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ORIGINAL ARTICLE

Low Oxygen Post Conditioning as an Efficient Non-pharmacological Strategy to Promote Motor Function After Stroke

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Abstract

Low oxygen post conditioning (LOPC) has shown promising results in terms of neuroprotection after stroke, but the effects on motor function have not been considered. Cortical stroke targeting the motor and sensory cortex was induced by photothrombotic occlusion and after 48 h allocated to LOPC (11% O_2) for 2 weeks. Motor impairment was assessed using the cylinder and grid walk tests during the exposure period and for two further weeks upon completion of the intervention. Neuroprotection was evaluated by histological and molecular analysis at two time points. Two weeks of LOPC was sufficient to significantly reduce motor deficits and tissue loss after stroke. This functional improvement was associated with increased capillary density, enhanced levels of BDNF, decreased neuronal loss and decreased microglia activation. These improvements, in most instances, were maintained up to 2 weeks after the end of the treatment. To our knowledge, this is the first study to demonstrate that LOPC induces a persistent improvement in motor function and neuroprotection after stroke, and in doing so provides evidence to support a case for considering taking LOPC forward to early stage clinical research.

Keywords Stroke · Motor function · Neuroprotection · BDNF · Hypoxia · Angiogenesis · Inflammation

Introduction

For several decades, it has been recognised that reducing the concentration of inspired oxygen over the course of several weeks can robustly improve athletic performance at sea level [1–3]. Performance improvements have been largely attributed to the pronounced improvements in cardiovascular function associated with intervention [4]. The benefits in stimulating cardiovascular fitness in otherwise healthy individuals have started to attract interest from those working on promoting cardiovascular fitness and repair in the context of pathology. Notably, Nakada et al. have recently identified that exposing adult mice to a reduced oxygen environment exerts a pronounced regenerative effect when deployed after myocardial infarction [5].

Several preclinical studies have now begun to consider the therapeutic potential of exposure to low oxygen environments after stroke. Low oxygen post conditioning (LOPC), or intermittent hypoxic post conditioning, has been shown to robustly promote neurogenesis and vasculogenesis [6–8]. Extending from this, LOPC has been shown to improve cognitive function, an effect that appears to be associated with a reduction in neuroinflammation [9]. LOPC has also been found to limit thalamic atrophy after stroke, with a neuroprotective effect confirmed in vitro [10]. Another major advantage of LOPC is that the equipment necessary to deploy the intervention is already commercially available, has a recognised safety profile, is available from several manufactures and is moderately priced.

Although it has not yet been clinically considered in the context of stroke, LOPC has been shown to be beneficial in

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patients with spinal cord injury [11–13], improving ankle strength [14], walking speed and endurance [15, 16] and hand function [17]. Given the promising results shown in improving motor function after spinal cord injury, it is somewhat surprising that the intervention has not been evaluated for its promotor effects after stroke, especially considering the ability of the intervention to promote vasculogenesis and neurogenesis. Given this situation, the primary aim of the current study was to examine the extent to which LOPC could promote motor function following exposure to LOPC.

LOPC involved exposure 48 h post stroke to normobaric 11% oxygen for 8 h/day for 14 days. To induce stroke in the motor and somatosensory cortex, we chose the photothrombotic (PT) model because it is well recognised to induce highly controllable vascular occlusion and, importantly, it produces only a modest penumbral area. Therefore, for longer term repair and rehabilitation studies that are concerned with cellular events long after penumbral conversion, the PT model represents an ideal model. To assess motor deficits, we used the cylinder test and the foot fault test (as per [18]). The behaviour of the mice was evaluated before, during and after intervention with a total follow-up period of 30 days. This allowed us to examine the immediate and persistent influence of LOPC on motor function. Prompted by the promising results, we investigated whether these improvements were associated with decreased level of tissue loss within the ipsilateral hemisphere along with improvements in the level of brain-derived neurotrophic factor, vasculogenesis and decreased microgliosis, all features that have been shown to be associated with a neuroprotective phenotype. This study itself involved four groups where we examined the impact of LOPC compared to controls in stroked animals at 2 weeks (immediately following the end of LOPC exposure) and then again at 4 weeks (2 weeks after the end of exposure).

Material and Methods

All the experimental groups were randomised and coded by an independent team member to blind the experimenter to the treatment condition.

Study Design

This study was planned and conducted according to the ARRIVE guidelines. In all experiments, mice were acclimatised to the environment and to the experimenter to avoid stress. Two to four mice were housed for cage. Mice were maintained in a temperature- (21 °C ± 1) and humidity-controlled environment with food and water available ad libitum. Lighting was on a 12:12 h reverse light–dark cycle (lights on 19:00 h) with all procedures conducted in the dark phase. Sample size was estimated using the following formula [19]:

$$SS = \frac{2SD^2\left(z_{1-\frac{\alpha}{2}} + z_{1-\beta}\right)^2}{d^2}$$

Using previous and preliminary data on hypoxic treatment, we estimated the effect size. We allowed a type-1 error $\alpha = 0.05$ with power of 0.8, and we calculated a sample size of six animals/group for immunohistology, eight for western blot and seven for motor test. A total of 80 male C57/BL6 8 weeks old were allocated into groups dedicated to different follow-up experiment (western blot, immunohistology and behavioural test) and underwent stroke surgery. After 48 h, mice were randomly assigned to the control (stroke) or treated (LOPC) groups and randomly sacrificed at 15 or 30 days for all experiments (Fig. 1a). A total of 16 mice were used for motor test (stroke $n = 7$, LOPC $n = 9$). A total of 35 mice were used for western blot (stroke 15 days $n = 8$, LOPC 15 days $n = 8$, stroke 30 days $n = 9$ and LOPC 30 days $n = 10$) (Fig. 2a). A total of 27 mice were used for immunohistology (stroke 15 days $n = 6$, LOPC 15 days $n = 6$, stroke 30 days $n = 7$ and LOPC 30 days $n = 8$) (Fig. 2a). As exclusion criteria, mice were removed from the study if the stroke was not identified by histological evaluation.

