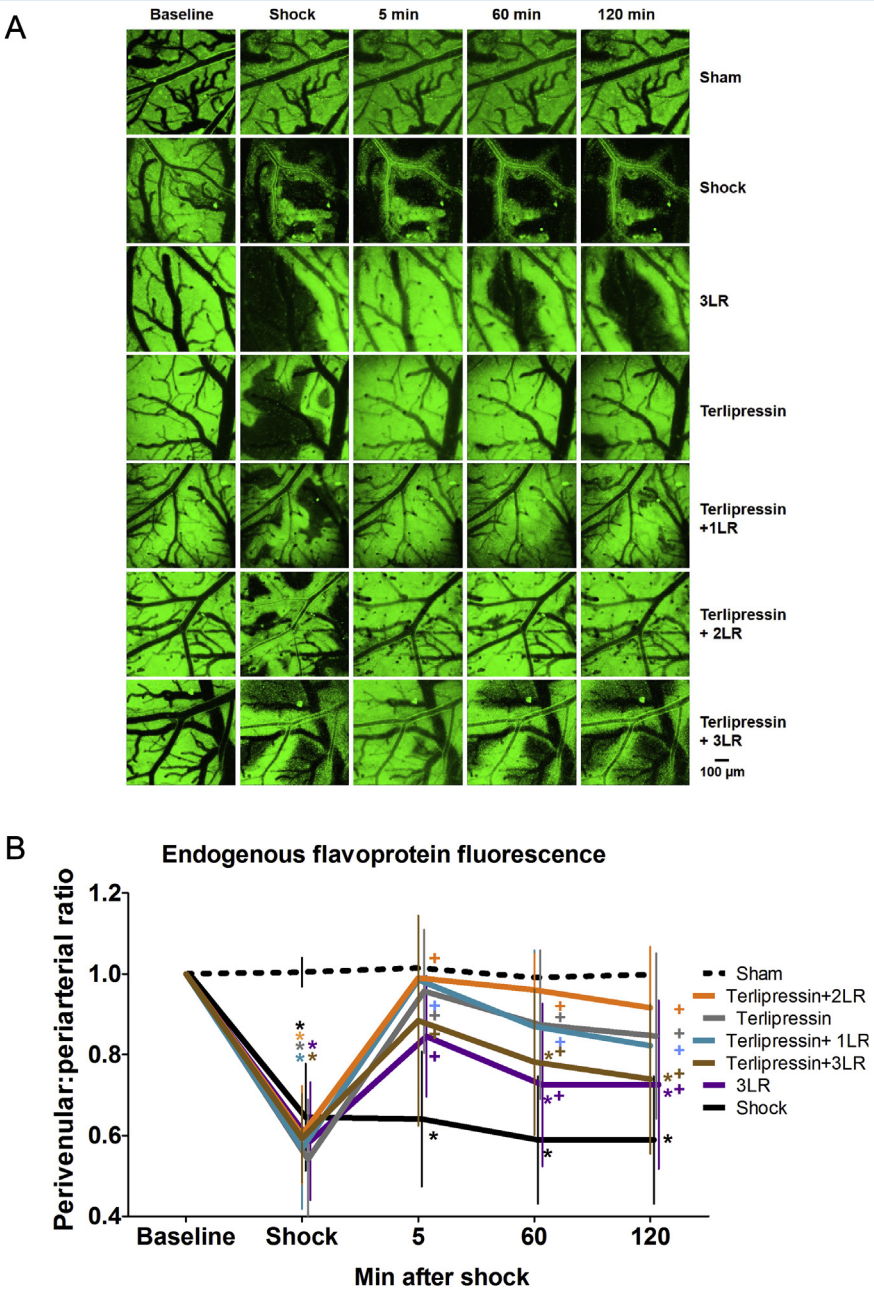


Fig 4. Endogenous flavoprotein fluorescence induced by haemorrhagic shock and the effect of different treatments. (A) Representative *in vivo* confocal images of rat cerebral cortex showing fluorescence of oxidized endogenous flavoprotein (green). Uniform fluorescence is seen before haemorrhagic shock, but lost except for a 'halo' around arteries after shock. Some recovery of fluorescence after treatment is seen at 5 min. At 120 min, fluorescence persists only in the Terli and Terli+2LR groups. (B) Changes in the perivenular:periarterial ratio of the endogenous flavoprotein fluorescence. * *vs* Sham (at least $P<0.05$); $^{+}$ *vs* untreated Shock group (at least $P<0.05$). LR, lactated Ringer's solution.

