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Head Down Tilt 15° in Acute Ischemic Stroke with Poor Collaterals: A Randomized Preclinical Trial

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Abstract—Cerebral collaterals are recruited after arterial occlusion with a protective effect on tissue outcome in acute ischemic stroke. Head down tilt 15° (HDT15) is a simple, low cost and accessible procedure that could be applied as an emergency treatment, before recanalization therapies, with the aim to increase cerebral collateral flow. Spontaneously hypertensive rats have been shown to display anatomical differences in morphology and function of cerebral collaterals, compared to other rat strains, resulting in an overall poor collateral circulation. We investigate the efficacy and safety of HDT15 in spontaneously hypertensive (SHR) rats, which were considered as an animal stroke model with poor collaterals. Cerebral ischemia was induced by 90 minute endovascular occlusion of the middle cerebral artery (MCA). SHR rats were randomized to HDT15 or flat position (n = 19). HDT15 was applied 30 minutes after occlusion and lasted 60 minutes, until reperfusion. HDT15 application increased cerebral perfusion (+16.6% versus +6.1%; $p = 0.0040$) and resulted in a small reduction of infarct size (83.6 versus 107.1 mm^3; − 21.89%; $p = 0.0272$), but it was not associated with early neurological improvement, compared to flat position. Our study suggests that the response to HDT15 during MCA occlusion is dependent on baseline collaterals. Nonetheless, HDT15 promoted a mild improvement of cerebral hemodynamics even in subjects with poor collaterals, without safety concerns.

Key words: acute ischemic stroke, cerebral collaterals, collateral therapeutics, head down tilt, ischemic penumbra.

INTRODUCTION

Acute ischemic stroke results from a time-dependent, critical reduction of regional cerebral blood flow (CBF) after arterial occlusion. Cerebral collateral circulation, which provides a subsidiary network of anastomotic arterial vessels at the skull base and along the surface of cortical regions, represent a major endogenous protective mechanism against ischemia and is present in the brain of many species, including humans and most mammals (Liebeskind, 2003; Shuaib et al., 2011; Jung et al., 2013).

Previous studies showed that a positional therapy with head down tilt 15° (HDT15) increased cerebral collateral flow and improved neurological outcome in the rat stroke model of transient middle cerebral artery (MCA) occlusion (Beretta et al., 2017; Diamanti et al., 2022; Zhao et al., 2022). In particular, HDT15 is a good candidate to be implemented as an emergency treatment, during the acute phase of ischemic stroke prior to recanalization therapies, in order to minimize ischemic core expansion and increase the benefit of recanalization.

Exploratory clinical studies indicate that HDT15 is well tolerated in healthy subjects and increased cerebral perfusion measured by transcranial Doppler in patients with acute ischemic stroke (Fortrat et al., 2001; Alexander et al., 2005; Mejdoub, 2020; Seiller, 2021). Nonetheless, the clinical benefit of HDT15 application in acute ischemic stroke, compared to usual positioning, remains to be proved (Alexandrov et al., 2018).

Spontaneously hypertensive (SHR) rats have been shown to display anatomical differences in morphology and function of cerebral collaterals, compared to Wistar

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Abbreviations: CBF, cerebral blood flow; HDT15, Head down tilt 15°; MCA, middle cerebral artery; SD, standard deviation; SHR, spontaneously hypertensive rat.

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rats, resulting in an overall poor collateral circulation (Coyle, 1987).

In the present study, we performed a randomized preclinical trial to evaluate efficacy and safety of HDT15 in SHR rats subjected to transient MCA occlusion, to assess the hemodynamic and neuroprotective effect of HDT15 in animals with a poor cerebral collateral circulation.

EXPERIMENTAL PROCEDURES

Experimental design, sample size determination and randomization

The experiments presented in this study were carried out under project license from the Italian Ministry of Health (81/2015-PR) and were approved by the Animal Care Committee of the University of Milano-Bicocca. The Italian and European guidelines on the use of laboratory animals (D.L. 26/2014; 2010/63/EU) were followed. This report was written according to the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines. We planned the study into two arms, flat group and HDT15 group, using infarct volume as the primary outcome. Previous results from our group showed that the size of ischemic lesions at 24 hours in untreated Wistar rats subjected to MCA occlusion for 90 minutes, followed by reperfusion, had a normal distribution and a within group standard deviation (SD) of 0.45. Since the expected effect size of ''collateral therapeutics'' was 0.62 (Beretta et al., 2017), we planned a randomized preclinical trial of 18 animals (9 for each treatment group), with an expected power of 0.80 and type I error of 0.05, with two-sided significance level. The control group included animals maintained in flat position during MCA occlusion for 90 minutes, followed by reperfusion. The treatment group included animals treated with HDT15 applied 30 minutes after MCA occlusion, which lasted 90 minutes and was followed by reperfusion. HDT15 treatment lasted 60 minutes and was terminated just before reperfusion. The experimental surgeon was blinded to treatment, since randomization was performed after MCA occlusion. Consecutive rats were randomized until the minimum predefined sample size of 9 animals per group was reached.

