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Title: Head down tilt 15° in experimental intracerebral hemorrhage: a randomized non-inferiority safety trial

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Abstract

Background: Head down tilt 15° (HDT15°), applied before recanalization, increases collateral flow and improves outcome in experimental ischemic stroke. For its simplicity and low cost, HDT15° holds considerable potential to be developed as an emergency treatment of acute stroke in the pre-hospital setting, where hemorrhagic stroke is the major mimic of ischemic stroke. In this study, we assessed safety of HDT15° in the acute phase of experimental intracerebral hemorrhage.

Methods: Intracerebral hemorrhage was produced by stereotaxic injection of collagenase in Wistar rats. A randomized non-inferiority trial design was used to assign rats to HDT15° or flat position (n=64). HDT15° was applied for 1 hour during the time window of hematoma expansion. The primary outcome was hematoma volume at 24 hours. Secondary outcomes were mass effect, mortality and functional deficit in the main study and acute changes of intracranial pressure, hematoma growth and cardiorespiratory parameters in separate sets of randomized animals (n=32).

Results: HDT15° achieved the specified criteria of non-inferiority for hematoma volume at 24 hours. Mass effect, mortality and functional deficit at 24 hours showed no difference in the two groups. HDT15° induced a mild increase in intracranial pressure with respect to the pre-treatment values (+ 2.91 +/- 1.76 mmHg). HDT15° had a neutral effect on MRI-based analysis of hematoma growth and cardiorespiratory parameters.

Conclusions: Application of HDT15° in the hyperacute phase of experimental intracerebral hemorrhage does not worsen early outcome. Further research is needed to implement HDT15° as an emergency collateral therapeutic for acute stroke.

Introduction

Cerebral collaterals in the acute phase of ischemic stroke are a major determinant of the "tissue time window", i.e. the subject-specific timing of penumbra evolution [1], which defines successful versus futile recanalization in patients treated with intravenous rtPA [2] or endovascular thrombectomy [3]. Leptomeningeal collaterals are an independent predictor of outcome even in untreated ischemic stroke patients with large vessel occlusion (LVO), providing residual blood flow to cortical ischemic areas despite persistent occlusion [4]. Recent experimental and clinical studies indicate that head down tilt 15° (HDT15°) increases collateral flow and improves outcome in acute ischemic stroke caused by LVO, by gravitational blood flow diversion from the lower body towards the head, without significant safety concerns [5,6,7,8]. HDT15° appears to be an extremely simple, low cost and feasible measure to be applied as a collateral therapeutic in the hyperacute (even pre-hospital) phase of acute ischemic stroke, prior to recanalization therapies. However, safety of HDT15° application needs to be investigated in hemorrhagic stroke, which is the major mimic of acute ischemic stroke in the pre-hospital setting [9], particularly in term of hematoma expansion which may potentially be favored by positional hemodynamic changes. We performed a preclinical randomized non-inferiority trial to evaluate the safety profile of HDT15° in a rat model of large intracerebral hemorrhage (ICH).

Methods

Experimental design, sample size determination and randomization

Experiments were carried out in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee on Animal Care of the University of Milano Bicocca, under project license from the Italian Ministry of Health (81/2015-PR).

We designed a non-inferiority trial to assess safety of HDT15° in ICH compared to flat position, which was considered as usual care. The primary outcome was hematoma volume at 24 hours, considered as a continuous variable. Secondary outcomes were mass effect, mortality and functional deficit at 24 hours. Given that our previous data indicated that the hematoma volume of collagenase-induce ICH in rats at 24 hours was normally distributed with a standard deviation of 0.24 and that the non-inferiority limit was set at 0.15, we obtained a sample size of 32 animals for each treatment group, with an expected power of 0.80 for a non-inferiority trial. Type I error to refuse the null hypothesis of no difference between the two groups was 0.05. The selection of the non-inferiority margin was based upon clinical judgement, considering a difference in the hematoma volume lower than 15% below the minimum clinically important difference.

A 1:1 block randomization, with block size 2, was performed using an online random number generator (www.random.org).

Randomization was performed after stereotaxic surgery, in order to guarantee allocation concealment to surgeon. Functional outcome assessment was performed by researchers blinded to treatment allocation (HDT15° versus flat position) and cause of any death. No person assisting the surgeon carried out assessment of functional outcome. Histological outcome, MRI image analysis and ICP tracing analysis were performed by researchers blinded to treatment allocation.

A preliminary set of untreated animals (n=18), not included in the trial, was used to perform a time course of early hematoma expansion at 30 min, 60 min and 120 min after collagenase injection (time course study).