Photothrombotic Occlusion

On day 0, mice were anesthetised by using 2% of isoflurane and injected intraperitoneally with 0.2 mL of 10 mg/mL of rose bengal. After 8 min, a cold light source with a fibre optic end of 4.5 mm diameter was placed 2.2 mm left lateral of the bregma onto the exposed skull for 15 min [20].

Low Oxygen Post Conditioning

Forty-eight hours after stroke induction (day 2), cages of animals were randomly allocated to the control (atmospheric oxygen) and LOPC groups (11% oxygen, 8 h/day) for 14 days (Figs. 1a and 2a), after which half of each group was euthanised for tissue collection. A customised hypoxic environment consisting of an independently ventilated cage system provided 11% oxygen, at atmospheric CO_2 concentration (300 ± 50 ppm) and atmospheric pressure (100 kPa). The remaining half of both groups was exposed to atmospheric air for further 14 days before the endpoint at day 30.

Assessment of Motor Deficit

Motor function was evaluated by cylinder test and foot fault test as described [18, 21]. We assessed the baseline for these tests 2 days before stroke induction (Figs. 1a and 2a). The

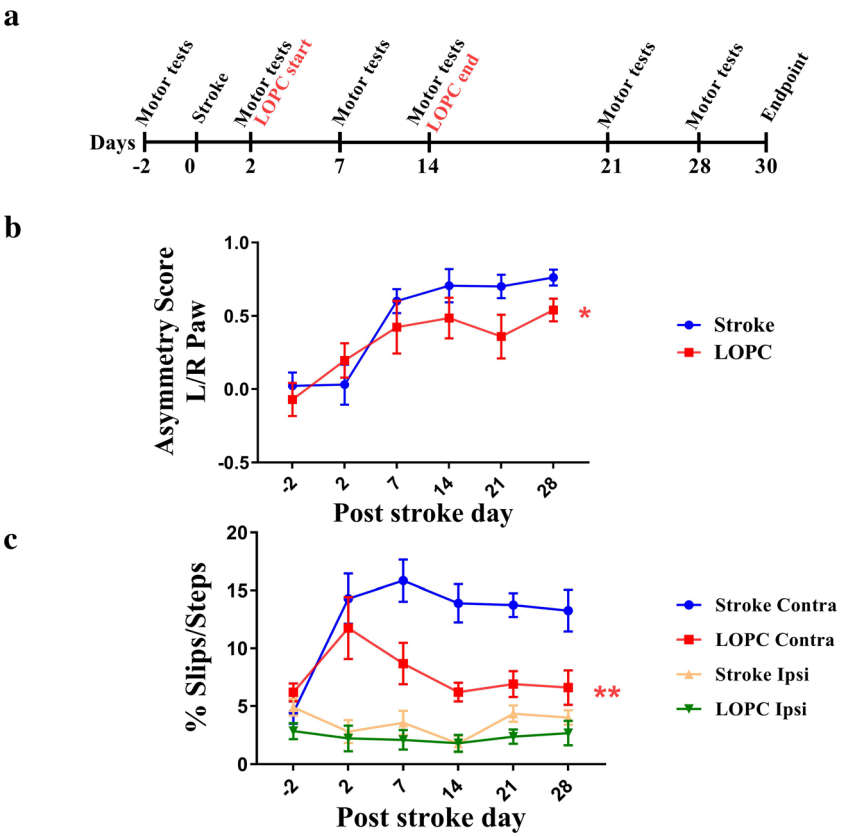

Fig. 1 LOPC prevents impaired motor function and tissue loss. **a** Experimental design for motor testing. Asymmetry score evaluated by cylinder test (**b**) and paw slips count in the grid walk test (**c**) show that mice that underwent LOPC significantly preserve their motor function. Ipsi, ipsilateral paw; Contra, contralateral paw. Results are shown as the mean ± SEM. Results are shown as the mean ± SD. $*p < 0.05$, $**p < 0.01$, two-way ANOVA

deficit was evaluated 2 days post stroke before the beginning of LOPC, during LOPC at days 7 and 14 and after the end of treatment at days 21 and 28. Control and LOPC groups underwent testing the same day and under the same conditions of light and noise.

Cylinder Test Two days before (day − 2) and days 2 (before the first LOPC session), 7, 14, 21 and 28 after stroke, each mouse was placed in a glass cylinder, and movements were recorded from both sides for 10 min. Afterwards, paw placement was determined by researcher blinded to the experimental condition. The first forelimb to contact the wall during a full rear determined one score for that side. The simultaneous contact of both the left and right forelimbs to the wall during a full rear was considered as one placement for both limbs. Up to 20 movements were scored, and mice below ten rears were excluded. A final asymmetry score was calculated as the ratio of non-impaired forelimb movement minus impaired forelimb movement to total forelimb movement. Total of $n = 16$ mice were used for this test.

Grid Walk This test determines sensorimotor function and motor coordination deficits during locomotion. Two days before (day − 2) and days 2 (before the first LOPC session), 7, 14, 21 and 28 after stroke, each mouse was placed on elevated grid of 2×2 cm^2. Healthy mice (− 2 days, before stroke) walk on the grid place paws precisely on the bars with a low percent of slips. After stroke, the mouse motor coordination of the contralateral paw is compromised while the ipsilateral paw is not affected. Each mouse was recorded for 5 min from below the grid and at an angle of − 20°, and a number of foot faults on each side, defined as limb crossing the grid, were calculated by a blinded researcher on a total of 50 steps after 30 s from the beginning of the test. Total of $n = 16$ mice were used for this test.

