

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

5 Small-volume resuscitation with terlipressin (short running  
6 title)

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## 34 **Abstract**

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

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

47 **Results:** Compared with sham, haemorrhagic shock reduced the mean (standard deviation)  
48 area of perfused vessels [82% (10%) vs 38% (12%);  $P<0.001$ ] and impaired oxygen  
49 concentration, mitochondrial redox state [99% (4%) vs 59% (15%) of baseline;  $P<0.001$ ],  
50 and somatosensory evoked potentials [97% (13%) vs 27% (19%) of baseline].

51 Administration terlipressin plus 1LR or 2LR was able to recover these measures, but  
52 terlipressin plus 3LR or 3LR alone were not as effective. Spinal cord dorsum potential was  
53 preserved in all groups, but no therapy protected electromyographic function.

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

57

58 **Key words:** brain ischaemia; confocal microscopy; electrophysiology

## 59 Editor's key points

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

69

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

84 Vasopressors can reduce the volume of crystalloid required to recover blood pressure  
85 after haemorrhagic shock and can rapidly recover cerebral perfusion pressure during  
86 prehospital care.<sup>3 13</sup> Terlipressin, a synthetic analogue of vasopressin, has been proposed for  
87 the treatment of haemorrhagic shock.<sup>14 15</sup> Compared with vasopressin, terlipressin is longer  
88 acting and has higher selectivity for the vasopressin V<sub>1</sub> receptor.<sup>15 16</sup> Although studies in  
89 models of haemorrhage have demonstrated that terlipressin can improve cerebral perfusion  
90 pressure and tissue oxygenation<sup>12 17</sup>, their efficacy in protecting brain microcirculatory,  
91 mitochondrial and electrophysiological function is unknown. We therefore used confocal  
92 imaging to study the circulation and metabolic state of the brain during shock *in vivo*, and in  
93 real time. We postulated that small-volume resuscitation with terlipressin would be superior  
94 to more aggressive fluid replacement therapy in protecting mitochondrial and  
95 electrophysiological function, and perfused vessel density, in a rodent model of  
96 haemorrhagic shock.

97

## 98 **Methods**

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

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

119

### 120 ***In vivo* confocal microscopy**

121 The skull was fixed to a custom-made titanium bar using dental cement  
122 (Contemporary Ortho-Jet Powder, Lang Dental Manufacturing Co., Wheeling, IL, USA)

123 mixed with cyanoacrylate glue. The dura was removed, and platinum(II)-5,10,15,20-  
124 tetrakis(2,3,4,5,6-pentafluorophenyl)porphyrin (PtPFPP)-based phosphorescent oxygen-  
125 sensitive microbeads (Luxcel Biosciences, Cork, Ireland) applied to the cortex. The  
126 craniotomy was then sealed with a glass coverslip and petroleum jelly. Time-lapse  
127 fluorescence images were acquired with a laser-scanning confocal microscope (512 by 512  
128 pixels, optical slice 37.1µm; LSM 5 Pascal, Zeiss, Jena, Germany) to assess mitochondrial  
129 redox state by imaging endogenous flavoprotein fluorescence (excitation: 488nm; emission:  
130 505-570nm), and changes in local oxygen concentration (ex: 543nm; em: 650nm). At  
131 termination, intravenous fluorescein isothiocyanate-dextran 70 kDA (FITC-dextran; 0.5mg  
132 i.v.; ex: 488nm; em: 505-570nm; Sigma-Aldrich, Poole, Dorset) and topical  
133 tetramethylrhodamine methyl ester (TMRM; 1µM; ex: 543nm; em: 585nm; T-668,  
134 Molecular Probes, Invitrogen, Paisley, UK) were imaged to establish perfused vessel density  
135 and mitochondrial membrane potential, respectively. Images were processed using  
136 Fiji/Image J 1.48v (NIH, Bethesda, MD, USA).

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## 138 **Electrophysiology**

139 The right tibial nerve was stimulated (DS2, Digitimer, Welwyn Garden City, Herts)  
140 percutaneously at the ankle (10Hz, twice supramaximal), with recording electrodes at the  
141 vertebral level T10/T11, and on the cortical dura (-2mm from bregma, 2.5mm from midline),  
142 with reference electrodes on nearby inactive tissue. Another recording electrode was placed  
143 over the ipsilateral metatarsal musculature, with a reference electrode in the third digit. The  
144 ground electrode was inserted under the lumbar skin. Recordings of the somatosensory  
145 evoked potentials, cord dorsum potentials and electromyographic signals were amplified  
146 (Neurolog System, Digitimer), and observed on an oscilloscope (Sigma 60, Nicolet,

147 Madison, WI, USA) and stored as averaged ( $n=20$ ) compound action potentials. They were  
148 monitored as measures of cortical, spinal and muscular function, respectively.

149

## 150 **Study design**

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

163 Haemorrhagic shock was achieved by removing blood from the arterial line, targeting  
164 a MAP of 40 mmHg, maintained for 30 minutes by withdrawing or re-infusing blood when  
165 necessary, before treatment.

166 Data were recorded at baseline, after 30 minutes with MAP of 40 mmHg (shock), and  
167 at 5, 60, and 120 minutes (T5, T60 and T120) after treatment. After 150 min all surviving  
168 rats were culled at the end of the experiment.