One rat in the HDT15 group died after MCA occlusion, before the end of the experimental procedure, for respiratory failure attributed to anesthetic overdose. Necropsy excluded hemorrhagic complications. This animal was excluded from analysis.

Animals and surgery model

The experimental cohort was composed by 19 adult male SHR Wistar rats (250–275 g; ENVIGO, Italy), n = 10 in the control group (flat) and n = 9 in the treatment group (HDT15). Animals were housed in a pathogen-free facility. Room temperature and light/darkness cycle (12/12 hours) were monitored and managed automatically. All the animals were subjected to the same anesthesia protocol, starting phase with 3% isoflurane in O2/N2O (1:3) induction followed by 1.5% isoflurane maintenance.

The right middle cerebral artery (MCA) was transiently occluded by a silicone-coated filament (diameter 0.37 ± 0.02 mm, Doccol Corporation, Redlands, CA, USA). Surgery technique involved the insertion of the filament in the external carotid artery (ECA), then progressing to the terminus of the internal carotid artery (ICA), to occlude the MCA. Prior the insertion of the filament into the ECA, the pterygopalatine artery (PPA) and ECA proximal branches were ligated and the common carotid artery (CCA) was temporarily clipped. After the MCA occlusion, CCA was reopened. In order to avoid displacement, the endovascular filament was secured to the ECA with a tight double knot. The occlusion time lasted 90 minutes and was followed by 24 hours of reperfusion. Body temperature was monitored during surgery using a rectal probe connected to a heating pad that maintained the core temperature to 37.0 ± 0.5 °C. After reperfusion, animals were returned to their cages to recover, with free access to food and water. If animal showed signs of distress, buprenorphine 0.05–0.1 mg/kg was be administered subcutaneously every 12 hours. Garcia neurobehavioral test was performed 24 hours after reperfusion (see below). After neurobehavioral assessment, animals were euthanized by CO_2 inhalation. Brains were extracted and fixed with 10% formalin.

Cerebral collateral perfusion monitoring

A blunt needle, single channel Laser doppler probe (moorVMS-LDFTM, Moor, Axminster, UK) was used to verify proper MCA occlusion (Beretta et al., 2013; Taninishi et al., 2015; Cuccione et al., 2017).

The probe was attached to the skull over the lateral MCA territory (bregma −1 AP; +5 ML) employing its specific support, in accordance with our previous work (Beretta et al., 2013).

During the whole anesthesia time window (including surgery, MCA occlusion and early reperfusion), cerebral perfusion values were recorded.

Data analysis was performed on five hemodynamic timepoints (expressed as perfusion unit, PU, % to baseline PU): *1. baseline*, the mean PU stabilized value prior the occlusion of CCA; *2. CCAO*, the mean PU stabilized value after CCA occlusion and before MCA occlusion; *3. MCAO*, the mean PU stabilized value after MCA occlusion and before treatment (0 to 30 minutes after MCA occlusion); *4. treatment*, the maximal PU stabilized value after treatment (30 to 90 minutes after MCA occlusion); 5. *reperfusion*, the mean PU stabilized value after early reperfusion (5 to 10 minutes after reperfusion).

Application of HDT15

According to our previous study, we applied HDT15 using a 15° tilted platform gently placed under the operating table. The whole body of the animal was tilted, including the neck and head. The treatment was applied 30 minutes after MCA occlusion and lasted 60 minutes, until reperfusion. After reperfusion, animals were

Table 1. Shapiro-Wilk normality test

Outcome	Perfusion groups		Perfusion Pre-post				Functional outcome		Infarct volume	
Group	HDT15	flat	HDT15		flat		HDT15	flat	HDT15	flat
			Pre	Post	Pre	post				
W	0.938	0.972	0.902	0.938	0.927	0.972	0.956	0.953	0.936	0.770
P value	0.566	0.916	0.265	0.566	0.423	0.916	0.765	0.704	0.543	0.006**

