Journal of
Cerebral Blood Flow
& Metabolism

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| Journal: | *Journal of Cerebral Blood Flow and Metabolism* |
| Manuscript ID | JCBFM-0031-21-ORIG.R1 |
| Manuscript Type: | Original Article |
| Date Submitted by the Author: | 23-Apr-2021 |
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# Selective intra-carotid blood cooling in acute ischemic stroke: a safety and feasibility study in an ovine stroke model

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**Running headline:** safety of intracarotid cooling device in stroke

**Abstract**

Selective therapeutic hypothermia (TH) showed promising preclinical results as a neuroprotective strategy in acute ischemic stroke. We aimed to assess safety and feasibility of an intracarotid cooling catheter conceived for fast and selective brain cooling during endovascular thrombectomy in an ovine stroke model.

Transient middle cerebral artery occlusion (MCAO, 3h) was performed in 20 sheep. In the hypothermia group (n=10), selective TH was initiated 20 minutes before recanalization, and was maintained for another 3h. In the normothermia control group (n=10), a standard 8 French catheter was used instead. Primary endpoints were intranasal cooling performance (feasibility) plus vessel patency assessed by digital subtraction angiography and carotid artery wall integrity (histopathology, both safety). Secondary endpoints were neurological outcome and infarct volumes.

Computed tomography perfusion demonstrated MCA territory hypoperfusion during MCAO in both groups. Intranasal temperature decreased by 1.1°C/3.1°C after 10/60 minutes in the TH group and 0.3°C/0.4°C in the normothermia group ($p<0.001$). Carotid artery and branching vessel patency as well as carotid wall integrity was indifferent between groups. Infarct volumes ($p=0.74$) and neurological outcome ($p=0.82$) were similar in both groups.

Selective TH was feasible and safe. However, a larger number of subjects might be required to demonstrate efficacy.

**Key words:** acute ischemic stroke, catheter, endovascular stroke therapy, hypothermia, selective brain cooling

## Introduction

Therapeutic hypothermia (TH) has proved neuroprotective effects in hypoxic-ischemic brain damage related to cardiac arrest.[1] Moreover, randomized clinical trials have shown improved functional outcome and reduced mortality after successful resuscitation when TH was applied systemically using extracorporal or intravenous blood cooling techniques. These techniques became therapeutic standards for cardiac arrest almost two decades ago.[2,3]

In acute ischemic stroke (AIS) caused by cerebral large vessel occlusion (LVO), TH might provide neuroprotection[4–6] with promising effects including lesion size reduction and preservation of white matter integrity being shown in several rodent models using brain cooling techniques.[7–10] Early clinical studies indicated feasibility and safety of systemic TH induced by intravenous and extracorporeal cooling devices.[11,12] However, a recent multicenter, randomized clinical trial designed to assess efficacy of an intravenous cooling device (ICTusS 2 trial) in patients eligible for intravenous thrombolysis (IVT) was stopped prematurely, reporting increased systemic complications such as pneumonia.[13] It was also postulated that effective cooling of ischemic brain tissue may be impaired by the LVO itself.[14]

Concurrently, the advent of mechanical thrombectomy (MT) revolutionized the treatment of patients with LVO, replaced IVT as a primary treatment and finally raised the interest for new treatment options to synergistically integrate neuroprotective effects such as mediated by hypothermia in the frame of a MT procedure.[15] In particular, a major pathophysiological element related to IVT or MT is reperfusion damage emerging immediately after blood flow restoration[16], so systemic whole-body cooling may be too slow and hence too late for an effective counteraction after recanalization. Thus, rapid and early endovascular selective brain cooling combined with MT may be an appealing alternative TH approach for neuroprotection in LVO stroke.[4,6,17,18] A recent study including 113 LVO patients demonstrated safety of a combined therapy applying a standard MT plus a short (15 min) intra-arterial (‘selective’) cold saline infusion versus MT alone.[6] Moreover, a trend towards improved functional outcome was

observed in the selective TH group. However, the duration and impact of direct intra-arterial cold saline infusion may be limited by the applicable saline volume.[8]

We have recently reported preliminary *in vitro* and *in vivo* assessment of a closed-loop cooling catheter (CLCC) system for intra-carotid blood cooling[17,19,20] conceived to provide swift and selective TH in combination with MT for the treatment of LVO stroke. Herein, we aimed to assess feasibility and safety of the CLCC system in an ovine stroke model simulating MT by transient middle cerebral artery occlusion (MCAO). The study was designed as an exploratory approach with partially blinded endpoint assessment.

## Material and Methods

*Study design and ethics*

The study was performed according to the German animal protection law and the animal care and welfare guidelines of the European Community (2010/63/EU). Animal experiments were approved by the local ethics committee (Regierungspräsidium Freiburg, Germany; #39-9185.81/G-15/38). ARRIVE guidelines were applied.

Primary endpoints comprised intranasal temperature decrease of 2°C within the first 30 minutes of cooling in the hypothermia group (feasibility), and carotid artery injury in hypothermia compared to normothermia group, assessed on histological findings and angiographically (safety). Secondary endpoints were infarct volumes assessed by magnetic resonance imaging (MRI) and functional outcome (efficacy).

The study was designed to reveal an effect size of at least 1.33 regarding primary endpoints at 80% power and $p<0.05$. This required a minimum sample size of n=10 per group. Treatment allocation of animals into hypothermia and normothermia groups had to be done in a non-randomized order due to delaying technical issues which impeded the use of the CLCC from the beginning of the study start and concomitant, temporally restricted availability of animals. Thus, the normothermia group had to be performed first. We accepted this limitation due to the

exploratory nature of the study. Evaluators of primary and secondary endpoints were blinded to the treatment allocation. Figure 1(a) provides an overview on the study design.

*Animals*

The study involved twenty merino half breed ewes (age 10-20 months; weight 45-76 kg). Animals were kept in the Center for Experimental Models and Transgenic Service of the University of Freiburg under following conditions: group housing on straw bedding, daily outside grazing, water and hay *ad libitum*, plus concentrated feed pellets as reward and to foster human familiarization. Blood test and parasitological examination were conducted one day before surgery. Animals were dewormed when recruited into the study population and deworming was repeated at regular intervals as well as in case of individual parasitological findings. Sheep were physically examined for 30 days after the procedure, assessing body weight, respiratory and pulse rates, and body temperature. The Body Condition Score (BCS) was also applied as reported elsewhere.[21]

*Anesthesia*

Anesthesia was initiated by intramuscular injection of midazolam (0.5 mg/kg bodyweight (BW)) and ketamine hydrochloride (20 mg/kg BW), and was deepened by intravenous propofol administration (1-2 mg/kg BW). After endotracheal intubation, 12-15 breaths/min were provided by a volume-controlled ventilator at a 10-15 mL/kg BW tidal volume and 5-mbar positive end-expiratory pressure. Settings were adjusted to normalize oxygen and carbon dioxide tension, and pH values. Anesthesia for surgical and endovascular procedures was maintained by isoflurane in oxygen/air ($FiO_2$ >0.4), intravenous ketamine (10 mg/kg BW/h) and fentanyl (5-10 µg/kg BW/h) administration. For computed tomography (CT) perfusion and brain MRI examinations, anesthesia was maintained by intravenous propofol administration at 15-18 mg/kg/h.