169

## 170 **Statistical analysis**

171 The sample size was calculated in preliminary experiments using a power analysis that  
172 indicated a minimum of 26 rats/group was required for a 95% chance (with 5% risk) to detect a  
173 difference between groups of 60%, 40%, and 46% in the cortical somatosensory evoked potentials  
174 (n=6/group), mitochondrial redox state (n=10 rats/group), and changes in tissue oxygen concentration  
175 (n=10 rats/group), respectively, considering a standard deviation of 8%, 15%, and 5%, respectively.  
176 Endogenous flavoprotein fluorescence was analysed by determining the ratio between the  
177 mean intensity of areas adjacent to veins and arteries (perivenular:periarterial ratio).<sup>19 20</sup> The  
178 mean phosphorescence of the oxygen-sensitive beads was analysed by selecting up to six  
179 beads representing proximity to different vascular regions (the beads distribute randomly).  
180 The emission signals of flavoproteins and oxygen-sensitive beads analysed at each time-  
181 point were compared with their corresponding emission signal at baseline. The TMRM  
182 images were analysed 150 minutes after shock, as described for flavoproteins. The FITC-  
183 dextran fluorescence was analysed at 120 minutes after shock by determining the number of  
184 vessels crossing three equidistant horizontal and vertical lines, divided by the total length of  
185 the lines, and the area occupied by fluorescent vessels above a threshold brightness. In the  
186 electrophysiological recordings, the measurements at each time-point were compared with  
187 the measurement at baseline. Data were assessed for normality using Kolmogorov Smirnov  
188 test and were compared within and between groups using repeated measure two-way  
189 ANOVA followed by Tukey's *post hoc* testing (GraphPad Prism 5.03, GraphPad Software  
190 Inc., La Jolla, CA, USA). The 'last observation carried forward' method was used when  
191 animals died before the end of the study. Pearson's coefficient was calculated to assess  
192 correlation between variables. A 0-40 scoring system was calculated for each variable  
193 according to the percentage difference at T120/T150 compared with baseline/sham: (0) for 0-  
194 20%, (1) for 21-40%, (2) for 41-60%, (3) for 61-80%, and (4) for 81-100%. The sum of the



195 scores (0=worst, 40=best) was calculated to compare the effectiveness of each treatment.

196 Data were presented as mean and SD. Statistical significance was considered at  $P < 0.05$ .

197

## 198 **Results**

### 199 **Bleeding and survival**

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

208

### 209 **Cardiorespiratory variables**

210 Haemorrhagic shock caused a decrease in respiratory rate,  $\text{ETCO}_2$  and MAP in all  
211 groups compared with Sham ( $P < 0.001$ ; Fig. 1). This cardiorespiratory impairment did not  
212 recover at any time in the Shock group. MAP was significantly higher at T120 in all treated  
213 groups ( $P < 0.001$  vs Shock), although it remained lower compared with Sham ( $P < 0.001$ ). The  
214 respiratory rate was restored by all treatments at T120. However, the improvement in  $\text{ETCO}_2$   
215 was lower than in Sham, although higher than in Shock ( $P < 0.001$ ; Fig. 1).

216

### 217 **Cortical tissue oxygenation**

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

## 222 **Cerebral vascular density**

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

231

## 232 **Cerebral mitochondrial redox potential**

233           In all groups, haemorrhagic shock caused a significant decrease in flavoprotein  
234 fluorescence (i.e. increased reduced state) adjacent to veins, but fluorescence persisted in a  
235 'halo' around arteries (Fig. 4A), reflected by a reduction in the perivenular:periarterial  
236 fluorescence ratio ( $P<0.05$ ; Fig. 4B). In the Shock group, the ratio was lower at T120  
237 compared with Sham [59% (16%) vs 99% (4%) of baseline;  $P<0.001$ ; Fig. 4B]. All  
238 treatments were effective in increasing the fluorescence around veins at T5. At study end,  
239 administration of 3LR (73% (20%) of baseline;  $P<0.001$ ) and Terli+3LR [74% (18%) of  
240 baseline;  $P<0.001$ ] resulted in a lower perivenular:periarterial flavoprotein ratio compared  
241 with Sham, although higher than in Shock ( $P<0.05$ ). At T120, animals treated with Terli,  
242 Terli+1LR and Terli+2LR showed ratios not significantly different to Sham [87% (20%),  
243 82% (20%), and 92% (15%) of baseline, respectively], but higher than Shock ( $P<0.001$ ) (Fig.  
244 4).

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## 247 **Cortical mitochondrial membrane potential**

248 Mitochondrial membrane potential, indicated by TMRM fluorescence (Fig. 5),  
249 revealed that most mitochondria were depolarised (non-functional) following haemorrhagic  
250 shock. Only the mitochondria located near arteries remaining polarised, resulting in arterial  
251 ‘halos’ similar to those observed with flavoproteins. At 150 minutes after shock the  
252 perivenular:periarterial ratio was worse in the Shock group [0.28 (0.08);  $P<0.001$ ], 3LR  
253 [0.39 (0.13);  $P<0.001$ ] and Terli+3LR [0.34 (0.01);  $P<0.001$ ], compared with Sham [0.97  
254 (0.09)]. No significant differences in the perivenular:periarterial TMRM ratio were observed  
255 in the Terli [0.98 (0.31)], Terli+1LR [0.76 (0.22)] and Terli+2LR [0.71 (0.04)] groups  
256 compared with Sham; values were also better than in Shock (all  $P<0.001$ ).

257

## 258 **Electrophysiological function**

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

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272 **Correlations and scores for effectiveness of the treatments**

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

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

286

## 287 **Discussion**

288

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

295 All treatments improved survival within the time course of the study. While  
296 administration of terlipressin was generally associated with improvement in the measured  
297 variables, the combination of terlipressin and aggressive fluid (i.e. Terli+3LR) did not.  
298 Whether this is due to negative cardiovascular effects and/or other causes remains uncertain.  
299 If the amplitude of the cortical evoked potential is taken as the benchmark of a good  
300 outcome, this was optimally achieved by Terli+2LR, and associated with a better  
301 mitochondrial membrane potential (required for ATP production) and well-perfused blood  
302 vessels. Mitochondrial and neuronal dysfunction were found to correlate with impaired  
303 capillary perfusion, illuminating an earlier discrepancy described between perfusion and total  
304 cerebral flow.<sup>8</sup>

305 Of note, we found that mitochondrial function was selectively preserved in cortical  
306 tissue surrounding arterioles, which reveals a profound spatial inhomogeneity in the  
307 vulnerability of cortical tissue to a reduced cerebral microcirculation.<sup>19 20</sup> A major reduction  
308 in oxygen supply to tissues remote from arteries can compromise oxidative phosphorylation  
309 and thus cellular ATP availability. This may affect the ability to maintain neuronal  
310 excitability and signalling;<sup>22</sup> in agreement we also report a loss of cortical evoked potential

311 during shock. The status of both mitochondrial<sup>23</sup> and neuronal<sup>24-26</sup> function are known to  
312 have a close correlation with prognosis following shock.