**significant *P* value (< 0.05).

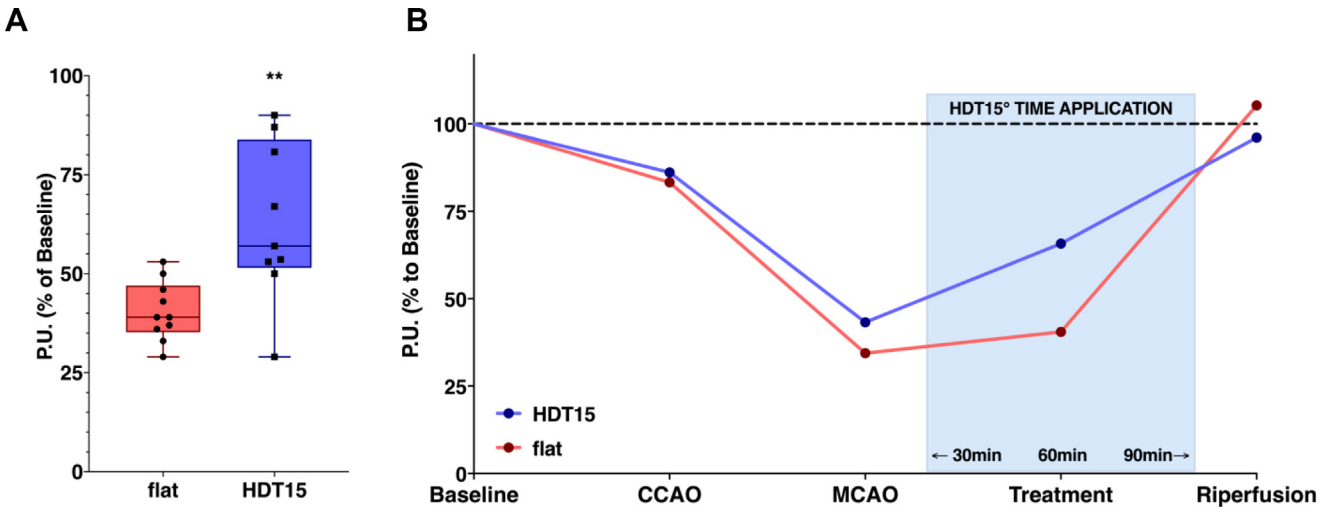

Fig. 1. Effect of HDT15 treatment on cerebral perfusion. (**A**) Maximal effect of HDT15 treatment in the lateral MCA territory, compared to the flat group. (**B**) Graphical representation of the hemodynamics changes at five experimental time points. Each time point represents the mean perfusion units of the animals included in each study arm. MCAO = middle cerebral artery occlusion. CCAO = common carotid artery occlusion. P. U. = perfusion units (% to baseline). Blue boxes represent the time window of treatment application. $^{**}p = 0.009$.

returned to flat position until they recovered from anesthesia.

Neurobehavioral test assessment

Garcia neurobehavioral test (Garcia et al., 1995) was performed to quantify neurologic outcome at 24 hours, assessing spontaneous movement, sensory function and motor function, and was expressed as an ordinal score (from 3 corresponding to worst neurologic outcome to 18 corresponding to normal neurologic behavior).

Histology analysis and infarct volume quantification

After the neurobehavioral test, rats were sacrificed by deep narcosis with CO_2. Brains were extracted and fixed in 10% formalin. After 72 hours of fixation, the brain was sectioned using Vibratome1000Plus (Leica) and all the coronal sections (100 um) were collected. Up to n = 19 consecutive sections with 200 um distance (bregma + 3.0 mm to −2.5 mm) were used for infarct volume quantification. Ischemic areas were defined using histological staining with Cresyl Violet 0.1% (Bioptica, Milano, Italy). Each section is mounted on a positively charged slide (SuperFrost Plus, Thermo Scientific) and rinsed in a saline solution (Dulbecco's Phosphate Solution w/Magnesium w/Calcium; Euroclone): only after 48 hours sections were stained with Cresyl Violet (Cresilvioletto Kluver Barrera 05-B16001; Bioptica) according to manufacturer's instructions. The staining protocol requires that the sections need to be rehydrated by immersing them in ethanol solutions with decreasing concentration (EtOH 95%, EtOH 70%, EtOH 50%; Sigma-Aldrich) and finally in demineralized water. Subsequently the sections are placed in Cresyl Violet for 5 minutes. After this, the sections are immersed again in demineralized water and then in alcohol solutions with increasing concentration (EtOH 50%, EtOH 70% + Glacial Acetic Acid 3%, EtOH 95%, EtOH 100%; Sigma-Aldrich) and finally in xylene (Sigma-Aldrich) to wash off the excess dye and dehydrate it, allowing assembly in dibutyl phthalate xylene (DPX non-aqueous mounting medium CL04.0401.0500; Chem_Lab NV). The volume of the ischemic lesions was quantified using ImageJ software (National Institute of Health, NIH, Bethesda, MD, USA) and expressed in mm^3.