Fluid homeostasis was maintained by intravenous infusion of Ringer solution (10 mg/kg BW/h). Infusion rates were increased in case of large fluid losses for instance by massive salivation (a common but benign phenomenon in anesthetized sheep), or to increase blood pressure non-pharmacologically. An intraoperative antibiotic treatment with ceftriaxone (2 g i.v.) was applied. Postsurgical antibiotic (dihydrostreptomycin sulfate 12.9 mg/kg, benzylpenicillin- procaine 8 mg/kg) and analgesic (carprofen 4 mg/kg) treatment was performed for at least 3 days following surgery.

*Physiological and temperature monitoring*

Physiological parameters (arterial oxygenation, heart rate and mean arterial blood pressure) were recorded within predefined intervals during the surgical procedure (30 to 0 minutes before cooling, as well as 5-30, 35-60, 65-90, 95-120, 125-150, 155-180 and 185-210 min post initiation of cooling). In order to avoid any interference of measurements with surgical procedures, exact time points of measurement were allowed to slightly differ between animals. Arterial blood gas analysis was performed at predefined time points (95 and 20 min before MCA recanalization, as well as 20, 50, 70, 115 and 150 min thereafter).

Body (rectal) and head (deep intranasal, right nostril) temperatures were recorded non-invasively and continuously (10-sec intervals) using temperature probes (MP00992; Draeger Medical GmbH, Lübeck, Germany). In a previously published analysis of intra-carotid blood cooling in sheep[20], ipsilateral nasal temperature was shown to correlate well with brain temperatures of the cooled hemisphere, exhibiting a stable mean gradient over the whole cooling period with nasal temperatures being about 0.4 to 0.5°C higher than brain temperatures. This gradient is likely related to mixing of cooled and non-cooled blood from bilateral external carotid artery supply to the nasal tissue. Thus, we decided to skip invasive brain temperature measurement in order to avoid potential sequelae from potential brain trauma, and used ipsilateral nasal measurements as a surrogate for brain temperature in the cooled hemisphere

instead. Temperature drops were calculated for both ipsilateral nasal and rectal measurements by subtracting each procedural measurement from an individual baseline temperature that was time-averaged over a 30 min interval prior to start of the cooling procedure for each animal and probe.

*Middle cerebral artery occlusion*

Transient MCAO by surgical clip application including confirmation of MCAO by CT perfusion imaging was performed as described previously.[22] In brief, sheep were positioned in supine position with the head turned to the left side. After a 5-7 cm long skin incision along the right superior temporal fossa, the fascia of the temporal muscle was opened and the muscle was stripped away laterally. The coronoid process was lateralized and craniectomy over the junction was performed using an electric high-speed drill (microspeed, Aesculap, Tuttlingen, Germany) to access the floor of the middle cranial fossa. After opening the dura and using an optic microscope (Möller-Wedel, Wedel, Germany), the distal branches of the MCA were followed proximally until the optic nerve and the terminal internal carotid artery (ICA) had been identified. An aneurysm clip (Yasargil transient titanium clip, Aesculap) was attached to the proximal MCA for transient occlusion and was removed after 3h. Directly after vessel occlusion, a transient wound closure was performed and animals were transferred to CT perfusion imaging for MCAO confirmation.[22] Thereafter, sheep were transferred back to the operating room for the endovascular procedure (see below) and clip removal followed by permanent cranial wound closure. An intravenous heparin bolus (70 IU/kg BW) was administered after clip removal and wound closure.

[Figure 1 around here]

*Endovascular procedure*

For endovascular access, the right femoral artery was punctured and a 12 French (F) sheath was introduced. In the hypothermia group, a CLCC was inserted into the right common carotid artery (CCA) by use of a coaxial 125 cm 5F vertebral or Simmons 2-shaped inner catheter for vessel selection. In the normothermia group, a 90 cm long 8F sheath (Flexor® Shuttle® Guiding Sheath, Cook Medical, Ireland) was inserted into the right CCA instead to simulate a standard MT procedure and potentially related vessel wall trauma. Outer diameter was similar in both CLCC and 8F sheath. A mono-planar C-arm angiography system (XA BV300, Philips Health Systems, Hamburg, Germany) was used to perform selective digital subtraction angiography (DSA) with contrast agent administration (Solutrast 300, Bracco Imaging Deutschland, Konstanz, Germany) into the right CCA. DSA imaging (anterior-posterior and lateral views) for assessment of vessel patency, CCA vasospasm and potential embolic occlusion was performed first during MCAO (prior to initiation of cooling), and after 90 min of cooling (70 min after recanalization by clip removal), and finally after 180 min of cooling prior to catheter removal.

*Closed-loop cooling catheter*

CLCCs (Figure 1(b)) were developed, manufactured and provided by the company Acandis GmbH (Pforzheim, Germany). The distal end of the CLCC consists of four balloons with a diameter of 4 mm and a length of 20 mm each, spaced from each other by 4 mm, resulting in a total length of 92 mm.[17,19,20] Two catheter lumina with an inner diameter of around 1 mm each enable a continuous closed-loop flow of cold saline into and out of the balloons, respectively. The catheter also features a central lumen compatible with a 6F catheter for MT procedure during cooling, resulting in a 3.4 mm outer diameter (corresponding to an 11F catheter or to an 8F sheath). The saline solution was cooled externally using a compression chiller (Ministat 125; Peter Huber Kältemaschinenbau, Offenburg, Germany) to provide a temperature of around 5°C at the catheter entrance, measured with a precision fine-wire thermocouple (5TC-KK-KI-24-2

m; Omega Engineering, Stamford, Connecticut, USA). A further thermocouple was used to measure the coolant temperature at the catheter outlet (data not shown). Flow was maintained by a roller pump (Behrotest PLP 220 with a PPH 303 pump head; Behr Labor-Technik, Düsseldorf, Germany) and measured by ultrasonic flow meters (M-2111; Malema Engineering, Boca Raton, Florida, USA). Coolant pressure was measured proximal to the catheter inlet (HPSA-B10DVAB-020-G; Althen, Kelkheim) to assure a maximal value of around 3 bar during the whole procedure, according to catheter and pump specifications. Custom-made, isolated, double wall PVD tubes allowed for coolant flow from the chiller to the pump and from the pump to the catheter. A further tube with integrated temperature and coolant flow probes enabled coolant flow-back from the catheter outlet to the chiller.

*Selective hypothermia*

In the hypothermia group, cooling with the CLCC was initiated 20 min prior to MCA recanalization by clip removal (initial coolant flow rate 100-120 ml/min; maximal inlet pressure 3 bar). Cooling was maintained for 180 min (or 160 minutes after MCA recanalization). Coolant flow rates were reduced towards the end of the cooling period in steps of 20 ml/min in order to maintain a maximal nasal temperature drop of 4°C and thus prevent heavy shivering during the post-operative period, which may compromise a controlled rewarming of the animals.

The CLCC was removed after 3 hours, the femoral artery was immediately ligated and the skin wound was closed. Procedures in the normothermia group were identical except for the long sheath being inserted into the right CCA instead of the CLCC, and omission of cooling.