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

324         Small volume resuscitation has been proposed to avoid tissue oedema resulting from  
325 aggressive fluid resuscitation.<sup>29</sup> Indeed, animal models of haemorrhage have demonstrated  
326 that terlipressin can restore cerebral perfusion pressure without increasing intracranial  
327 pressure,<sup>17</sup> namely conditions required for an adequate microcirculation. An alternative  
328 approach has been to provide perfusion by small-volume isotonic fluid such as LR in  
329 combination with terlipressin, which results in a more sustained improvement in arterial  
330 pressure. The vasoconstrictor effect of terlipressin, which can be given by a single bolus  
331 injection, makes it a simple and practical treatment for use until hospital care is available.  
332 Terlipressin improved survival in models of haemorrhagic shock,<sup>14-16</sup> as we observed in the  
333 present study. The main adverse effect of terlipressin is the increase in systemic vascular  
334 resistance that can further compromise both heart function and local tissue blood flow.<sup>30</sup>  
335 However, in a porcine model of haemorrhagic shock terlipressin was effective in

336 redistributing blood flow to recover cerebral perfusion pressure and oxygenation without  
337 deleterious effects on systemic perfusion.<sup>17</sup> Accordingly, in human patients with  
338 catecholamine-resistant shock, terlipressin has been successfully used to improve cerebral  
339 perfusion pressure and oxygenation in cases of septic shock,<sup>31</sup> acute liver failure,<sup>32</sup> and  
340 traumatic brain injury.<sup>33</sup>

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

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

355 The significant recovery of cerebral mitochondrial and electrophysiological function  
356 by administration of terlipressin and small volumes of LR was associated with restoration of  
357 a near-normal density of perfused cortical vessels and cortical mitochondrial function, at two  
358 hours, with recovery of cortical-evoked potentials. It is reasonable to expect that optimal  
359 resuscitation therapy may avoid the complications of haemorrhagic shock encephalopathy.



360 None of the other therapies tested were as effective as the combination of terlipressin and  
361 small volume LR.

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## 382 **Authors' contributions**

383           Designed the trial, obtained research funding, collected, analysed and interpreted the  
384 data, drafted the manuscript, and contributed substantially to its revision: K.K.I.

385           Contributed to experimental planning, to data analysis and interpretation, and  
386 contributed substantially to its revision: K.I.C.

387           Conceived the study, obtained research funding, and contributed substantially to its  
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389           Contributed to provision of experimental chemicals, to analysis and interpretation of  
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391           Provided senior advice on data analysis and interpretation and contributed  
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394 provided senior advice to study design, data analysis and interpretation, and contributed  
395 substantially to its revision: K.J.S.

396           Responsible for archiving the study files: K.K.I.

397           Read and approved the final manuscript: all authors.

398

## 399 **Declaration of interest**

400           The authors have no conflict of interest with any people or organization that could  
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404

405

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411

## 412 **References**

- 413 1. Kauvar DS, Lefering R, Wade CE. Impact of hemorrhage on trauma outcome: an overview of  
414 epidemiology, clinical presentations, and therapeutic considerations. *J Trauma* 2006; **60**: S3-11
- 415 2. Gutierrez G, Reines HD, Wulf-Gutierrez ME. Clinical review: hemorrhagic shock. *Crit Care* 2004;  
416 **8**: 373-381
- 417 3. Cavus E, Meybohm P, Doerges V, et al. Cerebral effects of three resuscitation protocols in  
418 uncontrolled haemorrhagic shock: a randomised controlled experimental study. *Resuscitation* 2009;  
419 **80**: 567-572
- 420 4. Vincent JL, De Backer D. Circulatory shock. *N Engl J Med* 2013; **369**: 1726-1734
- 421 5. Taccone FS, De Backer D. Is cerebral microcirculation really preserved in shock states? *Crit Care*  
422 *Med* 2010; **38**: 1008-1009
- 423 6. Meybohm P, Cavus E, Bein B, et al. Cerebral metabolism assessed with microdialysis in  
424 uncontrolled hemorrhagic shock after penetrating liver trauma. *Anesth Analg* 2006; **103**: 948-954
- 425 7. Meybohm P, Cavus E, Bein B, et al. Neurochemical monitoring using intracerebral microdialysis  
426 during systemic haemorrhage. *Acta Neurochir (Wien)* 2007; **149**: 691-698
- 427 8. Anwar M, Agarwal R, Rashduni D, et al. Effects of hemorrhagic hypotension on cerebral blood  
428 flow and perfused capillaries in newborn pigs. *Can J Physiol Pharmacol* 1996; **74**: 157-162
- 429 9. Ida KK ML, Otsuki DA, Chisholm KI, et al. Confocal imaging of impaired mitochondrial function  
430 in the cerebral cortex of rats during haemorrhagic shock in vivo. *Intensive Care Med Exp* 2014;  
431 **2(Suppl 1)**; O9