Statistical analysis

The data were analyzed using Prism 7 (version 7.04; GraphPad). Values were expressed as mean ± standard deviation. Shapiro Wilk normality test was performed to evaluate the sample distribution and F

test was performed to assess the equality of variances for all the analyses (Table 1). A p value < 0.05 was considered significant. Hedges' g test was used to evaluate the effect size. Unpaired Student t test was used for the two-group analysis on Garcia neurobehavioral test. Unpaired Student t test with Welch's correction was assessed (due to F test < 0.05) in cerebral perfusion comparing flat vs HDT15. Paired Student t test was used to evaluate pre- and post-treatment in perfusion analysis. Mann-Whitney test was performed in lesion volume analysis.

RESULTS

Effect of HDT15 treatment on cerebral perfusion

Cerebral perfusion was compared between HDT15 and flat groups during the treatment time point (Fig. 1(**A**)). We observed an increased cerebral perfusion within the ischemic territory in the HDT15 group, compared to flat group (absolute difference + 22.54%; 95% CI = 6.803 to 38.27; Hedges' g = 1.528; p = 0.009). Within-group analysis, comparing post versus pre-treatment (Fig. 1 (**B**)), showed a moderate increase in cerebral perfusion in HDT15 group (absolute difference + 16.63%; 95% CI = 4.889 to 28.37; Hedges' g = 0.737; p = 0.011), while a smaller effect was observed in the flat group (absolute difference + 6.10%; 95% CI = 2.338 to 9.862; Hedges' g = 0.852; p = 0.005).

Efficacy of HDT15 treatment on functional outcome

Application of HDT15 did not change early neurological outcome assessed by the Garcia neuroscore, compared to flat group (11.22 versus 10.40 points; absolute difference 0.82 points; 95% CI = -1.621 to 3.265; Hedges' g = 0.325; p = 0.487), as shown in Fig. 2.

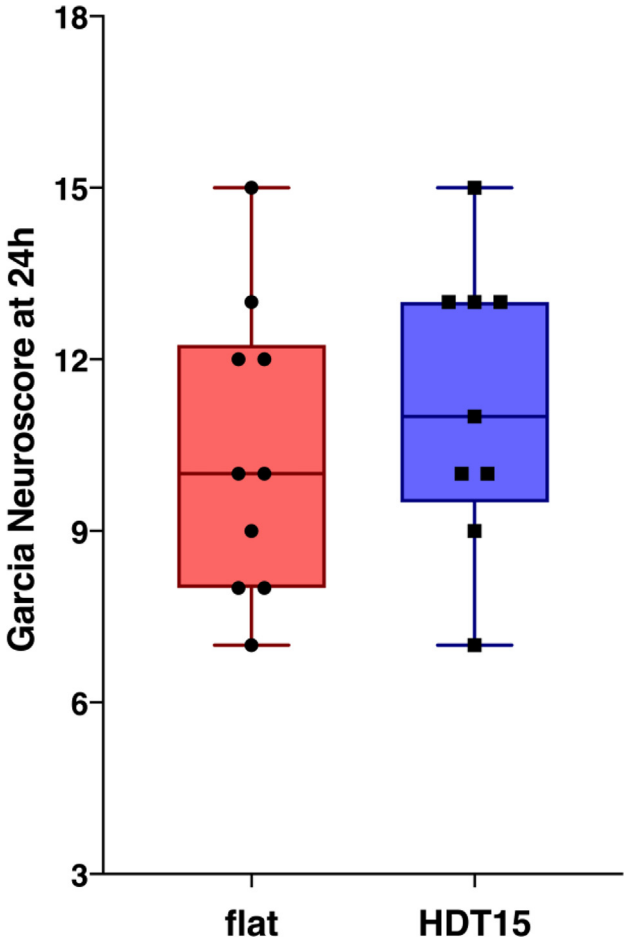

Fig. 2. Effect of HDT15 treatment on functional outcome. Representation of the Garcia neuroscore values, expressed as a continuous variable, in the two groups.