*Carotid blood flow measurements*

Carotid blood flow velocity within the right CCA was measured by experienced vascular neurologists (W.-D.N., C.S.) at mid cervical level using color Doppler ultrasound (Sonosite PX, FUJIFILM Sonosite, Amsterdam, Netherlands). Measurements were obtained first before

CLCC placement during MCAO, second after MCAO during cooling distal to the CLCC tip, and third after removal of the CLCC. Mean flow velocities were calculated from peak systolic and end diastolic flow velocity measurements.

*Animal imaging: CT perfusion and MRI*

CT perfusion was performed on a 16-slice CT scanner (Somatom Sensation 16, Siemens) immediately after surgical clip placement for confirmation of correct MCAO as previously described.[22] Standard perfusion image maps (CBV, CBF, and $T_{max}$) were processed using a dedicated commercial software package (SyngoVia, Siemens, Erlangen, Germany). Images were rated by an experienced neuroradiologist (S.M.) for the presence and degree of MCA territory hypoperfusion using the following semiquantitative score as previously reported[22] with 0: no hypoperfusion visible on $T_{max}$/CBF/CBV, 1: hypoperfusion visible on $T_{max}$ only, 2: hypoperfusion visible on $T_{max}$ and partially visible on CBF/CBV, 3: hypoperfusion visible on $T_{max}$/CBF and partially on CBV.

MRI was performed on a 3T MRI Scanner (Trio, Siemens, Erlangen, Germany) using a combined 12-channel head/neck coil on day 2 and day 30 after MCAO in each animal (see Supplementary Table 1 for details). Volumetric analyses of infarct (coronal DWI images, correlated with ADC maps) and edema (infarct plus surrounding vasogenic edema on coronal T2w images) were performed using manual segmentations on the medical imaging platform NORA (www.nora-imaging.com). From these segmentations, the total lesion volumes were automatically calculated (number of voxels in the segmentation x voxel size). Representative images are shown in Supplementary Figure 1.

*Neurological assessment*

All animals underwent neurological examination by an experienced veterinary physician (A.M.H., J.H.) pre-procedure and on days 1-5, 7, 10, 15, 20, 25, and 30 post MCAO using a

modified ovine neurological score for sheep (Supplementary Table 2) based on a previously reported one.[23]

*Histology of carotid arteries*

Sheep were sacrificed in deep anesthesia by an intravenous potassium chloride overdose following the MRT examination on day 30. Death by cardiac arrest was certified by an independent veterinarian. Long-segmental specimen (length, 15-21.5 cm) of bilateral CCAs were surgically removed and fixed in buffered 3.5% formaldehyde-solution (Otto Fischar GmbH & Co. KG, Saarbrücken, Germany) for histopathological evaluation. 40 samples per vessel were taken, and 4 samples were embedded together. Vessel samples were marked from cranial to caudal in order to allow later orientation at the incision. Of each embedding two slices of 2 µm were cut using a microtome (Leica RM2255®; Leica, Wetzlar, Germany). The slices were stained for hematoxylin-eosin (HE) and Verhoeff's van Gieson (EVG) by routine staining protocols of the Pathological Institute, University Hospital Freiburg. The following histopathological parameters accounting for vessel wall integrity were evaluated by two experienced pathologists (L.L., S.K.): thickening and inflammation of the tunica intima, thickening and cell abundance of the tunica media, fragmentation of the elastic fibers, presence of luminal or wall-adherent thrombus, fibrinoleukocytic scab, and presence of dissection. Samples were initially assessed using light microscopy (Leica DM2500® equipped with 2.5x objective). Only changes visible under these conditions were included in the semi-quantitative scoring analysis which was performed at 100x magnification. The extent of the vessel alterations was graded in a four-tier scale (0: no changes, 1: minimal changes; 2: moderate changes and 3: severe changes) as demonstrated in Supplementary Figure 2.

*Statistical Analysis*

All statistical analyses were planned and performed by a highly experienced senior biostatistician (G.I.). Descriptive data are presented as mean and standard deviations (SD) for normally distributed, continuous variables or median and interquartile ranges (IQR) for all other continuous variables, respectively. Frequency distributions are provided for binary or categorical variables. Continuous variables were checked for normality of data distribution using Shapiro-Wilk tests. Group comparisons were then performed with t-tests for normally distributed variables and Wilcoxon two-sample tests in case of non-normally distributed variables. Continuity adjusted chi-square tests were used for group comparisons of binary variables, and the Mantel-Haenszel chi-square test was applied for ordered categorical variables.

Since continuous temperature recording generated a large amount of individual data points, temperature course was analyzed in 5 min intervals. For temporal comparison of the neurological deficits, the area under the curve (AUC) from daily assessments was calculated and compared between groups. Spearman correlation coefficients between hypoperfusion score on CT and secondary outcomes (animal neuroscore, MRI infarct/edema volumes, and infarct size on histopathology) were calculated.

Statistical analysis was performed using SAS version 9.2 (SAS Institute Inc, Cary, NC, USA). Reported p-values are not adjusted for multiple testing and are therefore considered as descriptive information. To account for multiple testing time points, Bonferroni-type corrections are not useful as the measurements are highly dependent. A hierarchical testing procedure is applied, i.e. tests are ordered according to the time sequence. Tests are performed in this order at a significance level of $\alpha=0.05$. The procedure stops if a non-significant result is obtained, and no furthers tests are performed.[24]

## Results

*Physical and physiological parameters at baseline and during follow-up*

Mean body weight was 68.8 kg (SD 5.5) in normothermic animals and 57.9 kg (10.0) in hypothermic animals at baseline ($p<0.01$), and 68.3 kg (5.2) versus 56.3 kg (8.9) at day 30 follow-up ($p<0.01$), respectively. Results of pre-procedural blood and parasitological tests (Supplementary Table 3) showed a slightly higher presence of cestodes in the normothermia group ($p<0.05$), and higher blood bilirubin and fibrinogen levels in the hypothermia group ($p<0.01$) that, however, did not exceed the normal reference range. Assessment of physiological parameters (Supplementary Table 4) revealed that respiratory rates were significantly higher in the hypothermia group at days 1 to 4, and day 30 ($p<0.05$), but did not exceed the physiological reference range at any time. Body temperature did not differ significantly at any time despite for day 10 at which a minimal (0.3°C) difference was between the groups ($p<0.05$), again not violating physiological reference ranges. BCS was within normal limits in both groups but slightly higher in the normothermia group at days 1 to 3 ($p<0.05$).

*Procedural physiological monitoring during selective cooling*

Detailed intraprocedural physiological monitoring data is reported in Supplementary Table 5. Mean arterial blood pressure progressively lowered in the hypothermia group from 100.1 (SD 13.6) mmHg at baseline to 80.3 (11.7) mmHg at the end of the experiment versus 96.8 (29.1) mmHg to 98.3 (30.2) mmHg in the normothermia group. Blood pressure was maintained in normal ranges non-pharmacologically what required higher infusion volumes in hypothermia animals (data not shown), and was significantly lower in that group between 95 and 150 min after hypothermia onset. Although not reaching statistically significance at any time point, mean heart rate was lower in the hypothermia during cooling, sometimes dropping into mild bradycardia. Arterial oxygenation was normal and did not differ between groups.