Tissue Processing

At the scheduled endpoint (day 15 or 30), mice were deeply anaesthetised with sodium pentabarbitol and perfused via the ascending aorta with ice cold PBS followed by ice cold 4% paraformaldehyde (pH 7.4) for immunohistochemical analysis or with cold PBS only for western blotting. For immunohistochemical analysis, the brains were dissected, post fixed in 4% paraformaldehyde (4 h) and transferred to sucrose 12.5% in PBS for storage for a maximum of 1 month. Coronal sections of the brains were sectioned at a thickness of 30 μm with a freezing microtome (Leica). For cohorts dedicated to western blot analysis, the brains were dissected and flash-frozen in − 80 °C isopentane. The frozen brains were sliced using the cryostat at a thickness of 200 μm. The tissue was then punched using 2-mm tissue punch in the peri-infarct region. Samples were stored frozen in − 80 °C until further analysis.

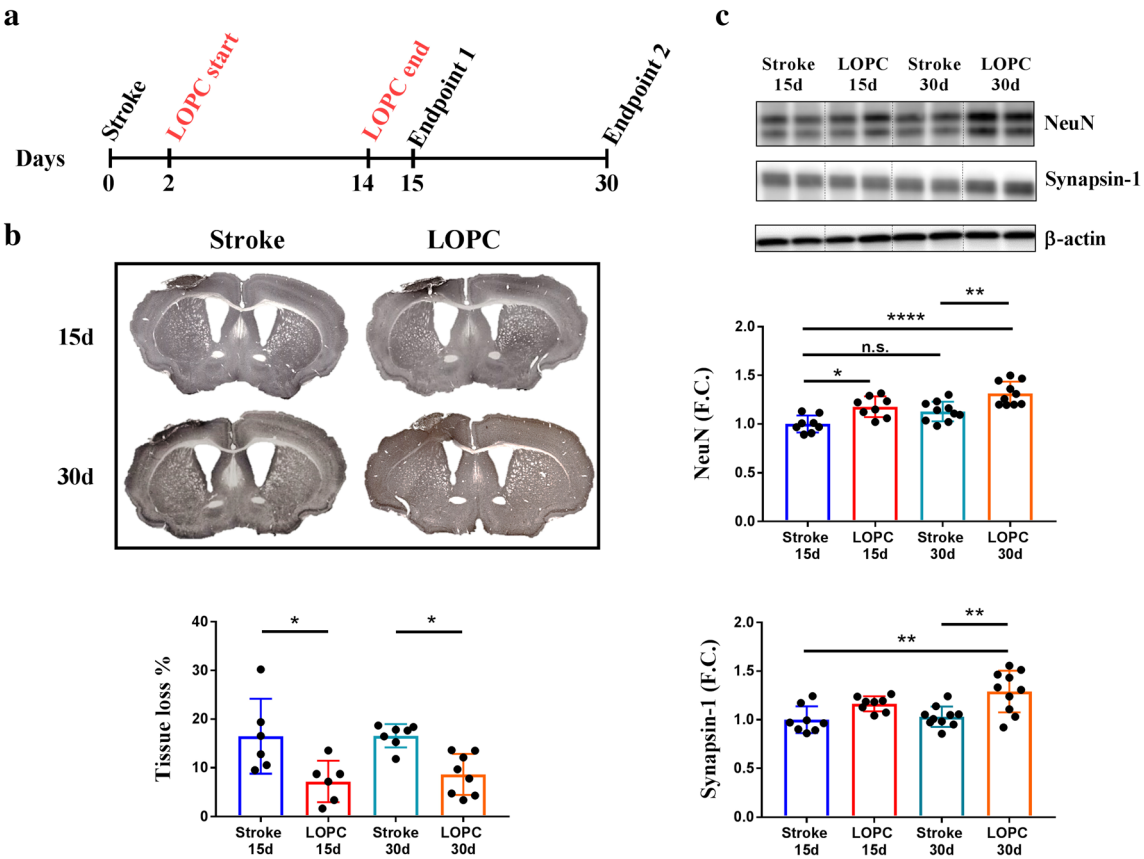

Fig. 2 LOPC is neuroprotective. **a** Experimental design of post condition and follow-up for histological and biochemical analysis. **b** NeuN staining at bregma 0. Evaluation of tissue loss as area (%) of the ipsilateral to the contralateral hemisphere shows decreased tissue loss in LOPC mice. **c** Representative blot shows increase of the neuronal marker NeuN and the presynaptic marker synapsin-1 after LOPC. Results are shown as the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s., not significant. Two-way ANOVA and Turkey's multiple comparison test

Molecular Analysis by Western Blot and ELISA

Punched samples were sonicated in 150-µl lysis buffer (50-mM tris buffer pH 7.4, 1-mM EDTA, 1-mM DTT, 80-µM ammonium molybdate, 1-mM sodium pyrophosphate, 1-mM sodium vanadate, 5-mM β-glycerolphosphate, 1% SDS, 1 protease inhibitor cocktail tablet, 1 phosphatase inhibitor cocktail tablet, final concentration) and centrifuged for 20 min at 4 °C. Samples for ELISA were sonicated in 50-mM tris buffer pH 7.4, 150-mM NaCl and 1% Triton X-100 with protease inhibitor cocktail according to manufacturer instruction. Next, supernatants were collected and protein levels were estimated by Pierce BCA protein assay kit according to the manufacturer instructions. For western blot analysis, 15 µg of lysate was loaded per lane while 20 µg/well was used for ELISA (Fig. 3). After transferring and blocking, the membrane was incubated overnight with the appropriate antibody: CD11b (cat#ab75476, Abcam), CD45 (cat#ab208022, Abcam), CD68 (cat#ab76308, Abcam), NeuN (cat#MAB377, Millipore), BDNF (cat#SC-546, Santa-Cruz), Synapsin-1 (cat#5297, Cell Signalling) and β-actin (cat#A3854, Sigma-Aldrich). Analysis was performed with the Amersham Imager 600 analysis software.