Cortical mitochondrial membrane potential

Mitochondrial membrane potential, indicated by TMRM fluorescence (Fig. 5), revealed that most mitochondria were depolarized (non-functional) after haemorrhagic shock. Only the mitochondria located near arteries remained polarized, resulting in arterial 'halos' similar to those observed with flavoproteins. At 150 min after shock the perivenular:periarterial ratio was worse in the Shock group [(0.28 (0.08); $P<0.001$), 3LR (0.39 (0.13); $P<0.001$)], and Terli+3LR [0.34 (0.01); $P<0.001$], compared with Sham [0.97 (0.09)]. No significant differences in the perivenular:periarterial TMRM ratio were observed in the Terli [0.98 (0.31)], Terli+1LR [0.76 (0.22)], and

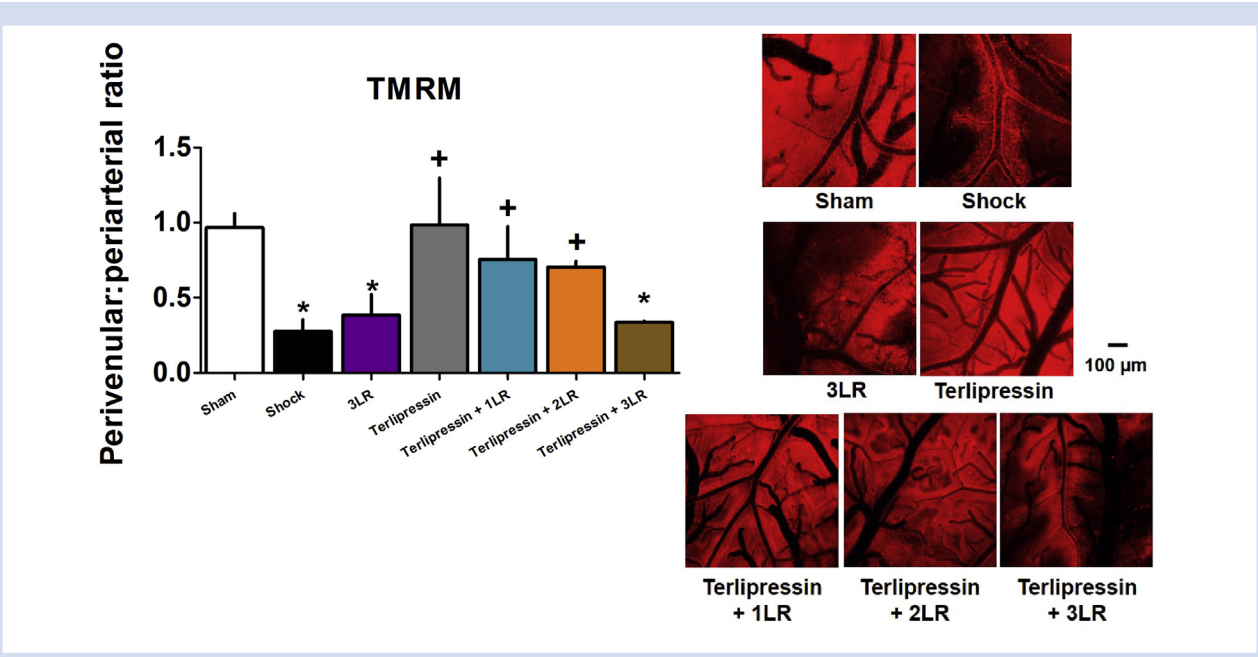


Fig 5. Graph and representative confocal *in vivo* images showing mitochondrial membrane potential in the cerebral cortex of rats at 150 min after haemorrhagic shock. In the untreated Shock group many mitochondria are depolarized, only mitochondria near arteries remain polarized, resulting in periarterial tetramethylrhodamine methyl ester (TMRM) 'halos'. A similar pattern of periarterial halos is seen in rats treated with 3LR and Terli+3LR, though polarized mitochondria were more widely distributed in rats treated with Terli, Terli+1LR and Terli+2LR and the distribution is not significantly different to Sham. * *vs* Sham (at least $P<0.05$); $^{+}$ *vs* untreated Shock group (at least $P<0.05$). LR, lactated Ringer's solution.

Terli+2LR [0.71 (0.04)] groups compared with Sham; values were also better than in Shock (all $P<0.001$).