Results of intraprocedural arterial blood gas analysis are provided in Supplementary Table 6. Arterial $pCO_2$ levels were elevated in the hypothermia group after cooling onset turning into mild hypercapnia (>47.0 mmHg, maximum 48.5 mmHg) towards the end of the measurement period (115 min and beyond). Blood oxygen was continuously elevated to non-physiological levels due to the $FiO_2$ >0.4 maintained throughout the experiment. Of note, a number of other parameters were intermittingly or permanently different from those in the hypothermia group, but did not violate physiological ranges in most cases. Sodium, calcium, potassium, lactate, and hematocrit fell below normal ranges in both groups, most likely due to the continuous fluid supply which tended to be higher in the hypothermia group to counter mild hypotension. Chloride was continuously elevated in the hypothermia group ($p<0.05$), but remained within the physiological range.

*Feasibility endpoint: intra-carotid blood cooling effect*

Catheter navigation to CCA using CLCC was feasible in all animals. Immediately after initiation of selective hypothermia, ipsilateral nasal temperature as a surrogate for hemispheric brain temperature started to decline and plateaued at approximately -4°C due to downregulation of CLCC cooling performance as described above (Figure 2(a)). Temperature differences became statistically significantly lower no later than 10 min after cooling onset (Table 1) compared to the normothermia group ($p<0.01$). The normothermia group showed a mild nasal (max. -0.7°C) and systemic temperature drop (max. -0.53°C) observed at 180 min, likely due to prolonged general anesthesia and loss of active temperature regulation. In the hypothermia group, ipsilateral nasal temperatures were significantly lower by 0.49 to 0.79°C ($p<0.01$) compared to systemic rectal temperatures during the initial 2 hours of cooling indicating, a selective cranial cooling effect (Supplementary Table 7). In the later cooling and early postcooling period, these differences levelled out due to active reduction of CLCC cooling rates (Figure 2(b)).

[Table 1 and Figure 2 around here]

*Safety endpoints: CCA ultrasound and angiography, and histological analysis of carotid arteries*

A moderate increase in CCA mean blood flow velocity compared to the baseline measurement was observed by Doppler ultrasound after removal of the CLCC (hypothermia) and the 8F sheath controls, respectively. There were no statistically significant differences between the groups, although a trend for higher flow velocities after MCAO (p=0.06) and CLCC/sheath removal (p=0.08; Table 2) was seen in the hypothermia group. On DSA, only mild CCA vasospasm occurred in 10-20% of cases after catheter insertion without statistically significant differences between both groups (p=1.0; Table 2). Major thrombus or vessel occlusion was not observed. Peripheral occlusions in superficial temporal CCA branches likely related to the surgical access for MCAO and were seen in both groups (p=0.37-1.0; Table 2). Post-mortem histological analysis of CCA specimens did not reveal evidence for decreased vessel wall integrity in the hypothermia versus the normothermia group (Table 3, Supplementary Figure 3). Moreover, no differences were observed between treated and non-treated CCAs in the hypothermia group.

[Tables 2 and 3 plus Supplementary Figure 3 around here]

*Secondary endpoints: MCAO imaging, MRI of infarcts, and neurological outcome*

Analysis of CT perfusion after MCAO revealed that the mean extent of MCA territory hypoperfusion was lower in the normothermia group, although without statistical significance (p=0.54; Table 2).

Temporal evolution of MCA infarcts by MRI showed no difference in volumes of early infarct and edema (T2) on day 2, as well as of chronic infarct on day 30 (T2) between both groups (p=0.56-0.74; Table 4 and Figure 3). All MCA vessels remained recanalized on TOF MRA at both MRI measurements.

A moderate correlation between the extent of MCA territory hypoperfusion during transient MCAO (hypoperfusion score 0-3) and the resulting MRI infarct (correlation coefficient, 0.58; $p<0.01$) and edema (correlation coefficient, 0.56; $p<0.05$) volumes on day 2 was observed in all animals irrespective of the mode of treatment.

[Table 4 and Figure 3 around here]

The course of neurological deficits within 30 days post MCAO showed no statistically significant difference between hypothermic and normothermic animals (p=0.82, Table 4), although a lower area under the curve AUC was seen in hypothermia animals. An additional subgroup analysis of animals with severe MCA territory hypoperfusion (n=8 and n=5 in hypothermia and normothermia groups, respectively) did not reveal statistically significant differences in functional outcome (neuroscore) or MRI edema and infarct volumes (p=0.47-0.76; Table 4).

## Discussion

TH is considered a promising approach for neuroprotection in the treatment of AIS. However, intravenous systemic cooling techniques being effective for cardiac arrest showed no benefit but an increased risk of pneumonia in the cooling arm in a prematurely stopped randomized multicenter trial when combined with IVT for acute AIS treatment.[13] In the era of MT being the primary treatment of AIS related to LVO, faster and more selective brain cooling via endovascular means combined with MT treatment becomes a valuable alternative strategy for TH.[4,25,26,6] Fast and selective brain cooling may also counteract secondary injury during the critical phase of reperfusion. This is emphasized by the strong effect of local arterial infusion of cold saline into the ischemic region of the brain: in rat models of transient MCAO, the perfusion of cold saline into the internal carotid artery shortly before or after reperfusion resulted in decreased infarct volumes.[27–29] However, these results may not be representative for AIS patients for three reasons. First, cooling of a larger brain volume might be required. Second, higher blood flow rates in the human CCA combined with proportionally lower coolant flow rates potentially reduce the cooling effect on brain parenchyma. Third, larger arterial wall surfaces may promote heat exchange with surrounding tissues, further limiting effective cooling. Large animal models better approximate the human situation than rodent models, warranting large animal experiments before moving on to clinical trials.

In two recent clinical studies with LVO stroke patients, selective TH was induced by direct infusion of 50 mL cold saline beyond an MCA-occluding thrombus. [30,6] Cold saline infusion was provided via a microcatheter for 5 minutes before MT was performed, followed by further 10 minutes of cooled saline infusion into the carotid artery. In both studies, no brain temperature measurement was provided. Besides showing the safety of the method, data suggest a trend towards a smaller infarct volume and a favorable functional outcome in patients receiving MT plus hypothermia compared to controls receiving only MT. Though these differences were not significant, results are promising and seem to support the rationale of early local TH despite a

potential delay of vessel recanalization by MT.[26,6] The CLCC tested in this study allows MT and simultaneous initiation of cooling directly at the site of the LVO. Pre-cooled blood would selectively reach the target brain tissue immediately after recanalization what may also mitigate local inflammatory processes.[31]

The cooling performance of the CLCC clearly matched the expectations: intranasal temperature dropped swiftly by 1.7°C within the first 20 minutes, i.e. prior to recanalization, compared to a systemic temperature reduction of 0.9°C, and further decreased to -2.1°C (systemic temperature -1.3°C) within the next 10 minutes. Given that the difference between measured nasal and brain temperature during cooling is about 0.4-0.5°C [20], an estimated brain temperature drop of 2.1-2.2°C occurred after 20 minutes. This reflects a reasonable time frame to navigate, reach the clot and successfully recanalize an occluded MCA in the clinical MT setting.