A further set of 1:1 randomized animals (n=10), not included in the trial, was used to perform an MRI-based analysis of hematoma growth and to assess acute changes in physiological parameters (MRI study).

A further set of 1:1 randomized animals (n=22), not included in the trial, was used to measure intracranial pressure (ICP) for 120 min after collagenase injection (ICP study).

A flow diagram of the study is shown in Supplementary Figure 1.

Animals and surgery

Animals were exposed to 12/12 hours light/dark cycle, at controlled room temperature, with free access to food and water, in a specific pathogen free facility. Adult male Wistar rats (weight 282 ± 32 g; total n=114) were anesthetized with intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). Rats were placed on the brain stereotaxic apparatus in prone position. One midline incision was made in the scalp and a burr hole was made to slowly inject collagenase type IA-S (Sigma-Aldrich, USA; 0.4 CDU in 1 microL of sterile saline solution) into the left putamen by a 27GA syringe (Hamilton Robotics, USA) at the cranial coordinates AP +0.2 mm, ML +3 mm left, DV +8 mm. Scalp was sutured and rats were placed in restrainers for 120 minutes where they recovered from anesthesia

and received treatment (HDT15° or flat position); then they were arranged in single cages. Temperature was maintained at 37°C. Animals showing signs of distress received subcutaneous buprenorphine 0.05-0.1 mg/kg every 12 hours.

Application of HDT15°

HDT15° was applied using a 15° tilted platform. HDT15° was applied during the time window of hematoma expansion, starting 60 minutes after collagenase injection (90 minutes in the MRI study). Duration of HDT15° treatment was 60 minutes.

Neurologic outcome assessment

Mortality was assessed 24 hours after collagenase injection and was expressed as a dichotomous variable. Garcia neuroscore [10] was used to assess global neurological functioning at 24 hours and was expressed as a dichotomous variable as follows: "good functional outcome" (scores 14 to 18) or "poor functional outcome" (scores < 13 or death). Corner turning test [11] was used to score sensorimotor asymmetries and visual-spatial deficits and was expressed as a dichotomous variable as follows: "good functional outcome" (scores > 30% right turns) or "poor functional outcome" (scores < or = 30% or death).

Hematoma volume and mass effect

Brains were fixed in formalin. Coronal sections (1 mm; n=10) were obtained using a rat brain matrix and were mounted unstained. Two methods were used to calculate hematoma volume (mm^3): volumetric histology by measurement of the hematoma area in all brain sections with visible hemorrhage, followed by volume calculation using ImageJ image processing software (NIH, USA); ABC/2 formula [12] where length (A) and width (B) of the hematoma on the slice with the largest hemorrhagic area are multiplied by the thickness of the slices where the hematoma was visible (C) and then divided by 2. Mass effect was calculated as the ratio of the volume of the entire hemorrhagic hemisphere to the volume of the entire healthy hemisphere and expressed as %. In particular, areas of all brain sections of each hemisphere were used to calculate separate hemispheric volumes (hemorrhagic hemisphere and healthy hemisphere) using ImageJ image processing software (NIH, USA).

MRI-based analysis of hematoma growth

Rats were placed in a horizontal-bore small animal 7T MRI scanner (BioSpec 70/20 USR, Bruker, Ettlingen, Germany) to acquire a Rapid Acquisition with Refocused Echoes (RARE) T2-weighted coronal sequence (TR = 4300 ms; TE = 33 ms; FA = 90° / 180°; FOV = 30*24 mm2; in-plane resolution 0.150*0.150 mm2, 40 continuous slices, slice thickness 0.500 mm; number of averages 10; time duration 14 minutes) under isoflurane anesthesia (1.5% in a 20% O2/80% air mixture). For each rat, MRI was performed at 3 predefined time points, with reference to collagenase injection: 60 minutes, 150 minutes and 24 hours. HDT15° was applied between 90 and 150 minutes. Hematoma volume was quantified using MIPAV (NIH Center for Information Technology, Bethesda, Maryland, USA) and expressed in mm3. Hematoma growth was calculated by direct subtraction of the

hematoma volume measured at each predefined time point as follows: (150 minutes – 60 minutes) and (24 hours – 60 minutes).

Physiological parameters

Arterial systolic blood pressure and heart rate were measured using a non-invasive blood pressure recorder (Ugo Basile, Italy) applied to the rat tail. Breath rate was measured by direct observation. Oxygen saturation was measured by pulse oximetry applied to rat tail (Ugo Basile, Italy). Blood glucose levels was measured by Accu-Chek clinical glucometer (Roche, Switzerland) in blood samples from a tail vein.