Electrophysiological function

All bled groups had decreased cortical function to approximately a third of baseline at shock ($P<0.001$ *vs* Sham). At T120, cortical function decreased further to 27% (19%) of baseline in Shock (Fig. 6); aggressive fluid (Terli+3LR and 3LR) groups were not significantly better than no treatment. The Terli+1LR [73% (32%) of baseline] and Terli+2LR [95% (30%) of baseline] groups were indistinguishable from Sham [97% (13%) from baseline], and higher than in Shock ($P<0.001$) at T120. A decrease was seen in peak-to-peak muscular function amplitude ($P<0.001$); no treatments restored this to baseline values (Fig. 6). In the Terli+1LR ($P<0.05$) and Terli+2LR ($P<0.001$) groups, the peak-to-peak amplitude was greater at T120 compared with Shock. The amplitude of the cord dorsum potential (Fig. 6) and the peak latency of all potentials did not change significantly in any group throughout the study (Table 2).

Correlations and scores for effectiveness of the treatments

A positive correlation was seen between the decrease in MAP, and changes in flavoprotein ($r^2=0.84$, $P=0.0006$) and TMRM signals ($r^2=0.57$, $P=0.0484$), total perfused vessel density ($r^2=0.69$, $P=0.0055$), area fraction of perfused vessels ($r^2=0.78$, $P=0.0019$), and changes in cortical function ($r^2=0.72$, $P=0.0032$), at T120. At T120, the changes in fluorescence of the perivenular oxygen-sensitive microbeads showed a weak but significant correlation with MAP ($r^2=0.54$; $P=0.0185$), and with changes in flavoprotein fluorescence ($r^2=0.60$; $P=0.0235$) and periarterial oxygen-sensitive microbeads ($r^2=0.63$; $P=0.0187$). In addition, changes in fraction of perfused vessels were correlated with changes in flavoprotein fluorescence ($r^2=0.90$, $P=0.0003$), TMRM signals ($r^2=0.89$, $P=0.0172$), and somatosensory evoked potentials ($r^2=0.82$, $P=0.0018$).

The Terli+2LR (score of 33), Terli+1LR (score of 27), and Terli (score of 27) were the most effective treatments to improve the variables assessed. Scores of effectiveness were 40 in Sham, 21 in 3LR, 20 in Terli+3LR, and 11 in Shock.

Discussion

We describe the consequences of different resuscitation regimens immediately after haemorrhagic shock, focusing on the vasculature, oxygenation, and function of the nervous system. Although the cerebral cortex is profoundly affected by haemorrhagic shock, with a dramatic reduction in perfusion of the smaller vessels accompanied by loss of mitochondrial function, it is nonetheless possible to restore perfusion, mitochondrial, and neurological function by the timely administration of effective therapy.

All treatments improved survival within the time course of the study. Whilst administration of terlipressin was generally associated with improvement in the measured variables, the combination of terlipressin and aggressive fluid (i.e. Terli+3LR) was not. Whether this is because of negative cardiovascular effects and/or other causes remains uncertain. If the amplitude of the cortical evoked potential is taken as the benchmark of a good outcome, this was optimally achieved by Terli+2LR,