Immunohistochemistry and Immunofluorescence

Free-floating sections were immunostained as described with minor modification [22]. All reactions for label markers were run at the same time, with the same reagents, at the same concentrations. Briefly, brain sections were incubated with 1-µg/ml pepsin in 0.01-M HCl for 2 min at 37 °C to retrieve antigen. Brain sections were then incubated with 1% hydrogen peroxidase for 30 min at RT followed by blocking with 3% horse serum for 30 min. Brain sections were incubated with primary antibodies: Iba1 (cat#019-19741, WAKO), NeuN (Millipore, cat#MAB377) and CD31 (cat#77699, Cell Signalling) for 72 h at 4 °C followed by appropriate secondary antibodies for 1 h at 25 °C. Next, brain sections were incubated for 2 h at 25 °C with avidin–biotin-peroxidase complex and finally developed using DAB peroxidase substrate. Brain sections were washed with PBS in between each incubation step. After the processing was complete, brain sections were mounted onto polylysine-coated slides and cover slipped. For lectin staining, after blocking as described above, brain sections were incubated in PBS-T with GFP labelled tomato-lectin (cat#L0401, Sigma) at 1:1000 for 2 h followed by repeated washings. Images were taken at × 20 with an Aperio

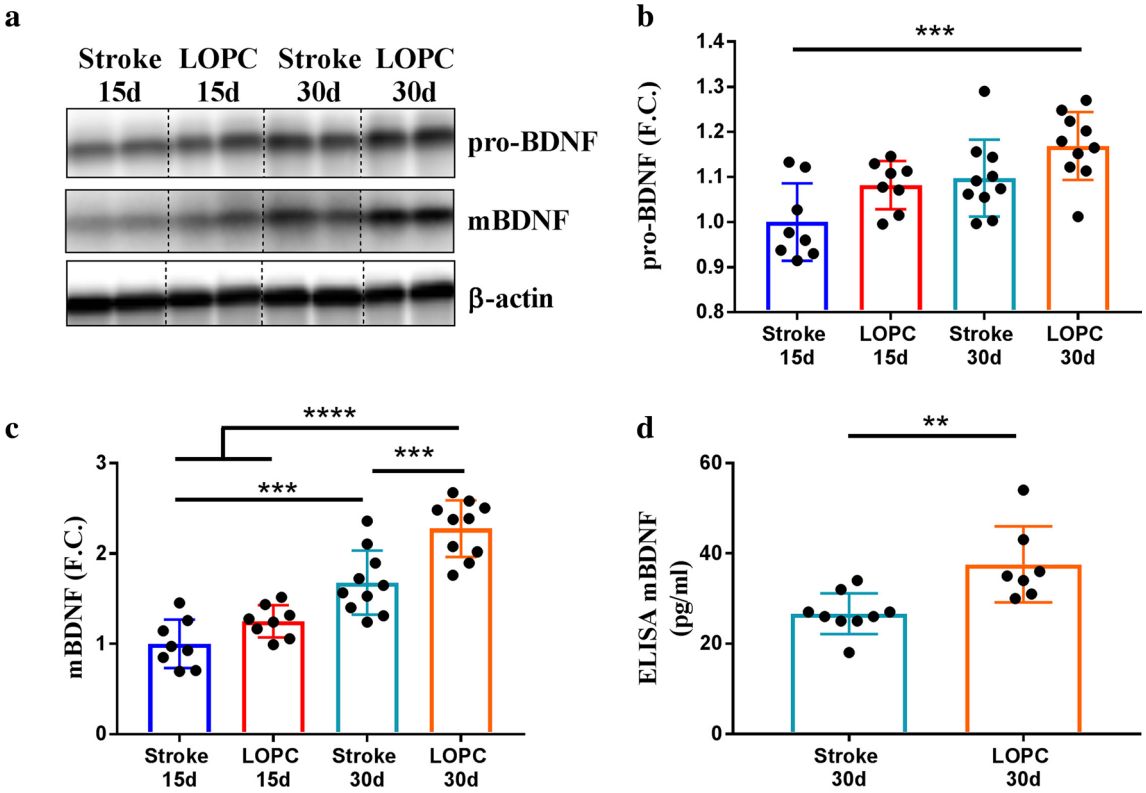

Fig. 3 LOPC increases proBDNF and mBDNF in the peri-infarct area. **a** Representative example of blot of proBDNF and BDNF at 15 and 30 days after LOPC. Quantification by western blot of proBDNF (**b**) and mBDNF (**c**). **d** Absolute quantification by ELISA of mBDNF. Results are shown as the mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Two-way ANOVA, Turkey's multiple comparison test and Mann-Whitney test

AT2 (Leica), and fluorescent images were taken at confocal microscope (× 40). ImageJ software (1.50, NIH) or Matlab custom script (R2015a, MathWorks) was used to estimate tissue loss, area coverage of immunolabelling and microglia morphology [22]. Analysis of vessel density and microglia morphology was focused on the area near the edge of the injury, referred to as peri- infarct (Fig. 4a).

Biochemical Analysis

Protein homogenates from the peri-infarct samples were obtained as described [21, 23]. The peri-infarct supernatants were prepared for ELISA according to manufacturer instruction and measured for mBDNF using commercially available human/mouse/rat mature BDNF Rapid ELISA kit (cat#BEK-2211-1P, Biosensis). Western blotting was performed as described [21, 23].

Statistics

Data were analysed using the Prism for Windows Version 7 (GraphPad Software) with either Mann-Whitney test or two-way ANOVA followed by the Turkey multiple comparison post test. $P < 0.05$ was considered significant.