Invasive ICP monitoring

Invasive ICP monitoring was performed after collagenase injection, under isoflurane 1% anesthesia (see above). ICP was monitored using a fiber optic pressure sensor (OPP-M250, Opsens, Canada) connected to a single channel signal conditioner (LifeSens, Opsens, Canada), gently introduced in the same burr hole made for the injection of collagenase for approximately 5 mm or until a stable ICP tracing was obtained. The probe was then fastened to the operating table and ICP measurements were recorded with a frequency of 1 Hz using a dedicated software, then they were exported for analysis. Timing from collagenase injection to ICP recording start was variable, but in all cases was < 30 min. ICP values from 30 minutes to 120 minutes after collagenase injection were included in the analysis.

Statistical analysis

The data was analyzed using the SPSS Statistics (IBM Corporation, USA). Values were expressed as mean +/- standard deviation (SD). The two-group analysis on hematoma volume and mass effect was done by means of unpaired Student's t test. The two-group analysis on hematoma growth, physiological parameters and ICP was done by means of Mann Whitney test. The two-group analysis on mortality, Garcia neuroscore and corner turning were done by means of Fisher's exact test. A p-value of less than 0.05 was considered significant.

Results

Timing of hematoma expansion

A preliminary study assessed the timing of hematoma expansion in our experimental conditions at 30 min, 60 min and 120 min after collagenase injection. An evolving hematoma in the left putamen was visible at all time points, with an expansion rate of 0.55 mm^3/min in the first 120 min after collagenase injection (Figure 1). The time window from 60 min to 120 min was chosen to investigate the safety of HDT15°, since the hematoma at 60 min was well delineated and rapidly expanding. No animal died within 120 min of collagenase injection.

Non-inferiority trial: hematoma volume, mass effect, functional outcome and mortality

Hematoma volume at 24 hours was not different in rats treated with HDT15° compared to flat position, either calculated by volumetric histology (106+/-27 mm^3 versus 106+/-29 mm^3, respectively; p = 0.980) or by ABC/2 method (72.6+/-26 mm^3 versus 72.5+/-17 mm^3, respectively; p = 0.982) (Figure 2). Mass effect at 24 hours was similar in the two groups (17+/-7% HDT15° versus 19+/-5% flat position; p = 0.123; Figure 3). Mortality at 24 hours

was of 4 animals (12.5%) in both groups. No difference in functional outcome at 24 hours, assessed with Garcia neuroscore and corner turning test, was observed in rats treated with HDT15° compared to flat position (Table 1).

MRI study of hematoma growth

MRI-based analysis of hematoma growth showed a neutral effect of HDT15°, compared to flat position (60 min to 150 min, 36.8+/-30 mm^3 versus 35.8+/-17 mm^3, respectively, p = 0.818, Figure 4; 60 min to 24 hours, 52.5+/-22 mm^3 versus 50.7+/-28 mm^3, respectively, p = 0.872, data not shown). No animal died within 150 min of collagenase injection. Mortality at 24 hours was 20% in both groups (1 animal each). Physiological parameters, including systolic blood pressure, heart rate, respiratory rate, oxygen saturation and glycemia were comparable between the two groups at baseline, during surgery and after treatment (Supplementary Table 1).

ICP study

Treatment with HDT15° produced a mild increase of the ICP, compared to flat position (+2.91 +/- 1.76 mmHg versus +1.00 +/- 1.73 mmHg, respectively; p = 0.552) which occurred immediately after HDT15° application, followed by a gradual adaptation towards a steady state (Figure 5). No animal died in the ICP study, which was performed in the first 120 min after collagenase injection.

Discussion

Investigating, developing and implementing a "collateral therapeutic" is a major objective in stroke research [13,14,15]. The primary aim of increasing collateral flow is slowing down the pace of penumbra evolution and expanding the "tissue time window" to increase the efficacy of recanalization therapies. The ideal timing for application of a collateral therapeutic is the hyperacute phase of ischemic stroke, even in the pre-hospital setting, to enhance cerebral collateral blood flow before the delivery of recanalization therapies. A collateral therapeutic should not be only effective, but also easy to apply (even by paramedics), rapidly active and - most importantly - safe. For its simplicity and low cost, HDT15° represents a good candidate as a collateral therapeutic to be applied by Emergency Services, as soon as an acute ischemic stroke is suspected, even a few minutes after arterial occlusion.