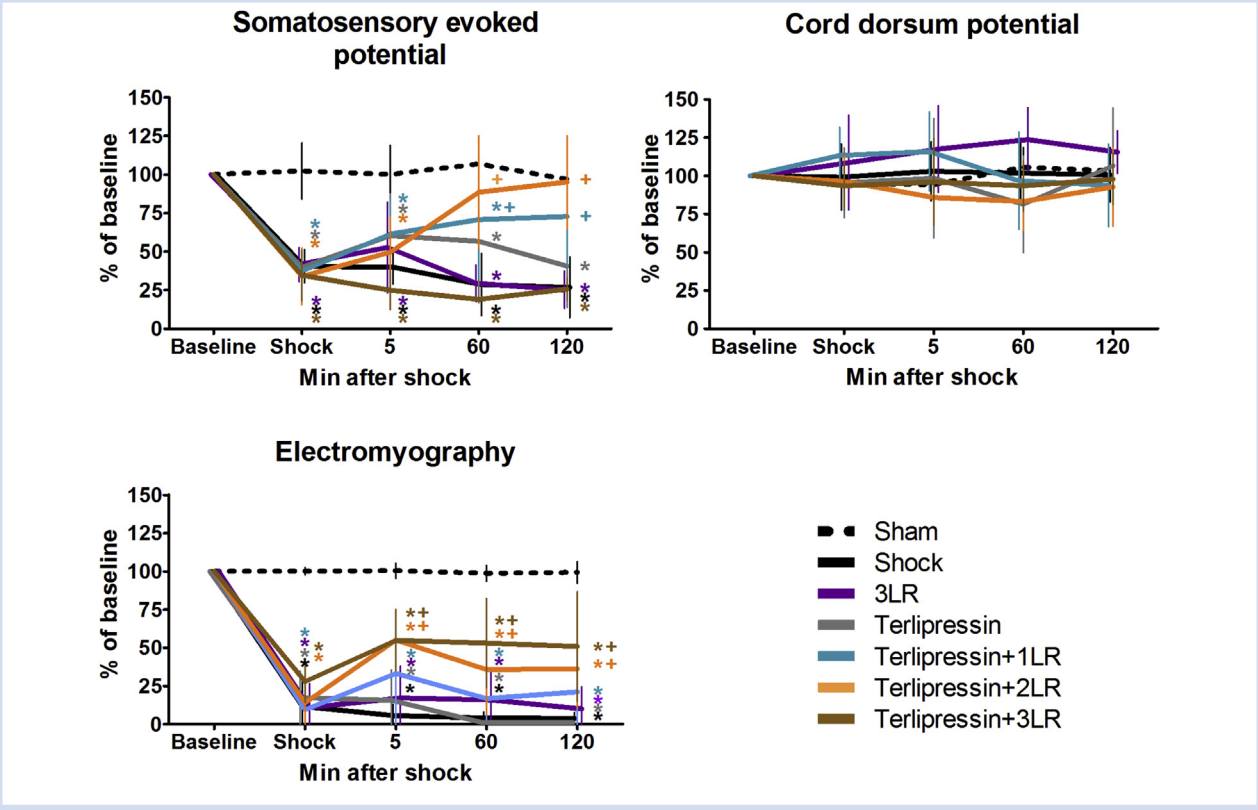


Fig 6. Changes in the amplitude of the somatosensory cortical evoked potential, cord dorsum potential, and electromyography in response to haemorrhagic shock and the different treatments. * *vs* Sham (at least $P<0.05$); + *vs* untreated Shock group (at least $P<0.05$). LR, lactated Ringer's solution.

and associated with a better mitochondrial membrane potential (required for ATP production) and well-perfused blood vessels. Mitochondrial and neuronal dysfunction were found to correlate with impaired capillary perfusion, illuminating an earlier discrepancy described between perfusion and total cerebral flow.[8]

Of note, we found that mitochondrial function was selectively preserved in cortical tissue surrounding arterioles, which reveals a profound spatial inhomogeneity in the vulnerability of cortical tissue to a reduced cerebral microcirculation.[19,20] A major reduction in oxygen supply to tissues remote from arteries can compromise oxidative

Table 2 Peak latency of all potentials. *ms*, miliseconds; LR, lactated Ringer' solution

Groups	Peak Latency in *ms* and *% of baseline* at T120		
	Somatosensory evoked potential	Cord dorsum potential	Electromyography
Sham	1.46 (0.45) ms 104 (13%)	0.54 (0.17) ms 96 (4%)	0.31 (0.07) ms 99 (4%)
Shock	1.21 (0.49) ms 129 (31%)	0.46 (0.15) ms 95 (22%)	0.33 (0.08) ms 132 (55%)
3LR	1.85 (0.66) ms 101 (31%)	0.53 (0.18) ms 106 (20%)	0.31 (0.07) ms 85 (14%)
Terlipressin	1.72 (0.32) ms 119 (37%)	0.58 (0.24) ms 89 (14%)	0.28 (0.06) ms 138 (59%)
Terlipressin + 1LR	1.55 (0.08) ms 85 (29%)	0.39 (0.09) ms 98 (7%)	0.28 (0.03) ms 128 (35%)
Terlipressin + 2LR	1.70 (0.19) ms 91 (13%)	0.37 (0.03) ms 93 (8%)	0.24 (0.05) ms 101 (30%)
Terlipressin + 3LR	1.51 (0.15) ms 91 (22%)	0.47 (0.16) ms 110 (28%)	0.24 (0.05) ms 129 (25%)

phosphorylation and thus cellular ATP availability. This may affect the ability to maintain neuronal excitability and signalling;[22] in agreement, we also report a loss of cortical evoked potential during shock. The status of both mitochondrial[23] and neuronal[24–26] function are known to have a close correlation with prognosis after shock.