After one hour of cooling, intranasal temperature dropped by 3.1°C as compared to baseline. Systemic (rectal) temperature lacked behind until 2 hours of cooling, but body temperature in the hypothermia group finally dropped by more than 4°C at 150 min. This means that systemic side effects of cooling cannot be excluded when the CLCC is applied for longer periods. These findings warrant exploration of short-time selective brain cooling approaches to limit the systemic temperature drop and thus possible side effects. In this regard, a recent *in vitro* study demonstrated a positive effect of a shorter cooling duration on neuron activity.[32]

Safety investigations did not reveal any inter-group differences that would indicate a detrimental impact of the CLCC. Of note, CCA mean flow velocity was higher prior to and after cooling although formal statistical significance was missed (p=0.06 and 0.08, respectively). The reasons are uncertain and it cannot be excluded that missing statistical significance is a matter of low statistical power. However, the fact that CCA flow velocity was already higher prior to the placement of CLCC may relate this difference to group inhomogeneity rather than to a direct effect from the cooling procedure. Another potential

explanation for the elevation of CCA flow velocities at the end of the cooling procedure could be a hypothermia-induced vasodilatation of large central arteries distal to the CLCC.[20,33] Histological assessments of the CCA did not indicate any tissue damage or other detrimental influence of the catheter or the treatment. Media thickening was even lowest in the hypothermia group although nominal statistical significance was missed (p=0.08). There were, however, significantly increased respiratory and heart rates in the hypothermia group in the first days after the cooling procedure. This can be considered uncritical, as the rates never violated physiological ranges. Moreover, heart rate was already higher in the hypothermia group prior to the procedure ($p<0.05$) in the hypothermia group whereas formal statistical significance was closely missed for a higher respiratory rate at that time point (p=0.07). Third, there were never any signs for infections or other indications of reduced wellbeing as compared to the normothermia group. Nevertheless, future studies should include similar safety endpoints to exclude the possibility that potential adverse effects with low effect size or frequency have been missed. In summary, this study revealed a favorable safety and feasibility profile of the assessed CLCC.

Secondary efficacy endpoints were not met in this study. The mean AUC of the neuroscore measurements was lower in the hypothermia group (n=10; 34), including those animals exhibiting severe hypoperfusion (n=8; 34), compared to the entire control group (n=10; 53) and to those control animals with severe hypoperfusion (n=5; 70). However, both comparisons did not reach statistical significance (p=0.83 for the entire groups; p=0.54 for animals with severe hypoperfusion), not indicating improved functional outcome. Lesion size on MRI at day 30 was also comparable between the groups. Potential reasons for not meeting the secondary endpoints are numerous. First, the inter-individual variability in both efficacy endpoints was high. Largest SD on lesion volumes as measured by MRI was 77.8%. Given the sample size (n=10), a mean intergroup-difference of 0.975 (i.e., a lesion size reduction by 97.5% in the hypothermia group) would be needed to reach statistical significance. This is highly unrealistic giving a 3 hour

MCAO in both groups. Large animals such as the sheep used in this study are outbred animals and inter-subject variability after stroke is higher than in inbred rodent strains due to individual differences in collateralization and blood vessel anatomy.[22,34–36] This also increases inter-subject variability in functional endpoints. This situation is similar to what is observed in human stroke patients and therefore more realistic than more standardized rodent models. On the other hand, the higher variability may also statistically obscure potential therapeutic effects of small to moderate size as could be expected in neuroprotection. Next to these general considerations, it cannot excluded that there was a "baseline disadvantage" to the hypothermia group. Although not reaching statistical significance, mean lesion and edema volumes were larger in the hypothermia group early after MCAO, and there were more animals with severe hypoperfusion (n=8 versus 5). The fact that we found a correlation between the extent of hypoperfusion during MCAO with edema and infarct volumes on MRI at day 2 irrespective of the treatment mode indicates an association between collateralization and final infarct in the ovine MCAO model in analogy to human MCA stroke. Hypothermia animals were also older and heavier than those in the normothermia group. Thus, there might have been a higher stroke burden in the hypothermia group underlining the need for well-powered efficacy studies using the ovine model.

Recently, the performance of an insulated cooling catheter was investigated in a canine MCAO stroke model, with a targeted 31–32°C brain temperature being reached within 25 min by cooled saline solution delivered directly into the ICA blood stream (flow rate of 22 mL/min). After transient MCAO for 45 min, infarct volume at 30 days was markedly reduced in treated animals compared to the control group, underpinning the neuroprotective effect of a fast and short cooling. Important differences in the study set-up could have contributed to better performance in terms of reduction of infarct size of the insulated catheter compared to the CLCC: first, the catheter was placed directly within the ICA, what is not possible in sheep due to the rete mirabile.[23] The achieved intracerebral temperature drop despite the relatively low

coolant flow rate clearly indicates, from an energetic point of view, a limited blood flow within the ICA. Since the saline temperature was around 12°C and assuming an ipsilateral brain temperature of 31°C, an ICA flow rate of around 66 mL/min can be presumed, which is considerably lower than in human physiology (~250 mL/min)[37] as well as in the ovine CCA (~780 mL/min, calculated from the mean velocity 39.9 cm/s and a CCA diameter of 6.7 mm measured by ultrasound[19]). Moreover, an absent systemic effect, which is related to the thermal energy amount "extracted" by the cooling system, indicates a small volume of the cooled brain compared to body mass.

We noted a number of statistically significant intra-procedural blood gas and blood chemistry parameter differences in hypothermia versus control animals. Some of those including $pCO_2$, chloride, tHB and MetHB did not relevantly violate physiological ranges in sheep, whereas others did. However, most of these individual differences may be considered uncritical in the light of multiple testing of parameters in a time-ordered sequence (e.g. repetitive blood tests during physiological monitoring) according to a hierarchical testing procedure algorithm for time-ordered data as used in our study (see also above).[24] It is also unlikely that these differences were a TH consequence because the violation occurred in both groups. Constellations such as low hematocrit but only slightly reduced sodium, potassium plus slightly increased lactate indicate that the reason was the fluid supply to both groups by Ringer lactate throughout the procedure. Future studies should therefore rely on pharmacological blood pressure support during prolonged anesthesia.

Our study has a number of limitations. The most obvious and severe one is the lacking randomization being required for logistical reasons. Although we could not avoid this limitation, it have subjected the study outcome to batch effects. We therefore conducted a number of *post-hoc* analyses revealing that groups were not statistically different from each other early with respect to important baseline parameters of the study, with even a minor mean value skew in favor of the control group. Thus, the omission of randomization may not have

biased the study in favor of the catheter-based selective brain cooling intervention, and we consider a false-positive result regarding the primary endpoints as unlikely. Moreover, the study was designed as an exploratory feasibility and safety investigation not necessarily requiring randomization. However, the lack of randomization clearly had consequences including the higher age and weight of the hypothermia animals what impressively underlines the need for proper randomization paradigms in future confirmative studies. A second limitation of our study is the relatively large inter-individual variability in key efficacy endpoints such as lesion volume and behavioral outcome. Downstream, efficacy-oriented research may therefore require the implementation of thorough measures to reduce this variability in order to detect a clinically meaningful benefit in the range of 10 to 20%.[38] A third limitation may arise form ovine brain anatomy which differs from human in terms of mass, blood supply and surrounding tissues. All parameters potentially influence the intraparenchymal heat transfer processes. However, recent numerical simulations estimating temperature decrease in the human brain using the same CLCC revealed a similar temperature as reported in our study.[39–41]

## Conclusions

In an ovine stroke model using transient MCAO, feasibility and safety of a novel closed-loop cooling catheter for selective cerebral hypothermia was demonstrated. Reduction of infarct size and improved functional outcome could not be shown, presumably related to small sample size given a high stroke variability between animals. A larger number of subjects might be required to demonstrate efficacy.