Hemorrhagic stroke, in particular primary intracerebral hemorrhage, represents the major ischemic stroke mimic in the pre-hospital setting before performing a head CT scan and it accounts for approximately 10-15% of suspected acute stroke cases [9]. For this reason, exploring the effects of HDT15° application in case of intracerebral hemorrhage, particularly in the acute phase of hematoma expansion, is highly relevant for the translation of HDT15° into a collateral therapeutic for human stroke.

Our randomized non-inferiority preclinical trial investigated the safety of HDT15° compared to flat position in a rat model of severe ICH. We applied HDT15° during the phase of active hematoma expansion to assess safety in the worst possible scenario. We used a brief application of HDT15°, for 1 hour, because the time window for collateral therapeutics is likely to be limited to the hyperacute phase of stroke, before recanalization therapies.

Our findings showed that application HDT15° for 1 hour did not worsen neurologic outcome in experimental ICH, compared to flat position, in terms of hematoma volume, mass effect, mortality and functional deficit at 24 hours.

HDT15° had a neutral effect on hematoma growth and physiological parameters. ICP was increased by only a few mmHg after HDT15° application.

There is a lack of consensus in the current clinical practice regarding the best head position for patients affected by acute stroke, albeit the most common is the sitting position of about +30° [16]. Lower head positions, in particular the supine one and the HDT15°, are simple non-pharmacological therapies proven to be effective in increasing cerebral collateral blood flow in ischemic stroke models [5,6] and in transcranial Doppler studies on human ischemic stroke [17,18]. In the HeadPoST trial, flat position had a neutral effect on outcome in acute stroke patients [19]. However, this trial has been criticized because it recruited mostly non-LVO ischemic stroke patients and positional therapy was applied several hours after symptom onset [20]. Notably, a recent observational study in consecutive LVO acute stroke patients treated with HDT15° for the first 12 hours showed better clinical outcome and no adverse events (including hemorrhagic complications, intracranial hypertension and aspiration pneumonia), compared to standard +30° positioning [7].

A first limitation of our study is that functional outcome was limited to the first 24 hours. However, our study was focused on safety and particularly on the risks of acute hematoma expansion and acute ICP changes associated with a brief application of HDT15°. Moreover, long-term outcome is directly dependent on acute hematoma expansion, early hematoma volume and early neurologic status in rats as in humans [21,22].

A second limitation is related to the non-inferiority trial design addressing a safety issue, since no clear-cut data are available to justify the margin for safety [23]. Nonetheless, the study was conceived under a strong clinical perspective, where "flat position" corresponded to usual care and a non-inferiority margin of 15% was judged acceptable by clinical experts in Vascular Neurology involved in the study design.

A third limitation is that generalizability of our results to clinical setting requires caution. In particular, when planning clinical translation of HDT15° as a pre-hospital treatment of acute stroke patients, specific exclusion criteria (headache, decreased level of consciousness) will be necessary to exclude subarachnoid hemorrhage and posterior fossa hematoma, both of which have not been modelled in our safety study. Moreover, our study has not explored the safety of HDT15° beyond the hyperacute phase of ICH.

To conclude, our experimental study supports the safety of a brief application of HDT15° in the hyperacute phase of primary supratentorial ICH. Further experimental and clinical studies are needed to translate HDT15° as an emergency treatment of human stroke.

Acknowledgment

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Author Contributions

S.B., A.V. and E.R. designed the study; S.B., A.V., B.M, M.V., S.D., C.P., G.P., D.C., J.M., L.M., M.R., G.P., G.C., F.P., D.A. and I.G. performed the experiments; S.B., B.M., E.R., G.C., C.G., E.S. and C.F. analyzed and interpreted data; S.B. wrote the manuscript, which was critically revised by the other authors.

Data availability statement

The data that supports the findings of this study are available from the corresponding author upon request.

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Figure captions

Figure 1. Time course of hematoma expansion. (**a**) Hematoma volumes at 30 min (n=6), 60 min (n=6) and 120 min (n=6) after collagenase injection, compared to the final hematoma volume at 24 hours (n=32). The light blues box represents the timing of HDT15° application. (**b**) Representative histological slices showing progressive growing of the hematoma in the left basal ganglia, at different time points**.**

Figure 2. Hematoma volume at 24 hours in rats treated with HDT15° (n=28) compared to flat position (n=28). Hematoma volume was not quantified in animals died within 24 hours (4 animals per group, 12.5%). (**a**) Quantification of hematoma volume by volumetric histology (left) and ABC/2 method (right). (**b**) Representative histological slices of a rat brain with final hematoma at 24 hours, showing the consecutive slices used for volumetric histology (region of interest is shown in light blue over the rat brain matrice) and the single slice with the largest area of the hematoma (in dark blue) used for ABC/2 method.