- 432 10. Guven H, Amanvermez R, Malazgirt Z, et al. Moderate hypothermia prevents brain stem  
433 oxidative stress injury after hemorrhagic shock. *J Trauma* 2002; **53**: 66-72
- 434 11. Meybohm P, Hoffmann G, Renner J, et al. Measurement of blood flow index during antegrade  
435 selective cerebral perfusion with near-infrared spectroscopy in newborn piglets. *Anesth Analg* 2008  
436 **106**: 795-803
- 437 12. Urbano J, Lopez-Herce J, Solana MJ, et al. Comparison of normal saline, hypertonic saline and  
438 hypertonic saline colloid resuscitation fluids in an infant animal model of hypovolemic shock.  
439 *Resuscitation* 2012; **83**: 1159-1165
- 440 13. Meybohm P, Cavus E, Bein B, et al. Small volume resuscitation: a randomized controlled trial  
441 with either norepinephrine or vasopressin during severe hemorrhage. *J Trauma* 2007; **62**: 640-646
- 442 14. Bayram B, Hocaoglu N, Atilla R, et al. Effects of terlipressin in a rat model of severe uncontrolled  
443 hemorrhage via liver injury. *Am J Emerg Med* 2012; **30**: 1176-1182
- 444 15. Lee CC, Lee MT, Chang SS, et al. A comparison of vasopressin, terlipressin, and lactated ringers  
445 for resuscitation of uncontrolled hemorrhagic shock in an animal model. *PLoS One* 2014; **9**: e95821
- 446 16. Cossu AP, Mura P, De Giudici LM, et al. Vasopressin in hemorrhagic shock: a systematic review  
447 and meta-analysis of randomized animal trials. *Biomed Res Int* 2014; **2014**: 421291
- 448 17. Ida KK, Otsuki DA, Sasaki AT, et al. Effects of terlipressin as early treatment for protection of  
449 brain in a model of haemorrhagic shock. *Crit Care* 2015; **19**: 107
- 450 18. Dyson A, Stidwill R, Taylor V, et al. The impact of inspired oxygen concentration on tissue  
451 oxygenation during progressive haemorrhage. *Intensive Care Med* 2009; **35**: 1783-1791
- 452 19. Chisholm KI, Ida KK, Davies AL, et al. In vivo imaging of flavoprotein fluorescence during  
453 hypoxia reveals the importance of direct arterial oxygen supply to cerebral cortex tissue. *Adv Exp Med*  
454 *Biol* 2016; **876**: 233-239
- 455 20. Chisholm KI, Ida KK, Davies AL, et al. Hypothermia protects brain mitochondrial function from  
456 hypoxemia in a murine model of sepsis. *J Cereb Blood Flow Metab* 2015; **36**: 1955-1964
- 457 21. Lee HB, Blaufox MD. Blood volume in the rat. *J Nucl Med* 1985; **26**: 72-76

- 458 22. Erecinska M, Silver IA. Tissue oxygen tension and brain sensitivity to hypoxia. *Resp Physiol*  
459 2001; **128**: 263-276
- 460 23. Fullerton JN, Singer M. Organ failure in the ICU: cellular alterations. *Semin Respir Crit Care*  
461 *Med* 2011; **32**: 581-586
- 462 24. Gregory PC, McGeorge AP, Fitch W, et al. Effects of hemorrhagic hypotension on the cerebral-  
463 circulation .2. electrocortical function. *Stroke* 1979; **10**: 719-723
- 464 25. Meldrum BS, Brierley JB. Brain damage in the rhesus monkey resulting from profound arterial  
465 hypotension. II. Changes in the spontaneous and evoked electrical activity of the neocortex. *Brain Res*  
466 1969; **13**: 101-118
- 467 26. Graham DI, Fitch W, MacKenzie ET, et al. Effects of hemorrhagic hypotension on the cerebral  
468 circulation. III. Neuropathology. *Stroke* 1979; **10**: 724-727
- 469 27. Cotton BA, Guy JS, Morris JA, Jr., et al. The cellular, metabolic, and systemic consequences of  
470 aggressive fluid resuscitation strategies. *Shock* 2006; **26**: 115-121
- 471 28. Umbrello M, Dyson A, Feelisch M, et al. The key role of nitric oxide in hypoxia: hypoxic  
472 vasodilation and energy supply-demand matching. *Antioxid Redox Signal* 2013; **19**: 1690-1710
- 473 29. Tan PG, Cincotta M, Clavisi O, et al. Review article: Prehospital fluid management in traumatic  
474 brain injury. *Emerg Med Austr* 2011; **23**: 665-676
- 475 30. Beloncle F, Meziani F, Lerolle N, et al. Does vasopressor therapy have an indication in  
476 hemorrhagic shock? *Ann Intensive Care* 2013; **3**: 13-19
- 477 31. O'Brien A, Clapp L, Singer M. Terlipressin for norepinephrine-resistant septic shock. *Lancet*  
478 **2002**; 359: 1209-1210
- 479 32. Eefsen M, Dethloff T, Frederiksen HJ, et al. Comparison of terlipressin and noradrenalin on  
480 cerebral perfusion, intracranial pressure and cerebral extracellular concentrations of lactate and  
481 pyruvate in patients with acute liver failure in need of inotropic support. *J Hepatol* 2007; **47**: 381-386
- 482 33. Salluh JJ, Martins GA, Santino MS, et al. Early use of terlipressin in catecholamine-resistant  
483 shock improves cerebral perfusion pressure in severe traumatic brain injury. *Acta Anaesthesiol Scand*  
484 2007; **51**: 505-508

485 34. Cavus E, Meybohm P, Dorges V, et al. Regional and local brain oxygenation during hemorrhagic  
486 shock: a prospective experimental study on the effects of small-volume resuscitation with  
487 norepinephrine. *J Trauma* 2008; **64**: 641-648