Results

Low Oxygen Post Conditioning Reduces the Severity of Motor Deficits

Mice ($n = 16$) were tested for motor deficits before (− 2 days) and at 2, 7, 14, 21 and 28 days post stroke. Locomotor asymmetry was assessed using the cylinder test. Time had a significant effect on the development of the impairment (two-way ANOVA $F_{(5,\ 84)} = 12.26$, $p < 0.0001$). Overall, LOPC treatment significantly increased spontaneous use of the stroke-affected limb (Fig. 1b) (two-way ANOVA $F_{(1,\ 84)} = 5.172$, $p < 0.05$), showing that LOPC persistently improves post stroke function for at least 2 weeks after the end of the treatment. Motor impairment was also evaluated by the foot fault test. In this case, LOPC reduced motor function deterioration, and the effect was observed to persist for 2 weeks after the end of the treatment (Fig. 1c) (two-way ANOVA $F_{(1,\ 83)} = 9.456$, $p < 0.01$). As expected, the ipsilateral paw was not affected by either stroke or LOPC.

Low Oxygen Post Conditioning Decreases Ipsilateral Tissue Loss

The tissue loss was quantified as (contralateral hemisphere area − ipsilateral hemisphere area) / contralateral hemisphere

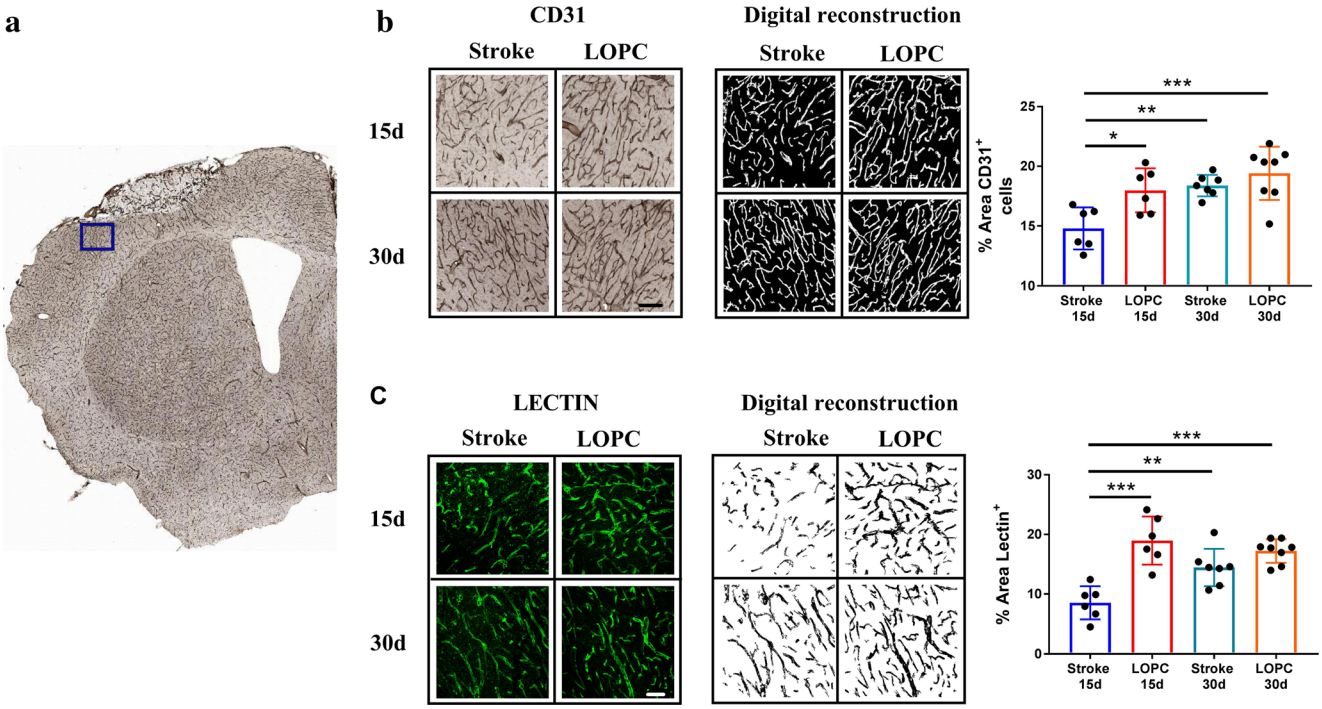

Fig. 4 LOPC increases vessel density in peri-infarct area. **a** Location of the peri-infarct region examined. Blood vessels were identified by immunolabelling targeting CD31 (scalebar = 100 μm) (**b**) or with fluorescent (GFP-conjugated) tomato-lectin (scalebar = 50 μm) (**c**). After digital reconstruction, the area covered by vessel was quantified as (%) of total area (right panels) showing a proangiogenetic effect of LOPC. Results are shown as the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Two-way ANOVA and Turkey's multiple comparison test

area × 100 at bregma = 0, as per [24] in NeuN-stained sections at 15 ($n = 12$) and 30 ($n = 15$) days after stroke. Results expressed as fold change (FC). Analysis identified that LOPC prevents tissue loss at days 15 (Fig. 2b) (0.56 FC, $p < 0.05$) and 30 (0.47 FC, $p < 0.05$).

Low Oxygen Post Conditioning Prevents Neuronal Death Within the Peri-Infarct Area and Promotes Expression of Presynaptic Markers

To investigate the neuroprotective activity of LOPC, we evaluated the expression of the mature neuronal marker NeuN and the presynaptic protein synapsin-1 (Fig. 2c). Our analysis indicated that increased levels of NeuN in the LOPC groups were present at both 15 (1.17 FC, $p < 0.01$) and 30 (1.11 FC, $p < 0.01$) days, while the increase on synapsin-1 was significant at 30 days (1.26 FC, $p < 0.01$).