Figure 3. Mass effect at 24 hours. (**a**) Quantification of mass effect in rats treated with HDT15° (n=32) compared to flat position (n=32). (**b**) Graphical representation of mass effect (green area) as the ratio of the volume of the hemorrhagic hemisphere (dashed line) to the volume of the healthy hemisphere (black dotted line; grey dotted line represents the mirror image over the hemorrhagic hemisphere). The region of interest for volume calculation extended beyond the hemorrhagic lesion, as shown in light green over the rat brain matrice.

Figure 4. MRI-based analysis of hematoma growth. (**a**) Quantification of hematoma growth (60 to 150 min) in rats treated with HDT15° (n=5) compared to flat position (n=5). Representative brain MRI slices of a HDT15°-treated rat showing hematoma growth between 60 min (**b**) and 150 min (**c**). HDT15° was applied for 1 hour between 90 min and 150 min.

Figure 5. Effect of HDT15° application on intracranial pressure. (**a**) Representative native tracing of intracranial pressure recording in a rat, before and during HDT15° application. X axis shows time after collagenase injection. (**b**) Mean and SD of intracranial pressure (n=11) per minute before and during HDT15° application. X axis shows time after collagenase injection. (**c**) Net changes in intracranial pressure before and after HDT15° application (n=11), compared to flat position (n=11).

Legends of supplementary files

Supplementary Figure 1. Flow diagram of the main study (non-inferiority safety trial) and ancillary studies (MRI study and ICP study).

Supplementary Table 1. Physiological parameters at baseline, during surgery and after treatment (MRI study).