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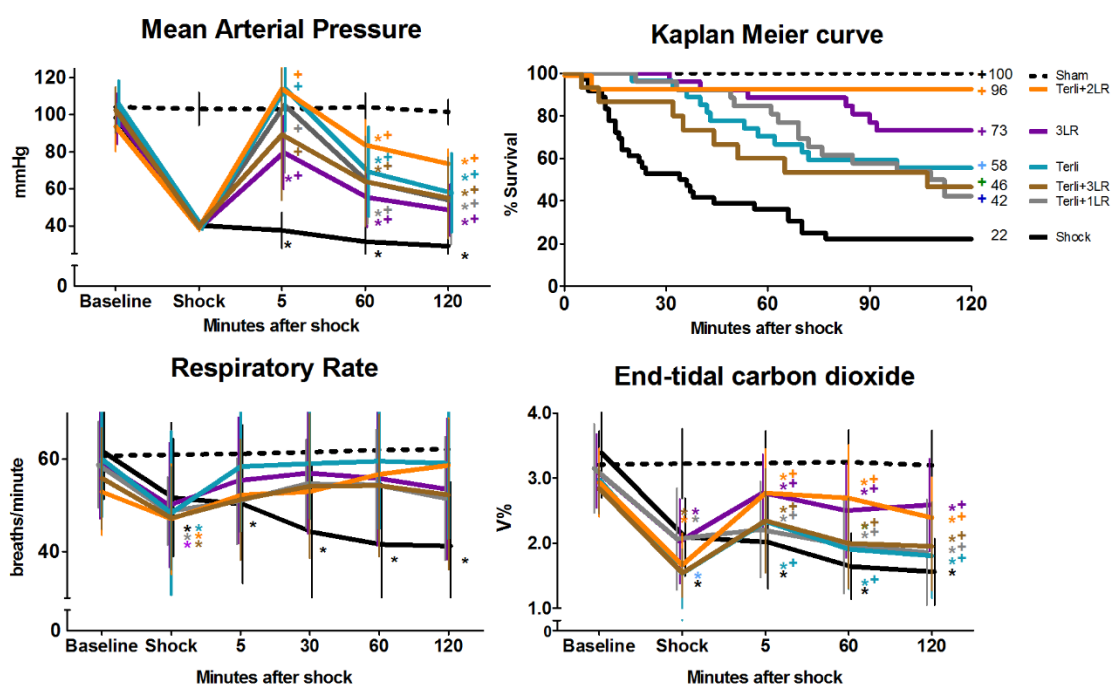
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508 **Figures**

(a)

	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 <sup>+</sup>
Terlipressin	162.9 ± 24.8	37.8 ± 8.2	52 ± 21
Terlipressin + 1LR	148.5 ± 15.7	43.6 ± 6.9	72 ± 27 <sup>*</sup>
Terlipressin + 2LR	160.7 ± 36.9	42.8 ± 6.7	8
Terlipressin + 3LR	160.6 ± 26.3	43.2 ± 11.4	44 ± 32

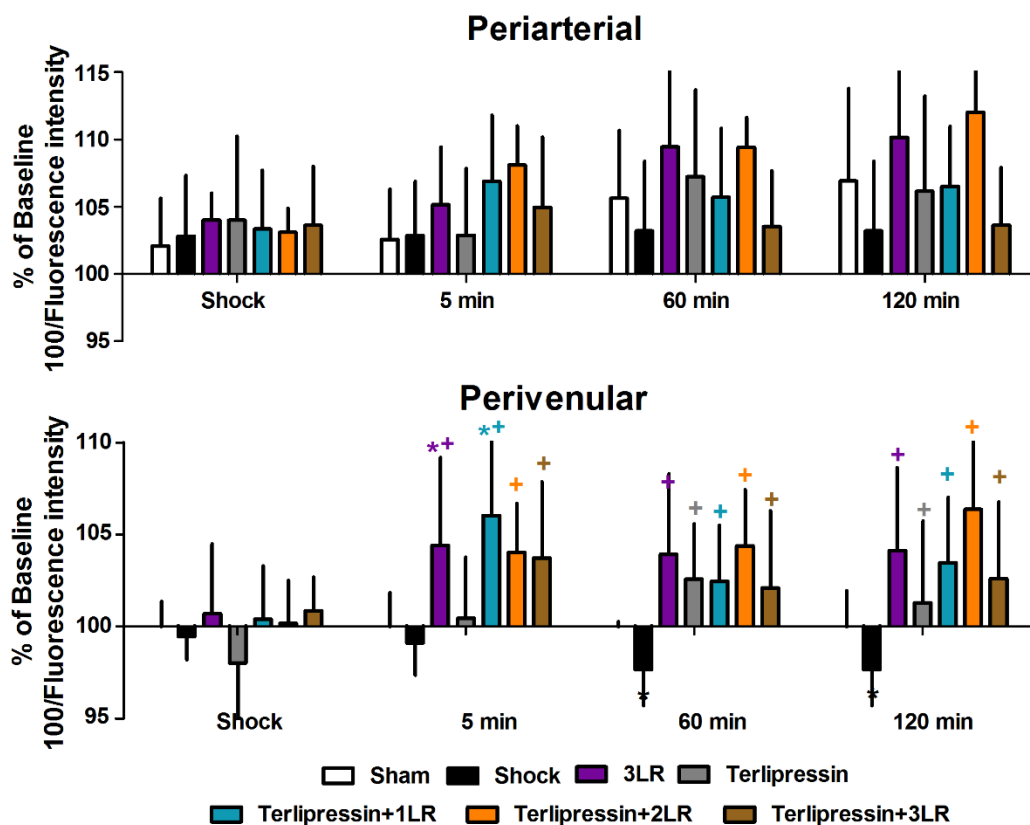
(b)



509

510 **Figure 1.** (a) Data showing mean bodyweight, mean estimated blood volume withdrawn to  
 511 induce haemorrhagic shock, and mean time until death after shock. EBV: estimated blood  
 512 volume. (b) Kaplan Meier curve and changes in mean arterial pressure, respiratory rate and  
 513 end-tidal carbon dioxide induced by haemorrhagic shock, and treatment with LR, terlipressin  
 514 and combined treatments of LR plus terlipressin. \*vs Sham (at least  $P < 0.05$ ); <sup>+</sup> vs Not treated  
 515 Shock group (at least  $P < 0.05$ ).