## Acknowledgements

This work was supported by the German Ministry of Research and Education (grant number 13GW0015A). We thank Acandis GmbH (Pforzheim, Germany) for the collaboration

in the frame of the jointly granted project 13GW0015B. We thank our research technician Hansjörg Mast for valuable support with the MRI measurements of all animals.

**Author contribution statement**

GFMC, HU, JB and SM designed the study. GFMC, AMH, SAE, MW, EK, SD, PS, SK, LL, CMa, JH, CS, WDN, MJS and SM performed the experiments. GFMC, JW, MB, and TJ developed the closed-loop cooling catheter prototype and related machinery. JW, MB, and TJ provided technical (device-related) support during the animal experiments. GFMC, AMH, GI, JW, BN, MB, TJ, HU, JB and SM analyzed the data, GFMC, AMH, MJG, CMü, JB and SM interpreted the results. GFMC, AMH, JB and SM drafted the manuscript. All authors contributed to manuscript revision and approved the final version of the manuscript.

**Availability of data and material**

The datasets generated and/or analyzed during the current study are available from the corresponding author on request.

**Competing interests**

GFMC was inventor of the device and employee of the Company Acandis GmbH during the course of the study. JW, TJ, and MB are still currently employed by the Acandis GmbH. All other authors do not report competing interests.

**Funding**

This work was supported by Federal Ministry of Education and Research, Germany (grants 13GW0015A and 13GW0015B), awarded to Acandis GmbH and University of Freiburg.

**Supplementary Information**

Supplementary material for this paper is available at

http://jcbfm.sagepub.com/content/by/supplemental-data

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## Figures legends

**Figure 1: Overview on experimental design and concept of the closed-loop cooling catheter**

(a) Overview on the experimental design. The upper timeline represents the overall study period while the lower timeline depicts the day of MCAO and cooling. '(X)' indicates that deworming was performed in case the parasitological screening revealed positive results. (b) Concept of CLCC implanted in the common carotid artery of a sheep. The serial four-balloon array at the catheter tip and the three-inner-lumen construction of the catheter is schematically depicted.

**Figure 2: Mean temperature drops and differences in the hypothermia versus the normothermia group**

(a) Mean (95%-CI) temperature drops (ΔT, °C) were calculated at 5-minute intervals throughout cooling (180 min) and post-cooling (30 min) periods. These are depicted for the rectal (●) and nasal (○) temperatures. (b) Mean (95%-CI) temperature differences between nasal and rectal temperature probes (ΔT, °C) were calculated at 5-minute intervals throughout cooling (180 min) and post-cooling (30 min) periods. Blue symbols represent the hypothermia and red symbols represent the normothermia group.

**Figure 3: CT perfusion images during MCAO and consecutive evolution of infarcts on MRI.**

(a) Animal from the hypothermia group. In the hypothermic animal, CTP images reveal mild right MCA hypoperfusion (hypoperfusion score: 1) during MCAO, which is only visible due to a slight $T_{max}$ prolongation (arrow) without any changes on CBF (not shown) and CBV maps. The consecutive MCA infarct is small on DWI (arrow) and T2 MRI (arrow) at day 2 (DWI volume, 1.1 mL; T2 volume, 1.7 mL). (b) Animal from the normothermia group. In the normothermic animal, severe MCA territory hypoperfusion (hypoperfusion score: 3) is

disclosed with a lesion being visible on $T_{max}$ (arrow), CBF (not shown), and CBV maps (arrow). The resulting MCA territory infarct is large (DWI volume 9.2 mL, arrow). T2 MRI shows a surrounding edema (total volume 13.4 mL, arrow) and a space-occupying effect (midline shift, arrowhead). False color scales indicate $T_{max}$ values from 0 (purple) to 12 s (red) and CBV values from 0 mL/100g (purple) to 6 mL/100g (red).

**Table 1: Comparison of temperature drops during selective intra-carotid blood cooling**

| time point† | ipsilateral nasal temperature ΔT °C (95%-CI) | | | systemic rectal temperature ΔT °C (95%-CI) | | |
|---|---|---|---|---|---|---|
| | hypothermia group | normothermia group | p-value | hypothermia group | normothermia group | p-value |
| 5 min | -0.72 (-0.97; -0.48) | -0.32 (-0.70; 0.06) | 0.0621 | -0.29 (-0.35; -0.23) | -0.09 (-0.15; -0.02) | **<0.0001*** |
| 10 min | -1.13 (-1.41; -0.86) | -0.30 (-0.64; 0.03) | **0.0004*** | -0.53 (-0.62; -0.44) | -0.11 (-0.18; -0.03) | **<0.0001*** |
| 20 min | -1.71 (-1.97; -1.45) | -0.23 (-0.42; -0.05) | **<0.0001*** | -0.93 (-1.05; -0.82) | -0.13 (-0.22; -0.04) | **<0.0001*** |
| 30 min | -2.06 (-2.33; -1.79) | -0.15 (-0.27; -0.03) | **<0.0001*** | -1.31 (-1.43; -1.20) | -0.12 (-0.22; -0.01) | **<0.0001*** |
| 60 min | -3.09 (-3.49; -2.69) | -0.35 (-0.57; -0.14) | **<0.0001*** | -2.33 (-2.53; -2.14) | -0.20 (-0.33; -0.06) | **<0.0001*** |
| 90 min | -3.74 (-4.08; -3.41) | -0.40 (-0.60; -0.21) | **<0.0001*** | -3.16 (-3.35; -2.98) | -0.31 (-0.49; -0.12) | **<0.0001*** |
| 120 min | -4.15 (-4.39; -3.91) | -0.62 (-0.91; -0.34) | **<0.0001*** | -3.74 (-3.92; -3.57) | -0.42 (-0.63; -0.21) | **<0.0001*** |
| 150 min | -4.22 (-4.36; -4.08) | -0.67 (-0.94; -0.41) | **<0.0001*** | -4.02 (-4.21; -3.84) | -0.50 (-0.74; -0.25) | **<0.0001*** |
| 180 min | -4.10 (-4.21; -4.00) | -0.70 (-1.01; -0.38) | **<0.0001*** | -4.09 (-4.30; -3.88) | -0.53 (-0.82; -0.24) | **<0.0001*** |

†refers to time elapsed from procedural start (cooling or sheath insertion in normothermia group)

ΔT refers to mean (95%-CI) temperature drop calculated by subtraction of measured procedural temperature from temperature at baseline which was time-averaged over the last 30 min prior to procedural start per probe and animal. Asterisk (*) indicates significant difference between hypothermia and normothermia group.