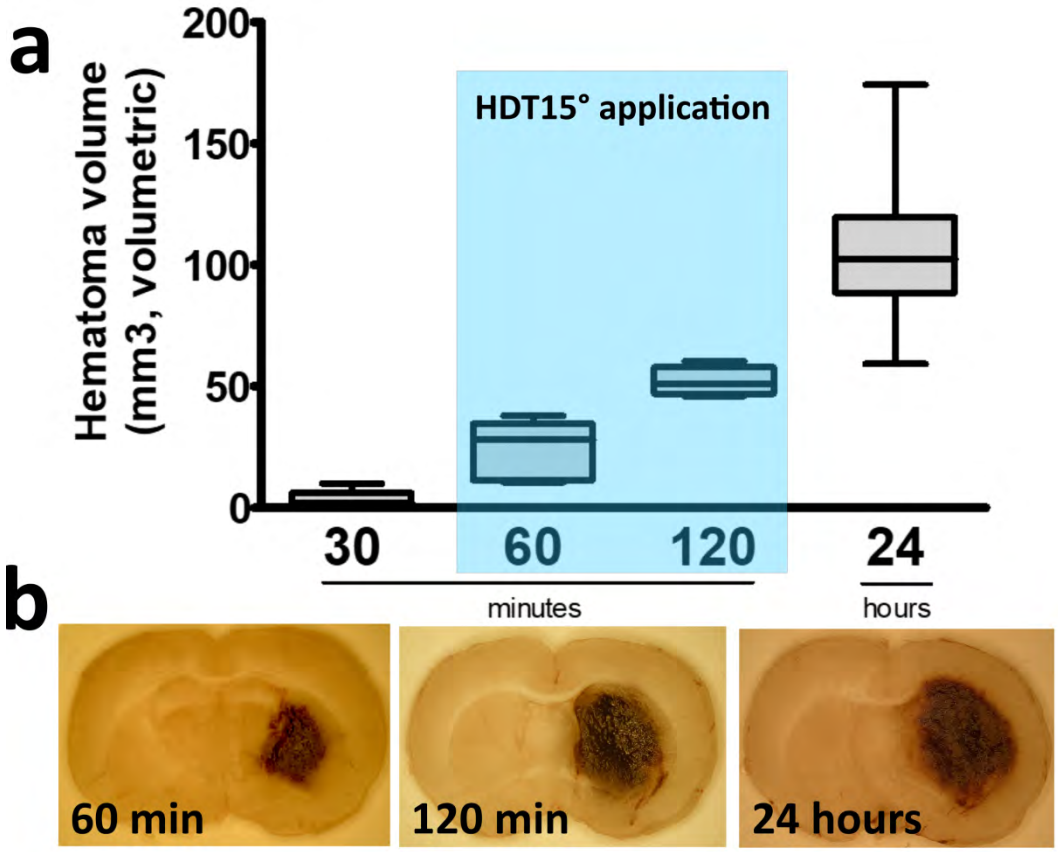
Graphical table of contents, highlights

- Exploring safety of head down 15° (HDT15°) application in the acute phase of intracerebral hemorrhage is highly relevant for its translation into a collateral therapeutic for pre-hospital treatment of acute ischemic stroke.
- Application of head down tilt 15° for 1 hour in a rat model of large supratentorial intracerebral hemorrhage does not worsen early outcome, compared to flat position, in terms of hematoma volume, hematoma growth, mass effect, mortality and functional deficit at 24 hours.
- Intracranial pressure was increased by a few mmHg (+ 2.91 +/- 1.76 mmHg) after HDT15° application.

Table 1. Functional outcome 24 hours after induction of ICH

Outcome	Groups	Success n (%)	Failure n (%)	p-value
Mortality	Flat position	28 (87.5)	4 (12.5)	1.000
	HDT15°	28 (87.5)	4 (12.5)	
Garcia neuroscore	Flat position	4 (12.5)	28 (87.5)	1.000
	HDT15°	3 (9.4)	29 (90.6)	
Corner turning test	Flat position	16 (50.0)	16 (50.0)	0.802
	HDT15°	14 (43.8)	18 (56.2)	

"Success" indicates a good functional outcome at 24 hours (alive; Garcia neuroscore 14 to 18; % of right turns > 30%). "Failure" indicates a poor functional outcome at 24 hours (dead; Garcia neuroscore < 14; % of right turns 30% or less).



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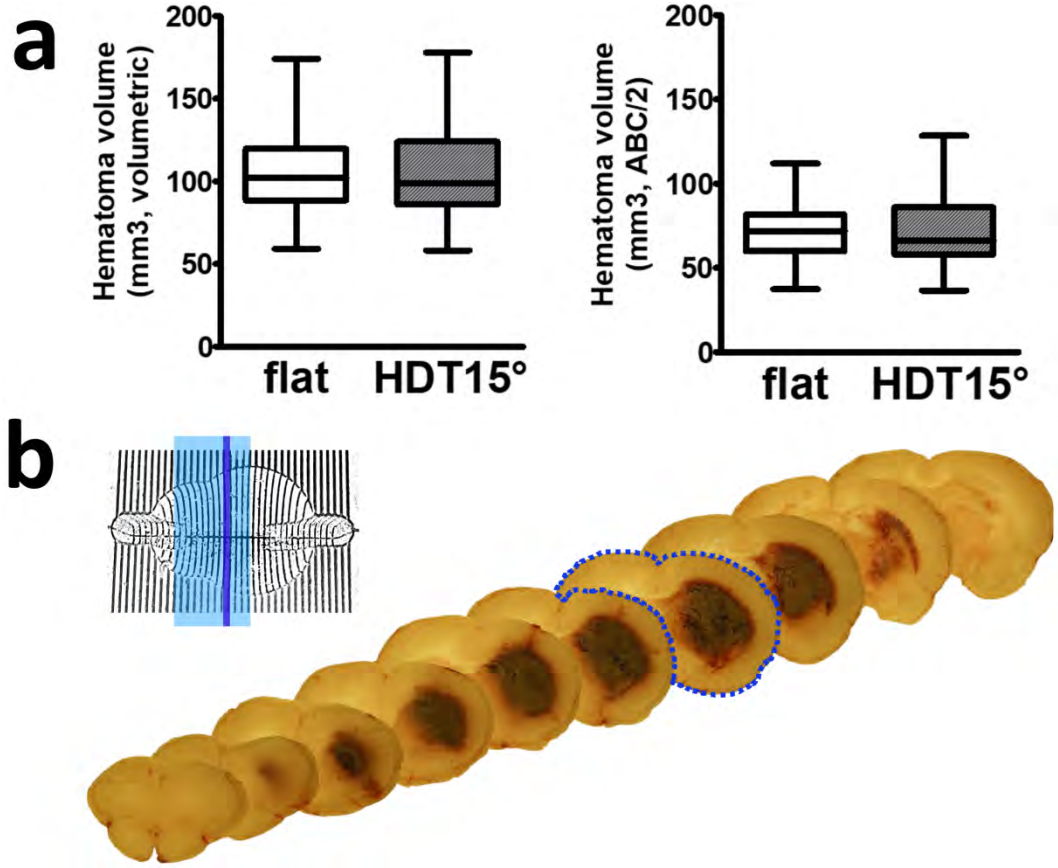