Efficacy of HDT15 treatment on infarct volume

HDT15 application reduced infarct volume at 24 hours, compared to flat group (86.39 versus 97.70 mm^3; −11.31 mm^3 absolute difference; −11.57% relative difference; 95% CI = 2.140 to 42.43; Hedges' g = 1.112; p = 0.043), as shown in Fig. 3.

Treatment-related mortality

HDT15 had a neutral effect on mortality at 24 hours. No rats died due to either HDT15 treatment or flat positioning.

DISCUSSION

Penumbra is defined as brain tissue that is doomed to infarction in the absence of early reperfusion (Leigh et al., 2018). The speed of infarct progression largely depends on the compensatory collateral perfusion and the amount of salvageable territory, the penumbra, correlates with the efficiency of the collateral circulation (Liebeskind, 2012; Beretta, 2015; Ginsberg, 2016).

The hemodynamic performance of cerebral collaterals represents a key factor for tissue outcome in the acute phase of ischemic stroke (Brunner et al., 2014; Hwang et al., 2015), defining successful versus futile recanalization therapies, including endovascular thrombectomy (Fanou et al., 2015; Leng et al., 2015; Sheth and Liebeskind, 2015; Van den Wijngaard et al., 2015).

Investigating and developing an effective strategy to enhance cerebral collateral circulation is a top priority in stroke research (Liebeskind, 2010). Previous experimental studies (Beretta et al., 2017; Diamanti et al., 2022; Zhao et al., 2022) has demonstrated that HDT15 is an effective and safe collateral therapeutic, resulting in a rapid recruitment of cerebral collaterals after MCA occlusion in Wistar and Sprague–Dawley rats.

In the present study, HDT15 treatment was tested for efficacy and safety in the worst possible scenario, i.e. an animal stroke model with a poor collateral circulation, such as SHR rats. Our findings showed that HDT15 moderately increased cerebral perfusion in the MCA territory and slightly reduced infarct size in SHR rats, compared to flat positioning. However, no difference in early functional outcome was observed between HDT15 and flat positioning in SHR rats. Notably, HDT15 proved to be safe, in terms of mortality, in SHR rats similarly to what observed in Wistar and Sprague-Dawley rats.