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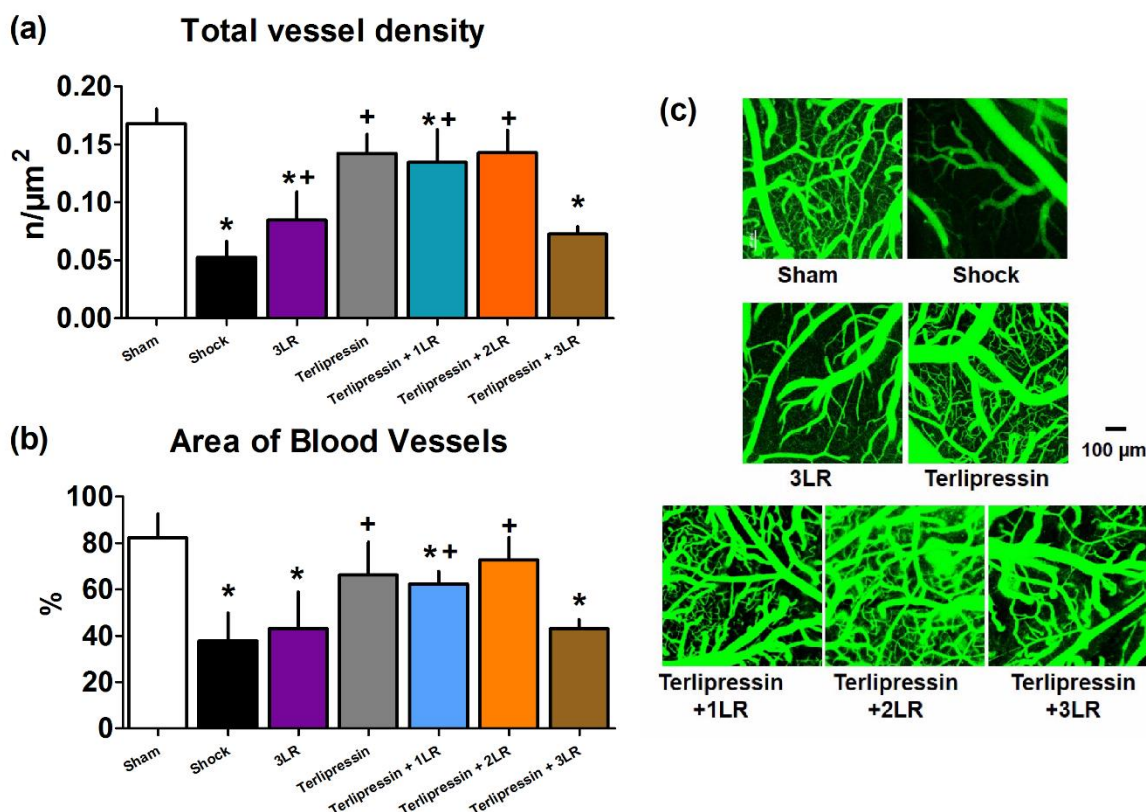


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518 **Figure 2.** Changes in phosphorescence of oxygen-sensitive microbeads according to their  
 519 location relative to the veins and arteries throughout the study. \* vs Sham of the same time-  
 520 point (at least  $P < 0.05$ ); + vs Not treated Shock group of the same time-point (at least  $P < 0.05$ ).

521





522

523 **Figure 3.** Vasculature of the cerebral cortex assessed by FITC-dextran administered

524 intravenously in rats after 120 minutes of haemorrhagic shock. (a) Total perfused vessel

525 density reflects the quantity of blood vessels with flow. (b) Representative *in vivo* confocal

526 images of the cortical vasculature revealed by the FITC-dextran. (c) Area fraction of blood

527 vessels represents the relative area covered by the FITC-dextran fluorescence. The marker