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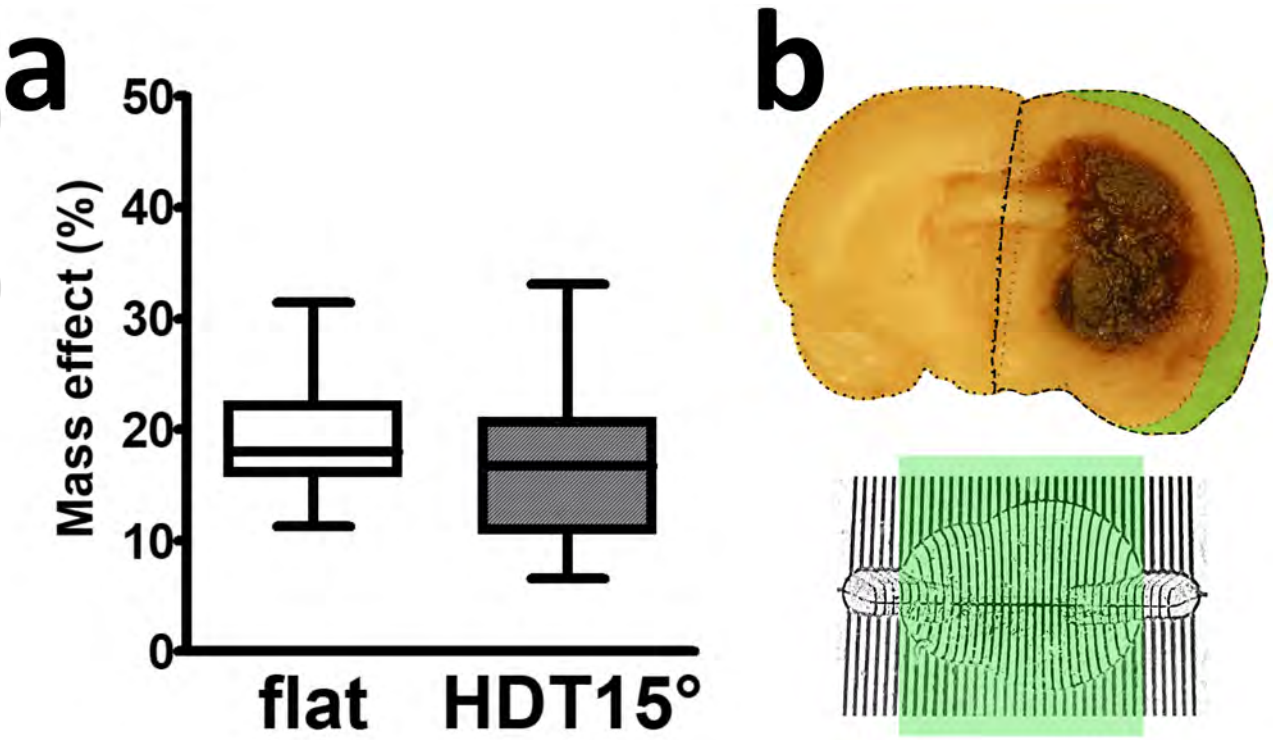

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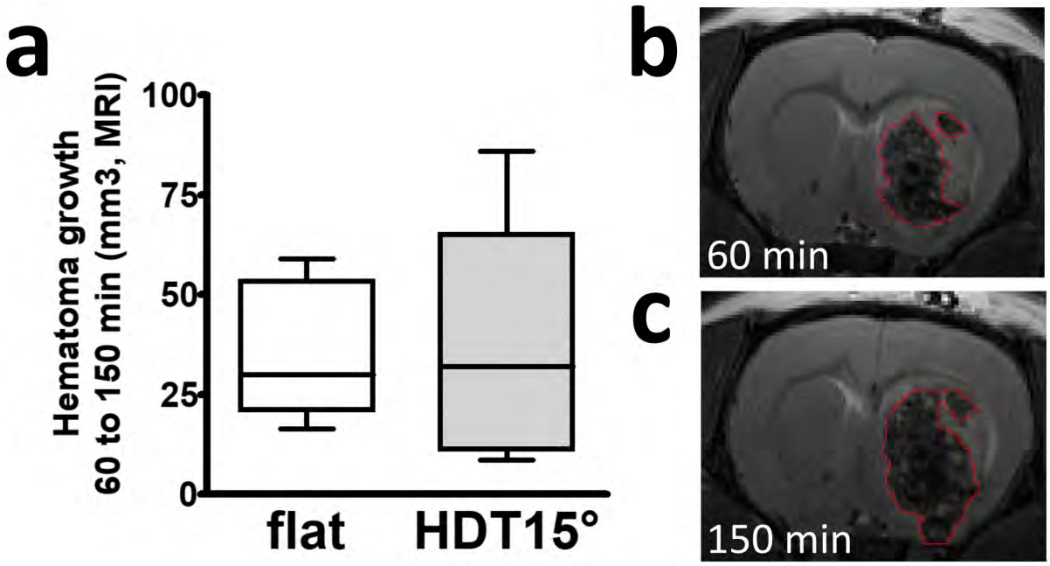