Increases Expression of Mature Brain-Derived Neurotrophic Factor

The improvement observed in the motor tests suggests that LOPC can have a neuroprotective effect. We decided to investigate this aspect by measuring the precursor and the mature form of BDNF, one of the most important neuroprotective and neurotropic factors. To investigate the effect of LOPC on BDNF expression, we considered the levels of proBDNF and mBDNF immediately after the treatment (15 days, $n = 16$) and 15 days later (i.e. day 30 of the study, $n = 19$) (Fig. 3a). Two-way ANOVA analysis revealed that for proBDNF (Fig. 3b), there was a main effect for time ($F_{(1,\ 32)} = 12.81$, $p < 0.005$) and LOPC ($F_{(1,\ 32)} = 8.864$, $p < 0.01$), with the LOPC group at 30 days showing a significant increase to the stroke group at 15 days (1.17 FC, $p < 0.0005$). Also for mBDNF (Fig. 3c), both time ($F_{(1,\ 32)} = 75.36$, $p < 0.0001$) and LOPC ($F_{(1,\ 32)} = 18.65$, $p = 0.0001$) were identified as significant. Multiple comparison analysis showed no difference among the groups at 15 days, while at 30 days, the increase in mBDNF was significant between groups (1.35 FC, $p < 0.0005$) and between time points (15 vs 30 days, stroke 1.67 FC, $p < 0.0005$; LOPC 1.82 FC, $p < 0.0001$). To validate this result, we used an ELISA kit specific for the mature form of BDNF with minimal cross reactivity with the precursor form to analyse homogenates from peri-infarct area in the cohorts that underwent the motor function test (30 days, $n = 16$). We confirmed that at 30 days, there is indeed a significant increase of mBDNF (Fig. 3d) in the LOPC group compared to the stroke group alone (1.36 FC, $p < 0.01$).

Low Oxygen Post Conditioning Increases Vessel Density in Peri-Infarct

Exposure to transiently reduced oxygen levels has been shown to have a proangiogenetic effect. To assess if our

LOPC protocol had a proangiogenetic effect, we analysed the changes in peri-infarct vessels with two independent approaches. First, we used immunohistochemistry targeting CD31 (Fig. 4b), a specific marker expressed on the membrane of endothelial cells. Second, we used immunofluorescent labelled tomato-lectin, which binds to carbohydrate components of the endothelial plasmalemma (Fig. 4c). In both cases, we observed a higher density of vessels at 15 days in the LOPC group (CD31 1.2 FC, $p < 0.05$, lectin 2.2 FC, $p < 0.005$) while at 30 days, it was similar between the groups.

Expression of Microglia Activation Markers Is Reduced After LOPC

We analysed the expression of microglial activation markers in the peri-infarct area (Fig. 5a). Our two-way ANOVA analysis revealed that both time and LOPC had a significant effect on CD11b levels (time $F_{(1, 32)} = 19.43$, $p < 0.001$; LOPC $F_{(1, 32)} = 4.835$, $p < 0.05$), CD45 (time $F_{(1, 32)} = 37.54$, $p < 0.0001$; LOPC $F_{(1, 32)} = 12.47$, $p < 0.001$) and CD68 (time $F_{(1, 32)} = 5.956$, $p < 0.05$; LOPC $F_{(1, 32)} = 10.64$, $p < 0.005$). In particular, LOPC induced a significant decrease in the expression of CD45 (0.8 FC, $p < 0.05$) at 15 days, while at 30 days, there is no difference among the groups. With our analysis of CD68, we identified a significant decrease following LOPC at 15 days (0.73 FC, $p < 0.05$). However, post hoc analysis did not show significant differences in the single time points for CD11b. We further consider microglial changes by examining the morphological reconfiguration, which is recognised to change under conditions of neuroinflammation (Fig. 5b, c). This analysis indicated that the number of primary branches in the LOPC group at 30 days was elevated relative to the stroke alone group both at