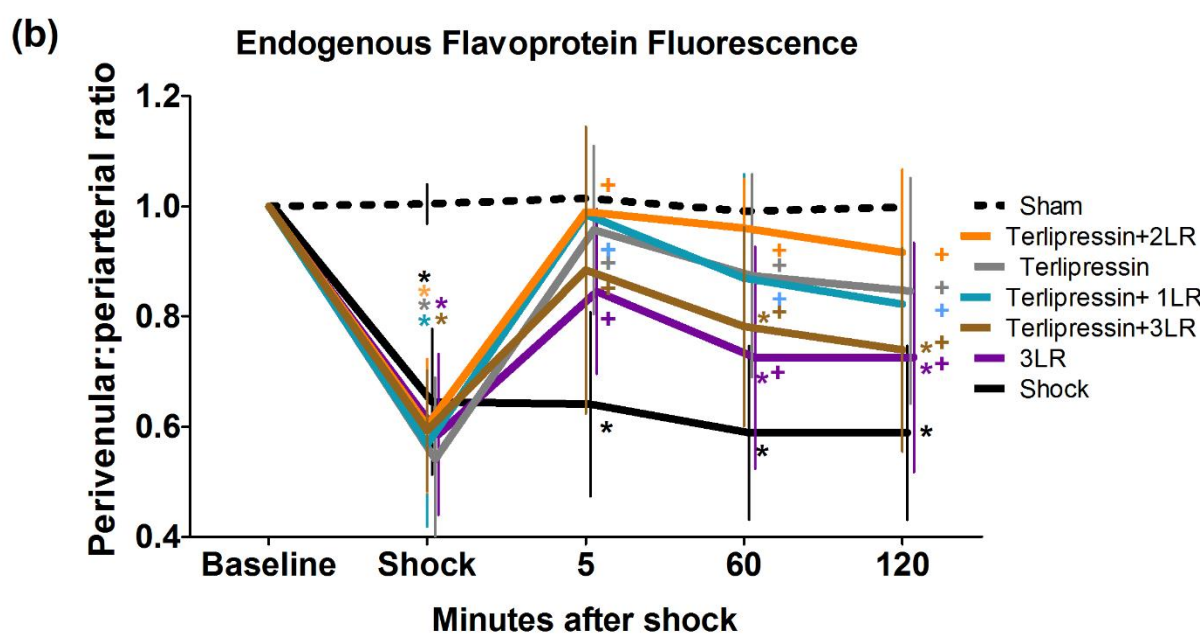
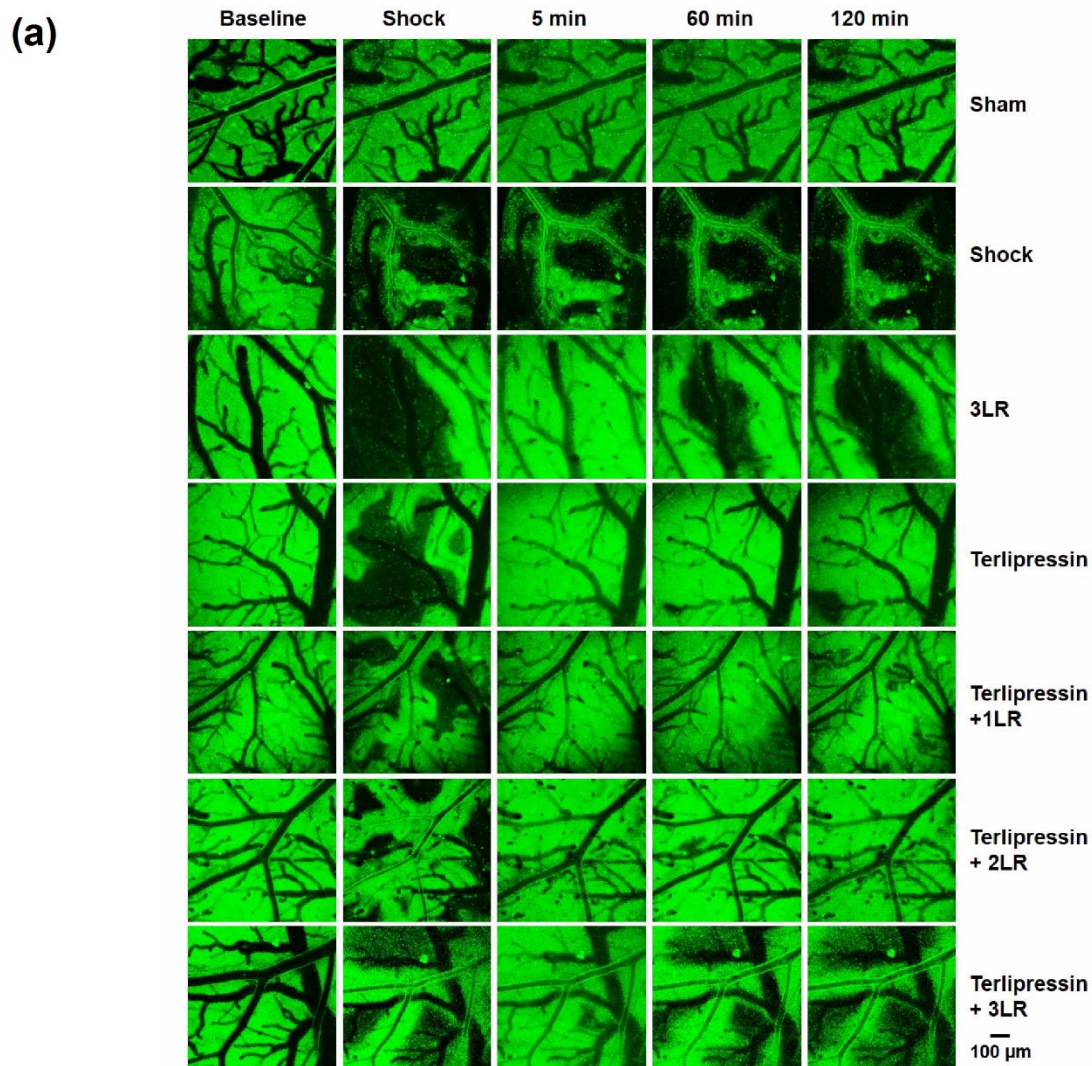
528 was administered intravenously and, therefore, was only present in perfused blood vessels.

529 The images of the Not treated Shock, 3LR and Terli+3LR groups show notably fewer vessels

530 than Sham. Images of rats receiving Terli and Terli+2LR showed no significant differences

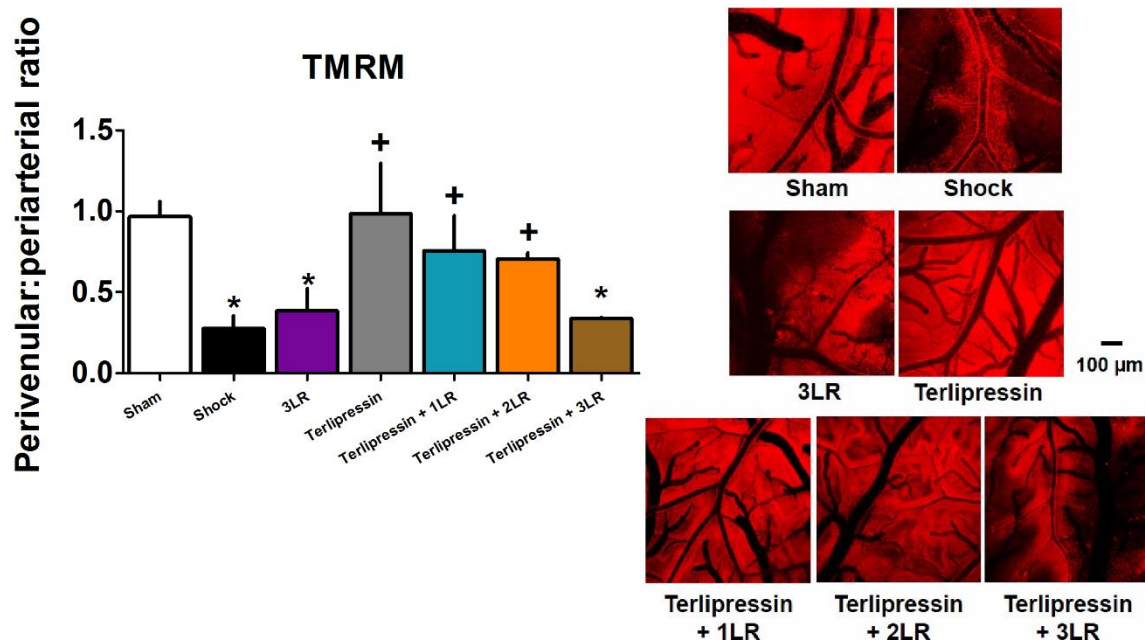
531 with Sham. \* vs Sham (at least  $P < 0.05$ ); <sup>+</sup> vs Not treated Shock group (at least  $P < 0.05$ ).