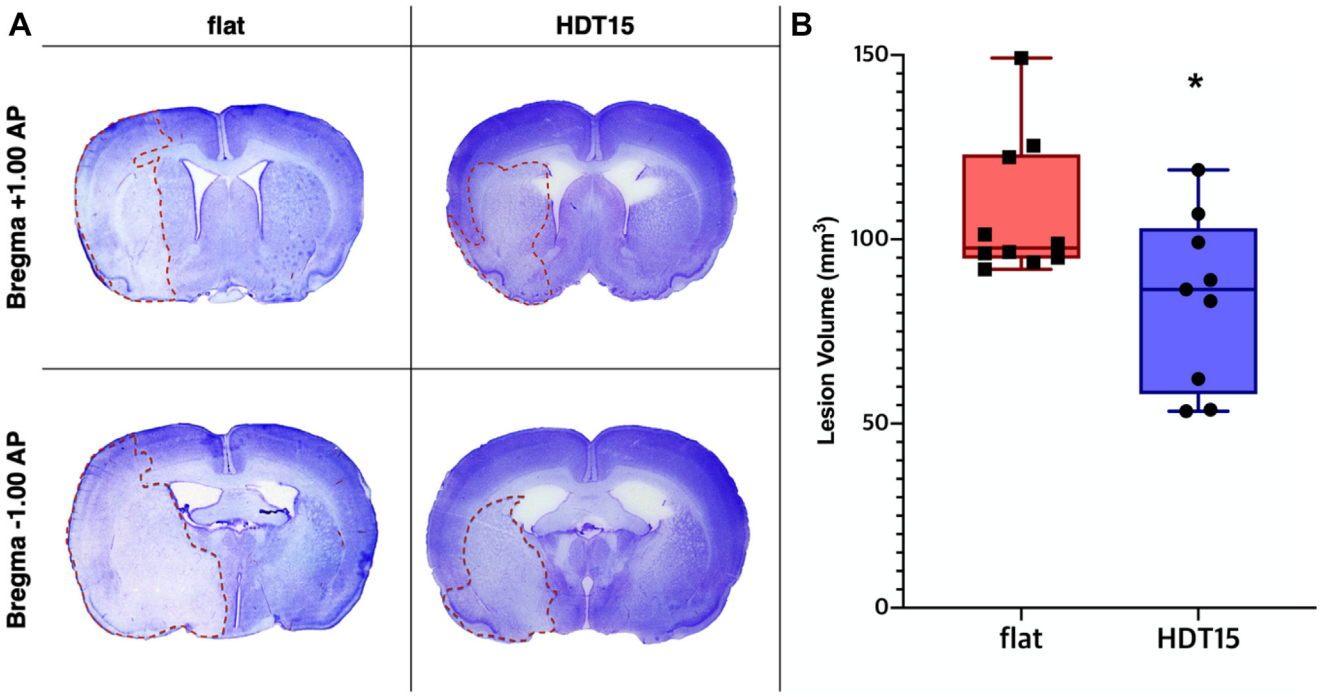

Fig. 3. Effect of HDT15 treatment on infarct volume. A) Histological coronal brain sections of representative rats, maintained in flat position (flat; left images) or treated with head down tilt 15° (HDT15; right images). Sections were stained with Cresyl Violet. Ischemic areas are marked by a red dot line. **B)** Infarct volumes in the two groups, expressed in mm^3. *$p = 0.043$.

HDT15 is a promising candidate as a collateral therapeutic for acute ischemic stroke, to be applied in the pre-hospital setting, as soon as possible after arterial occlusion to enhance the amount of salvageable tissue in the ischemic penumbra.

Our experimental results highlight important points for the translation of HDT15 in human stroke. First, HDT15 application could be beneficial even in subjects with poor collaterals, although this benefit is expected to be smaller compared to subjects with good collaterals. Second, HDT15 could be applied in subjects with poor collaterals without safety concerns. These safety results are complementary to the previous results in an experimental rat model of hemorrhagic stroke, where HDT15 application proved to be safe and did not worsen hematoma expansion and early outcome (Beretta et al., 2020). In fact, patients with acute ischemic stroke with good or poor collaterals, hemorrhagic stroke and stroke mimics are expected be recruited in a future clinical trial of HDT15 in the pre-hospital setting, since they could not be distinguished before neuroimaging.

Our study has limitations. First, Laser doppler has limitations, since it measures microcirculation in a small cortical region and does not provide a direct imaging of collaterals. For this reason, detailed data on anatomical variations, regional differences and flow directions were not addressed in the present study. However, Laser doppler provided a real-time assessment of cerebral hemodynamics in a cortical region of interest over time, encompassing the entire period from MCA occlusion to reperfusion, which is not possible with more sophisticated methods such as perfusion MRI or other imaging techniques. Further studies are needed using perfusion MRI and/or catheter angiography to assess the changes in regional CBF and flow direction associated with HDT15 during MCA occlusion.

Second, neurobehavioral outcome was assessed only at 24 hours and no female animals were used in the study. Although we can't exclude that a larger effect on outcome could have emerged using longer time points or in females, we decided to implement the same protocol used in our previous work on Wistar rats, to make a direct comparison (Beretta et al., 2017). Third, the usual positioning applied by Emergency Services for acute stroke patients in + 30°, while rats were positioned flat as a control group. However, rats are not bipedal animals and flat positioning was judged to be closer to the physiological rat positioning, compared to + 30°, and was adopted as the control arm.

Our results suggest that the response to HDT15 could be dependent on baseline collaterals. SHR rats with poor collaterals displayed a smaller response to HDT15, compared to normotensive rats from our previous work, that displayed well-functioning collaterals. In particular, the effect size of HDT15 was smaller in SHR rats, compared to normotensive rats (for infarct volume: absolute difference 23.41 versus 64.93 mm^3, respectively; Cohen's d 1.10 versus 1.96, respectively). Nonetheless, we observed that HDT15 promoted some degree of hemodynamic improvement even in subjects with poor collaterals, without safety concerns. Despite these results, the influence of the cerebral collaterals was not addressed in the present study. Further preclinical and clinical studies are needed to investigate the exact mechanism of action of HDT15 and promote

translation of HDT15 as an emergency collateral therapeutic for acute ischemic stroke.

AUTHOR CONTRIBUTION STATEMENT

S.B. and J.M. designed the study; J.M., S.B., A.V., S.D., M.V., L.C., B.M., E.C., L.M., D.C. performed the experiments; J.M., S.B., S.D., L.S., C.G., C.F. analyzed the data; J.M. and S.B. wrote the manuscript; all authors critically revised the manuscript.

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DECLARATIONS OF INTEREST

None.

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