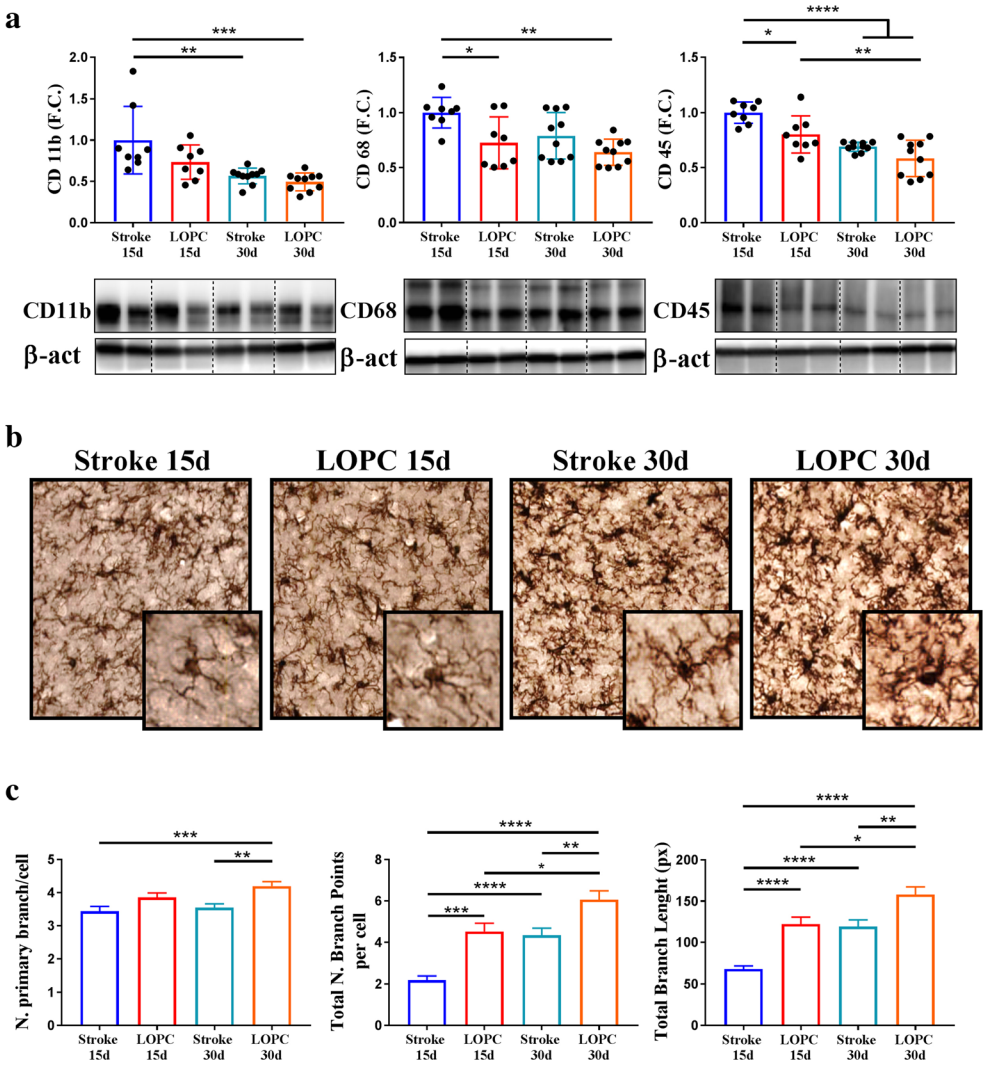

Fig. 5 LOPC decreases microglia activation in the peri-infarct area. **a** Quantification by western blot of activation markers CD11b, CD45 and CD68. **b** Representative images of Iba-1 immunostaining and high magnification detail (scalebar = 10 μm). **c** Parameters analysed to estimate microglia ramification. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Two-way ANOVA and Turkey's multiple comparison test

15 (1.22 FC, $p < 0.005$) and 30 days (1.18 FC, $p < 0.01$). The number of branch points was increased by LOPC at both 15 (2 FC, $p < 0.005$) and 30 days (1.39 FC, $p < 0.01$). Increased ramification was also noted to occur with time (15 vs 30 days, stroke 1.98 FC, $p < 0.001$; LOPC 1.33 FC, $p < 0.05$). The total branch length has a very similar pattern, being increased by LOPC (15 days 1.8FC, $p < 0.001$; 30 days 1.32 FC, $p < 0.01$) and with time (15 vs 30 days, stroke 1.75 FC, $p < 0.001$; LOPC 1.29 FC, $p < 0.05$). These morphological changes are consistent with microglia entering into a less inflamed phenotype.

Discussion

While LOPC has been shown to improve a number of deficits induced by stroke, the ability of the intervention to improve motor function had not, until now, been extensively investigated [10, 25]. As such, the primary aim or our study was to investigate whether LOPC could ameliorate motor impairment after stroke. Motor function was assessed using two different tests [18]; and in both tasks, we observed that mice exposed to LOPC demonstrated less severe motor deficits. To our knowledge, this is the first report to describe the ability LOPC to promote a sustained improvement in motor function after stroke in adult mice.

Specifically, in the current study, we utilised two separate tasks to consider the impact of LOPC on motor function, the cylinder task and the grid walk task. Both of the tasks were chosen because of the fact that they have been extensively used and validated in preclinical studies interested in considering motor impairments. The cylinder task considers the preference given to one forepaw over the other while stabilising the trunk during spontaneous rearing. Post stroke mice typically demonstrate a preference to bear weight on the paw that is ipsilateral to the brain injury (i.e. the paw not connected to the damaged hemisphere). We have previously reported that deficits following photo thrombotic occlusion directed to the motor cortex induce an asymmetry score of 0.5 after 2 weeks from stroke. Our data were completely consistent with these prior findings [21]; and at 7 days, the deficit is established. LOPC had a small but significant overall effect, ameliorating the preferential use of the non-affected limb. In terms of the animals' grid walk performance, we identified clear evidence of a stroke-induced increase in foot faults, as per our expectations. We further observed that LOPC was associated with a significant reduction in overall foot fault errors. Together, these results indicate that LOPC improves stroke-induced motor impairments, and the benefits persist for at least 2 weeks following the cessation of the intervention.

The positive effect of LOPC on motor function was directly supported by our examination of tissue loss from the peri-infarct territory. Specifically, we examined tissue loss using two separate and complementary approaches. Firstly, we used and examined the total area of tissue loss by measuring the hemispheric area (taking into consideration the negative area representative by the ventricular cavities). This analysis indicated that stroke produced a loss of approximately 15% of tissue from the infarcted hemisphere relative to the contralateral hemisphere, a finding consistent with prior work [21, 23]. We further identified that LOPC was associated with an improvement, exhibiting only an 8% loss of area. Interestingly, there were no identifiable differences in the amount of tissue loss in the LOPC group seen at day 15 and day 30; a result that suggests that most of the neuroprotective effect is attributable to benefits obtained during the primary treatment window. Secondly, we considered the abundance of NeuN and synapsin-1 using semi-quantitative western blotting. NeuN is marker for mature neurons, and syanpsin-1 is found with the membrane of synaptic vesicles. Our analysis indicated that LOPC increased NeuN levels at both 15 and 28 days post stroke and synapsin-1 levels at 30 days post stroke. These results in combination with the area measurements indicate that LOPC is robustly neuroprotective.