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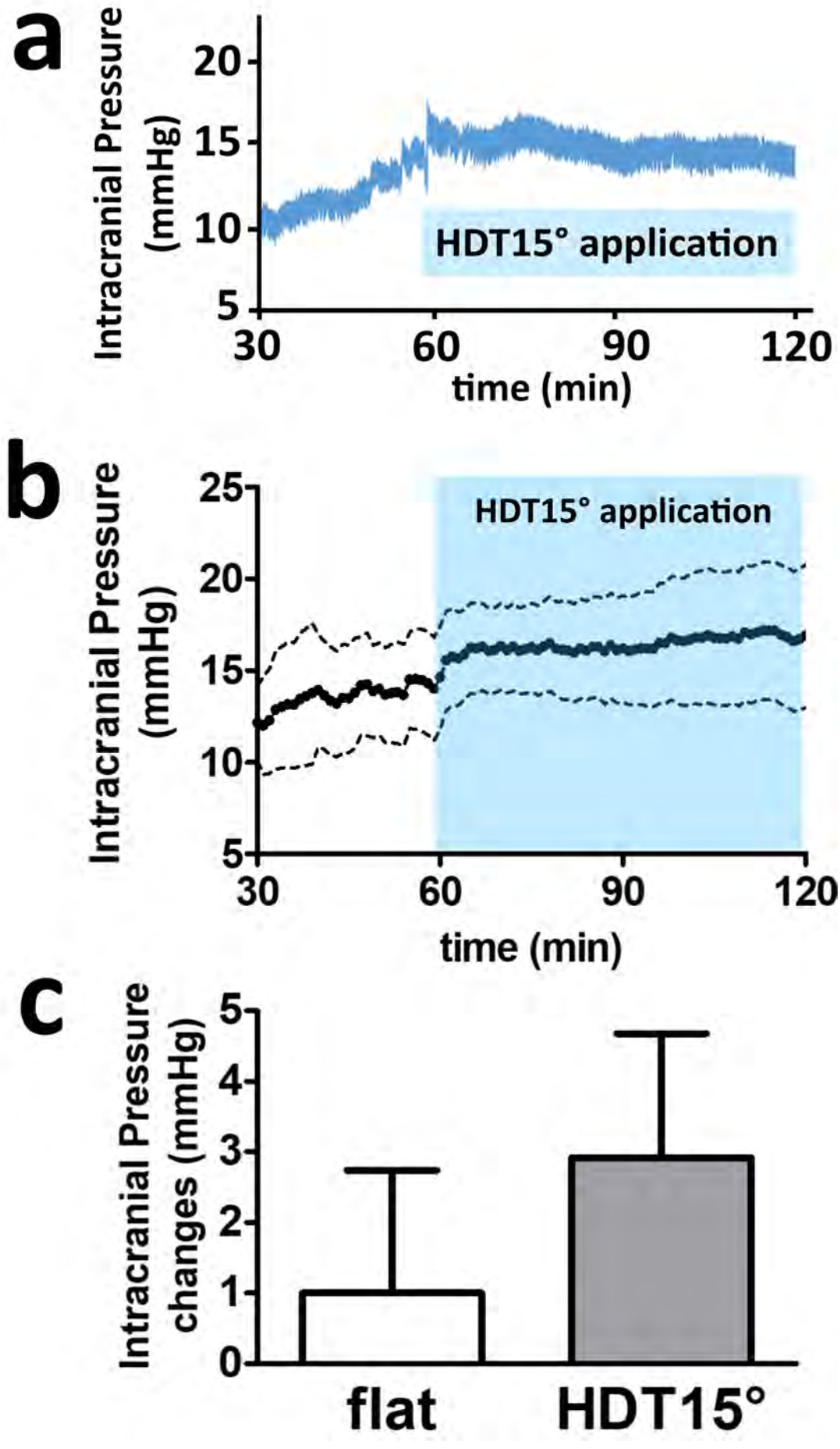

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