**Table 2: Ultrasound and angiography of CCA, and brain CT perfusion**

| | hypothermia group | normothermia group | p-value[†] |
|---|---|---|---|
| **CCA ultrasound** | | | |
| mean flow velocity; cm/s, mean (SD) | | | |
| during MCAO, before CLCC insertion | 35.4 (8.6) | 28.0 (7.7) | 0.06 |
| after MCAO, during cooling | 32.4 (9.8) | 29.5 (9.2) | 0.5 |
| end of cooling, after CLCC removal | 46.4 (16.0) | 35.4 (8.9) | 0.08 |
| **CCA angiography[#]** | | | |
| vasospasm, n (scores 0-2) / thromboembolism, n (scores 0-3) | | | |
| during MCAO, after CLCC insertion | 1 (score 1) / 4 (score 3) | 2 (score 1) / 5 (score 3) | 1.0 / 1.0 |
| after MCAO, during cooling | 1 (score 1) / 5 (score 3) | 1 (score 1) / 5 (score 3) | 1.0 / 1.0 |
| end of cooling (180 min) | 1 (score 1) / 7 (score 3) | 1 (score 1) / 4 (score 3) | 1.0 / 0.37 |
| **CT perfusion brain during MCAO** | | | |
| hypoperfusion score[§], median (IQR) | 2 (2-2) | 1.5 (1-2) | 0.5356 |
| mild hypoperfusion (0-1), n (%) | 2 (20%) | 5 (50%) | 0.3484[$] |
| severe hypoperfusion (2-3), n (%) | 8 (80%) | 5 (50%) | n/a |

[†]p-values refer to comparisons by t-test for results of CCA ultrasound flow velocities, continuity-adjusted Chi-Square Test for angiography results/degree of hypoperfusion, and Wilcoxon test for hypoperfusion scores (on CT perfusion).

[#]DSA images were analyzed for CCA vasospasm and thromboembolism using semi-quantitative scores: vasospasm score; 0: no vasospasm, 1: mild vasospasm, 2: severe vasospasm; thromboembolism score; 0: no thromboembolism, 1: mild thrombus without vessel occlusion, 2: severe thromboembolism with large artery occlusion, 3: external carotid artery occlusion related to surgical MCAO procedure. For both scores, frequencies (n) are provided solely for categories other than 0.

[§]hypoperfusion score is defined for rating of MCA territory: 0: no hypoperfusion visible on $T_{max}$/CBF/CBV, 1: hypoperfusion visible on $T_{max}$ only, 2: hypoperfusion visible on $T_{max}$ and partially visible on CBF/CBV, 3: hypoperfusion visible on $T_{max}$/CBF and partially on CBV.

[$]p-value refers to a difference in the categorized hypoperfusion scores: mild (0-1) versus severe (2-3) hypoperfusion between both groups.

**Table 3: Histopathological findings of carotid artery specimen**

| histopathological parameter | hypothermia group | | normothermia group | | comparisons p-value† | |
|---|---|---|---|---|---|---|
| | treated CCA | non-treated CCA | treated CCA | non-treated CCA | hypothermia versus normothermia (treated CCA) | treated versus non-treated CCA (hypothermia Group) |
| clot, wall-adherent | 0 / 10 | 0 / 10 | 0 / 10 | 0 / 10 | 1.0 | 1.0 |
| fibrinoleukocytic scab | 0 / 10 | 0 / 10 | 1 / 10 | 2 / 10 | 1.0 | 1.0 |
| luminal thrombus | 7 / 10 | 7 / 10 | 4 / 10 | 7 / 10 | 0.369 | 1.0 |
| dissection | 0 / 10 | 0 / 10 | 1 / 10 | 0 / 10 | 0.501 | 1.0 |
| intimal thickening | 7 / 10 | 7 / 10 | 7 / 10 | 8 / 10 | 1.0 | 1.0 |
| intimal inflammation | 0 / 10 | 0 / 10 | 0 / 10 | 1 / 10 | 1.0 | 1.0 |
| fragmentation of elastic fibers | 1 / 10 | 3 / 10 | 1 / 10 | 2 / 10 | 1.0 | 0.317 |
| thickening and cell abundance in media | 0 / 0 | 3 / 10 | 2 / 10 | 2 / 10 | 0.456 | 0.083 |

Analysis of histopathological findings from CCA specimen explanted 30 days after MCAO and cooling procedure.

†P-values refer to Chi-Quadrat-Test for comparison of findings between hypothermia and normothermia groups, and to McNemar test for comparison between treated (right) and untreated (left) CCAs within the hypothermia group.

**Table 4: Neurological outcome and infarcts on MRI**

| | hypothermia group | normothermia group | p-value† |
|---|---|---|---|
| Functional neurological outcome; all animals | n=10 | n=10 | |
| neuroscore# (AUC, day 1-30), median (IQR) | 34 (19-80) | 53 (15.5-123.5) | 0.82 |
| animals with severe hypoperfusion (score 2-3) | n=8 | n=5 | |
| neuroscore (AUC, day 1-30), median (IQR) | 34 (20-81.75) | 70 (36-123.5) | 0.54 |
| MRI on day 2, all animals | n=10 | n=10 | |
| Infarct (DWI) volume; mL, mean (SD) | 5.58 (3.21) | 4.79 (2.77) | 0.56 |
| Edema (T2) volume; mL, mean (SD) | 8.33 (3.78) | 6.07 (3.48) | 0.18 |
| MCA recanalization (TOF MRA) status; % | 100% | 100% | |
| animals with severe hypoperfusion (score 2-3) | n=8 | n=5 | |
| Infarct (DWI) volume; mL, mean (SD) | 6.04 (3.17) | 6.56 (2.22) | 0.76 |
| Edema (T2) volume; mL, mean (SD) | 9.20 (3.31) | 7.72 (3.66) | 0.47 |
| MRI on day 30, all animals | n=10 | n=10 | |
| Final infarct (T2) volume; mL, mean (SD) | 1.62 (1.26) | 1.80 (1.17) | 0.74 |
| MCA recanalization (TOF MRA) status (%) | 100% | 100% | |
| animals with severe hypoperfusion (score 2-3) | n=8 | n=5 | |
| | 1.95 (1.28) | 2.4 (1.24) | 0.55 |

†p-values refer to comparisons by t-test for MRI volumes and histopathology of chronic infarcts, and Wilcoxon test for neuroscores.