In terms of considering the mechanisms involved in mediating the neuroprotective effects conferred by LOPC, we considered several factors. An extensive literature has shown the importance of the neurotrophin brain-derived neurotrophic factor (BDNF) after stroke. Specifically, in preclinical studies, the intravenous delivery of BDNF has shown to improve the motor outcome after photothrombotic ischaemia [26], while blocking BDNF expression prevents improvement associated with rehabilitation [27]. Moreover, lower levels of circulating BDNF are associated with poorer long-term functional outcomes in patients after stroke [28]. BDNF is synthetised as a precursor (proBDNF) which is cleaved to generate mature BDNF (mBDNF). Using western blot, we investigated changes in both proBDNF and mBDNF. For both the precursor and the mature BDNF, we observed a trend to increase with time, which was enhanced by LOPC treatment. Only after the end of the treatment at 30 days did the LOPC group display a higher level mBDNF, whereas no effect on proBDNF was noted. To validate this result, we performed mBDNF absolute quantification by ELISA in samples at 30 days. Using this alternative approach, we confirmed that mBDNF was significantly increased in the LOPC group. BDNF delivery is a very promising treatment to promote motor function and neuroplasticity after stroke, but an efficient method of delivery in patients is yet to be found. Here, we show that LOPC can boost physiological expression of BDNF, circumventing problems associated with invasive delivery or blood brain barrier permeability. Future studies with a longer time horizon are needed to analyse how this increase translates in functional outcomes.

BDNF has been shown to be proangiogenetic, and in turn endothelial cells can secrete BDNF; therefore, these two factors are intertwined. Angiogenesis is considered to be central to promoting recovery [29]; and after stroke, both apoptosis and proliferation of capillary endothelial cells are observed, with a neuroprotective role of the latter [30]. In addition, LOPC has been shown to have a robust proangiogenetic effect in the brain [31]. Given these findings, we analysed vessel density in the peri-infarct area using the two different markers for endothelial cells that are commonly used to identify blood vessels, CD31 and lectin. Our results indicated that LOPC-treated animals exhibited greater vessel density at day 15 indicating that 2 weeks of LOPC strongly induces angiogenesis in the peri-infarct area. At day 30, the stroke group displays a higher vessel density than at 15 days, reflecting the endogenous activation of proangiogenetic signals after stroke. However, we did not observe significant difference at 30 days between the stroke and the LOPC group. The results show that 2 weeks of exposition to LOPC stimulates angiogenesis, and this stimulus fades after the end of treatment. The formation of new vessels provides oxygen and nutrients to the tissue and might have a supportive role for surviving neurons; thus, LOPC could exert its neuroprotective role by promoting neo-vascularisation. It will be the object of future studies whether prolonging the protocol of LOPC translates in a further enhancement of angiogenesis and neuroprotection.

The final mechanism that we explored, with a view to improving our understanding of the neuroprotective effects of LOPC, was neuroinflammation and especially microgliosis. Excessive microglial-mediated neuroinflammatory responses have been shown to be detrimental for the outcome after stroke, and approaches directed to specifically target inflammation have shown promising results in preclinical studies [32, 33] . In this study, we estimated neuroinflammatory status by assessing expression levels of microglia activation markers. We found that LOPC reduced the expression of CD68 and CD45 at 15 days post stroke, but no differences were observed in CD11b. We take this result to indicate that LOPC moderately dampens neuroinflammatory tone rather than suppressing it. As expected, the expression of these markers also decreased with time independently of the treatment, indicating the natural progression and resolution of neuroinflammation.

A second index often used to investigate changes in neuroinflammatory status is changes in microglial morphology and in particular changes in branching status (otherwise referred to as process ramification) [22, 34]. Several studies have now identified that ramified microglia is typically associated with neuroprotection [35]. Here, we show that LOPC induces an intriguing phenotype that to our knowledge has not been previously described. Specifically, we identify that stroke induces a typically activated morphology characterised by short ramifications with low complexity. Against this background, it appears that LOPC promotes ramification. Therefore, stroked mice treated with LOPC exhibit feature of activation and enhanced ramification. This effect was noted at both 15 and 30 days post stroke and was confirmed by analysis indicating that microglia post stroke and LOPC had longer processes with a greater number of branches. This result suggests that LOPC promotes microglia shift towards a less inflammatory state; however, further investigation of this unusual phenotype is required.

In conclusion, here, we describe for the first time the ability of LOPC to improve motor function post stroke. This observation is also robustly supported by a constellation of complementary mechanistic markers that collectively all point to the ability of LOPC in inducing a neuroprotective phenotype. Second, and an important finding from the current study is that LOPC not only produced positive effects during the delivery of the treatment but many persisted for 2 weeks following cessation. While this is only a modest window for persistence, it is certainly encouraging and it would be very worthwhile to further consider both how long the benefits last for as well as to consider how late the treatment could be started. Another promising angle has been suggested by several reports showing that exposure to low oxygen in hypobaric conditions can improve anxiety and depression-like symptoms in rats [36, 37]. We have previously shown that chronic stress inducing depression-like symptoms in mice is detrimental for their recovery after stroke. In particular, chronic stress promotes neuronal loss and reduction in vascular density in the peri-infarct area, accompanied by an exacerbation of motor impairment [23, 38]. Notably, in the current study, all these parameters were significantly improved by LOPC. Future studies are now needed to verify if LOPC can effectively prevent the deleterious effects associated with chronic stress after stroke, potentially adding translational value to LOPC treatment [39]. While our results are promising and support earlier promising results from other groups, further research should consider undertaking a full multi-organ safety audit as well as identifying the minimum length of exposure required to produce therapeutic effects. Despite the requirement for additional research, it is now important to recognise that LOPC is one of the first non-pharmacological interventions that can be deployed days after the initial stroke and still reduce tissue loss, promote neurogenesis, angiogenesis and cognitive and motor functions. Moreover, the technology to translate this intervention to the clinic already exists [13]. Therefore, we propose that LOPC appears to represent an excellent therapeutic intervention and should soon be considered for translation into early stage clinical research.

Authors' Contributions Experiments were designed by GP, MN and FRW and performed by GP, KZ and ZZ. Manuscript was written by GP and FRW and edited by GP, SJ, MN and FRW.

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Compliance with Ethical Standards

All experiments were conducted in accordance with the New South Wales Animals Research Act (1985) and the Australian Code of Practice for the use of animals for scientific purposes.

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval All experiments were approved by the University of Newcastle Animal Care and Ethics Committee.

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