532



534 **Figure 4.** Changes in endogenous flavoprotein fluorescence induced by haemorrhagic shock  
 535 followed by different treatments. (a) *In vivo* confocal images of rat cerebral cortex showing  
 536 mitochondrial function revealed by the intrinsic fluorescence of oxidized endogenous  
 537 flavoprotein (green). Whereas the normal brain displays a quite uniform green fluorescence  
 538 for flavoproteins before haemorrhagic shock, the fluorescence was lost almost everywhere  
 539 except for a 'halo' around arteries after shock. All treatments gave some recovery of  
 540 fluorescence at 5 minutes, but the recovery after some treatments was only temporary. At  
 541 120 minutes after shock, the flavoprotein fluorescence persisted only in the Terli and  
 542 Terli+2LR groups. (b) Changes in the perivenular:periarterial ratio of the endogenous  
 543 flavoprotein fluorescence throughout the study. \* vs Sham (at least  $P<0.05$ ); + vs Not treated  
 544 Shock group (at least  $P<0.05$ ).

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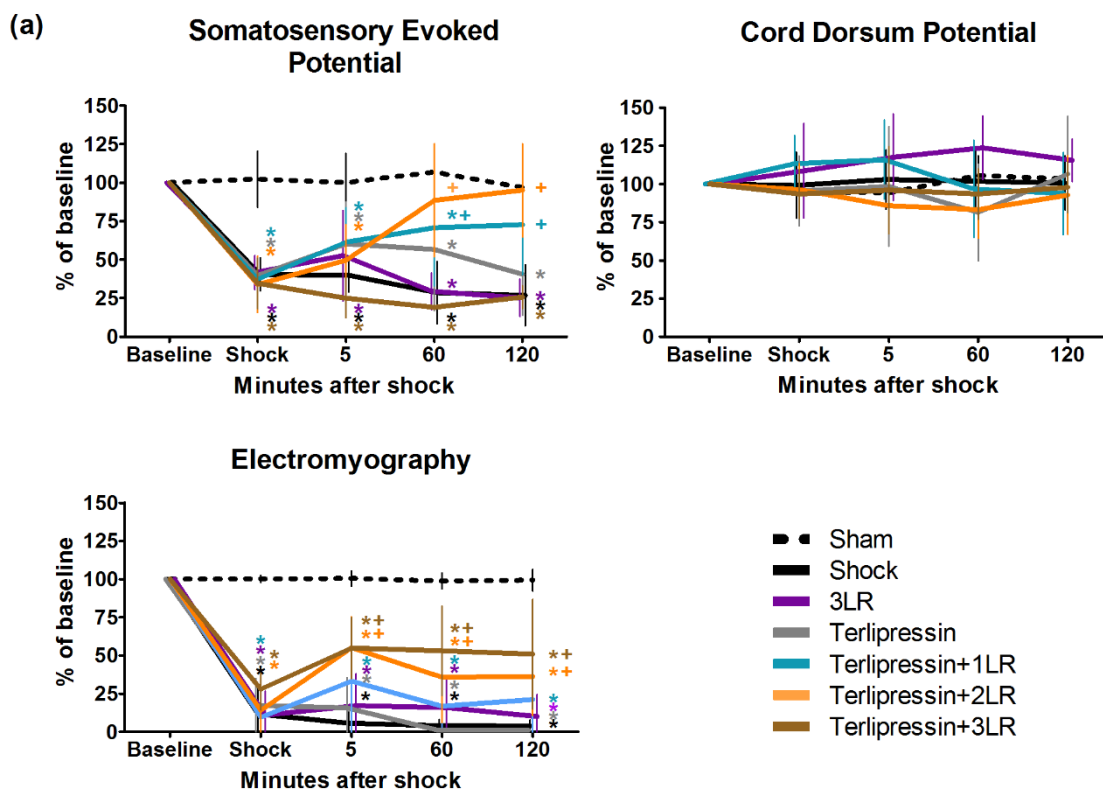


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547 **Figure 5.** Graph and confocal *in vivo* images showing changes in mitochondrial membrane  
 548 potential revealed by TMRM in the cerebral cortex of rats at 150 minutes after haemorrhagic

549 shock. TMRM fluorescence only accumulates within mitochondria possessing a membrane  
550 potential. In the not-treated Shock group many mitochondria were depolarized, and  
551 presumably non-functional; only mitochondria near arteries remained polarized, resulting in  
552 the formation of periarterial TMRM ‘halos’. A similar pattern of periarterial halos was  
553 observed in rats treated with 3LR and Terli+3LR, though polarized mitochondria were  
554 distributed more widely in rats treated with Terli, Terli+1LR and Terli+2LR: indeed, rats  
555 treated with these regimens had a spread of polarized mitochondria that was not significantly  
556 different to Sham, \* vs Sham (at least  $P<0.05$ ); + vs Not treated Shock group (at least  
557  $P<0.05$ ).

558



(b)

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

559

560 **Figure 6.** Changes in the amplitude of the somatosensory cortical evoked potential, cord  
 561 dorsum potential and electromyography in response to haemorrhagic shock and the different  
 562 treatments. \* vs Sham (at least  $P < 0.05$ ); + vs Not-treated Shock group (at least  $P < 0.05$ ).