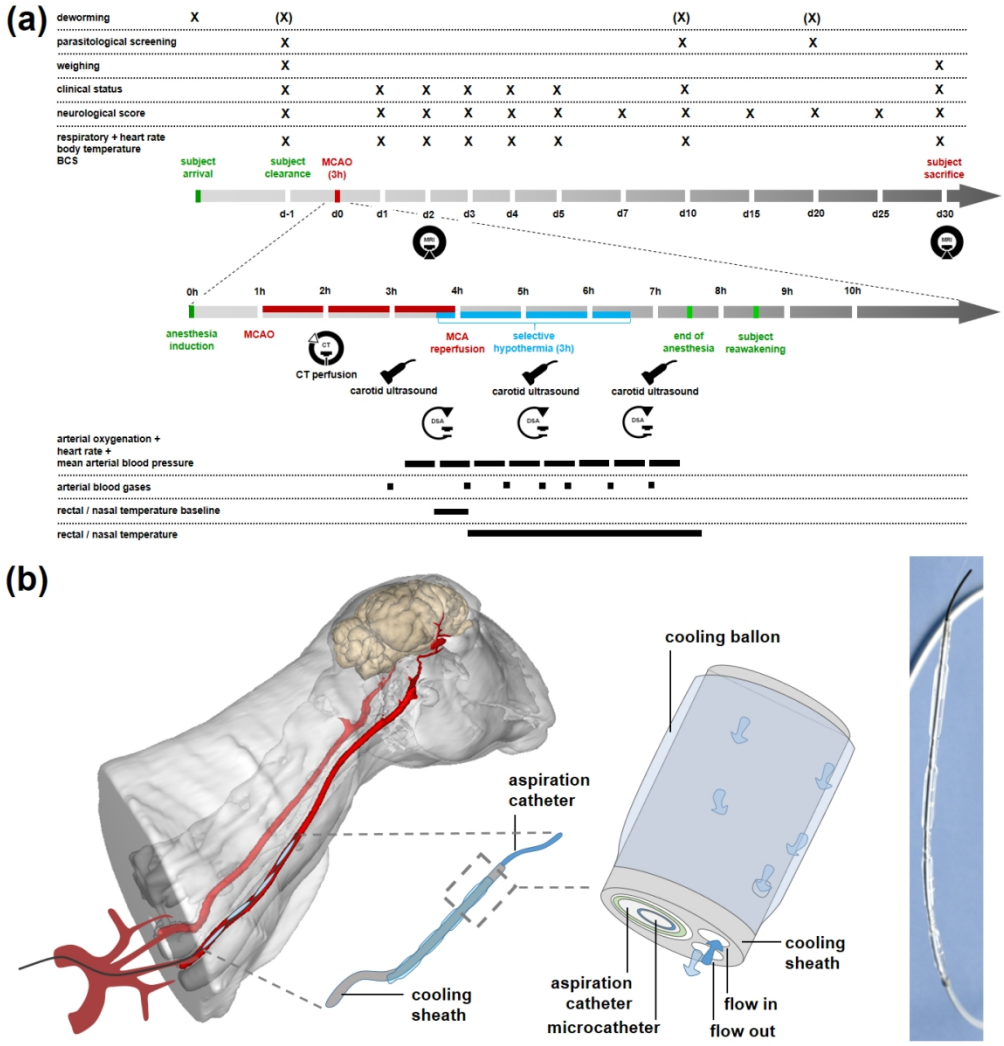


Figure 1: Overview on experimental design and concept of the closed-loop cooling catheter
(a) Overview on the experimental design. The upper timeline represents the overall study period while the lower timeline depicts the day of MCAO and cooling. '(X)' indicates that deworming was performed in case the parasitological screening revealed positive results. (b) Concept of CLCC implanted in the common carotid artery of a sheep. The serial four-balloon array at the catheter tip and the three-inner-lumen construction of the catheter is schematically depicted.

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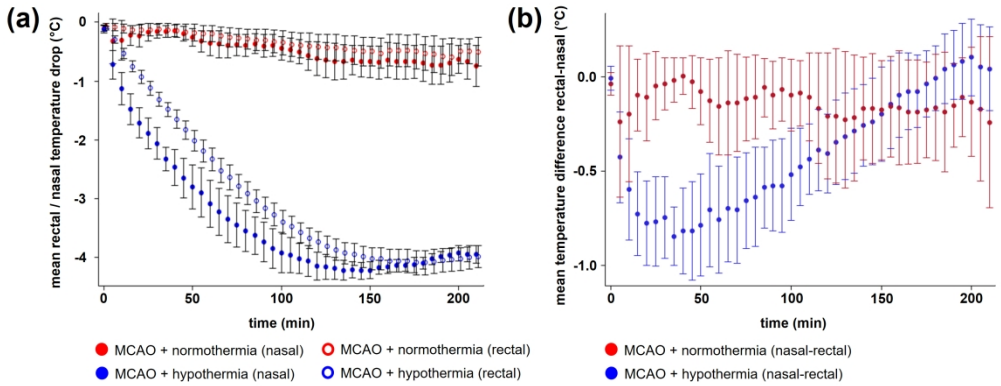


Figure 2: Mean temperature drops and differences in the hypothermia versus the normothermia group (a) Mean (95%-CI) temperature drops (ΔT, °C) were calculated at 5-minute intervals throughout cooling (180 min) and post-cooling (30 min) periods. These are depicted for the rectal (●) and nasal (○) temperatures. (b) Mean (95%-CI) temperature differences between nasal and rectal temperature probes (ΔT, °C) were calculated at 5-minute intervals throughout cooling (180 min) and post-cooling (30 min) periods. Blue symbols represent the hypothermia and red symbols represent the normothermia group.

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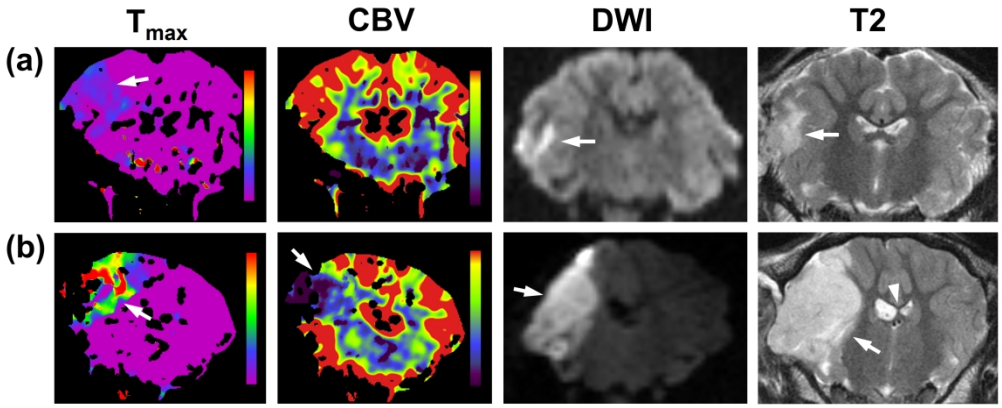


Figure 3: CT perfusion images during MCAO and consecutive evolution of infarcts on MRI. (a) Animal from the hypothermia group. In the hypothermic animal, CTP images reveal mild right MCA hypoperfusion (hypoperfusion score: 1) during MCAO, which is only visible due to a slight Tmax prolongation (arrow) without any changes on CBF (not shown) and CBV maps. The consecutive MCA infarct is small on DWI (arrow) and T2 MRI (arrow) at day 2 (DWI volume, 1.1 mL; T2 volume, 1.7 mL). (b) Animal from the normothermia group. In the normothermic animal, severe MCA territory hypoperfusion (hypoperfusion score: 3) is disclosed with a lesion being visible on Tmax (arrow), CBF (not shown), and CBV maps (arrow). The resulting MCA territory infarct is large (DWI volume 9.2 mL, arrow). T2 MRI shows a surrounding edema (total volume 13.4 mL, arrow) and a space-occupying effect (midline shift, arrowhead). False color scales indicate Tmax values from 0 (purple) to 12 s (red) and CBV values from 0 mL/100g (purple) to 6 mL/100g (red).

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