The failure to recover capillary perfusion by standard aggressive fluid resuscitation is perhaps not unexpected, given the failure of all therapies to achieve persistent restoration of arterial pressure. This failure may result from the extravasation into interstitial tissues of large amounts of isotonic crystalloids,[27] causing brain swelling and thus compression within the skull, diminishing the cerebral perfusion pressure gradient.[3,9] Arguably the most important consequence is mitochondrial dysfunction, perhaps related to increased nitric oxide production[28] combined with reduced oxygen transport to mitochondria. This is particularly pertinent in shock states, as nitric oxide competes with oxygen for the same binding site on mitochondrial Complex IV (cytochrome oxidase).[28] Thus, an increase in oxygenation does not necessarily signal a good outcome, but perhaps a failure of oxygen utilization.

Small volume resuscitation has been proposed to avoid tissue oedema resulting from aggressive fluid resuscitation.[29] Indeed, animal models of haemorrhage have demonstrated that terlipressin can restore cerebral perfusion pressure without increasing intracranial pressure,[17] namely conditions required for an adequate microcirculation. An alternative approach has been to provide perfusion by small-volume isotonic fluid such as LR in combination with terlipressin, which results in a more sustained improvement in arterial pressure. The vasoconstrictor effect of terlipressin, which can be given by a single bolus injection, makes it a simple and practical treatment for use until hospital care is available. Terlipressin improved survival in models of haemorrhagic shock,[14–16] as we observed in the present study. The main adverse effect of terlipressin is the increase in systemic vascular resistance that can further compromise both heart function and local tissue blood flow.[30] However, in a porcine model of haemorrhagic shock, terlipressin was effective in redistributing blood flow to recover cerebral perfusion pressure and oxygenation without deleterious effects on systemic perfusion.[17] Accordingly, in human patients with catecholamine-resistant shock, terlipressin has been successfully used to improve cerebral perfusion pressure and oxygenation in cases of septic shock,[31] acute liver failure,[32] and traumatic brain injury.[33]

A benefit of terlipressin on the brain was the higher number of perfused vessels when associated with LR in small volumes. This indicates that terlipressin can reduce the volume of LR necessary for resuscitation, thereby reducing the adverse effects of aggressive volume resuscitation, such as cerebral expansion and compression. Studies using other vasopressors, such as norepinephrine, did not report improved cerebral perfusion pressure and oxygenation in models of haemorrhagic shock.[7,34]

Study limitations include the fact that the study was focused on the effects of a vasopressor after haemorrhagic shock, which resulted in the absence of groups treated with LR in a volume of two and three times the volume of blood removed to induce haemorrhagic shock. The absence of correlation between vessel perfusion and tissue oxygenation could be attributed to the fact that the method used to assess vessel perfusion could not differentiate arteries from veins as in the tissue oxygenation assessment. Finally, the data were limited to 2 h after shock in an attempt to reflect a common prehospital resuscitation regimen, and long-term outcomes remain unknown.

The significant recovery of cerebral mitochondrial and electrophysiological function by administration of terlipressin and small volumes of LR was associated with restoration of a near-normal density of perfused cortical vessels and cortical mitochondrial function, at 2 h, with recovery of cortical-evoked potentials. It is reasonable to expect that optimal resuscitation therapy may avoid the complications of haemorrhagic shock encephalopathy. None of the other therapies tested were as effective as the combination of terlipressin and small volume LR.

Authors' contributions

Designed the trial, obtained research funding, collected, analysed, and interpreted the data, drafted the manuscript, and contributed substantially to its revision: K.K.I.
Contributed to experimental planning, to data analysis and interpretation, and contributed substantially to its revision: K.I.C.
Conceived the study, obtained research funding, and contributed substantially to its revision: L.M.S.M.
Contributed to provision of experimental chemicals, to analysis and interpretation of data, and contributed substantially to its revision: D.B.P.
Provided senior advice on data analysis and interpretation and contributed substantially to manuscript revision: A.D., M.S., M.R.D.
Obtained research funding, supervised the conduct of the trial and data collection, provided senior advice to study design, data analysis, and interpretation, and contributed substantially to its revision: K.J.S.
Responsible for archiving the study files: K.K.I.
Read and approved the final manuscript: all authors.

Declaration of interest

The authors have no conflict of interest with any people or organization that could inappropriately influence this work. No potential competing interest is declared. D.P. is a stakeholder of Luscel Biosciences which provided the oxygen-sensitive microbeads for this study.

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