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Combined treatment of lactate and rTPA for the management of acute ischemic strokes

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1. Introduction

1.1 Epidemiology

Stroke is the leading cause of handicap in developed countries and ranks as the third cause of death worldwide. According to European statistics it accounts for 1.1 million deaths each year and is responsible for 10% of all deaths in men and 15% of all deaths in women[1].

It is the second cause of lost disability-adjusted-life-years after ischemic heart diseases in developed countries. The incidence of stroke is variable depending on the country and increases with age[2].

1.2 Clinical aspects

Clinically, we differentiate two types of stroke, ischaemic and haemorrhagic. Ischaemia is by far the most common cause of stroke and accounts for about 80% of them[3]. In this paper, we're going to focus on them, their presentation and the different treatment options that we have. The most common causes of ischaemic strokes are atherosclerosis in large vessels, embolic thrombus from the heart and lacunar strokes (occlusion of one of the penetrating brain arteries by two mechanisms: Thickening of the arterial media of these vessels and the other is the obstruction of the penetrating arteries by intimal plaques from a larger intracranial artery)[59]. Rarer causes include patent foramen ovale and arterial dissection.

Haemorrhagic strokes account for the remaining 20% of all strokes, the vast majority are due to primary intracerebral haemorrhage, the rest to subarachnoid haemorrhages[3].

It is clinically important to differentiate the type of strokes as the acute management depends on it.

When a patient presents itself to the A&E with symptoms evoking an acute stroke, the evaluation and diagnosis should be started straightaway as the outcome depends on the rapid onset of the treatment. Cerebral tissue is especially sensible to hypoxia and the loss of functional brain tissue is proportional to the time of ischemia.

The diagnosis of strokes is based on a thorough history, a precise clinical exam and the use of

diagnostic imaging.

An history of sudden onset of neurological deficits associated with a positive neurological examination (Table 1, see appendix) suggesting a stroke allow physicians to diagnose correctly an acute stroke with a sensitivity of 86.4% and a specificity of 99.1% according to one study[4]. In addition, the physician will perform several diagnostic tests to exclude other causes of acute neurological impairment or to highlight a condition that may be the reason of the stroke (Table 2 see appendix).

Several scoring tests have been validated to evaluate the severity and the prognosis of a stroke as well as its response to treatment. Among them the NIHSS (National Institutes of Health Stroke Scale) score is the most commonly used. The NIHSS is based on examination findings (Table 3, see appendix) and a score < 10 is associated with a better survival rate at one year. Patients with an overall score above 20 present poorer outcomes at one year[5]. As mentioned above, it also allows the clinician to evaluate the response to thrombolytic agent treatment, especially to detect patients at risk of haemorrhagic transformation, a typical complication of rTPA treatment. A score of > 20 on the NIHSS has been shown to increase the risk of secondary intracerebral haemorrhage of 17%, according to one study[6].

Once the suspicion of stroke has been established, neuroimaging should be performed as soon as possible as it permits to identify candidates for rTPA treatment[7]. Modern imaging techniques have allowed incredible ameliorations in the care of the patients. Non Contrast-Enhanced Computed Tomography Scan of the brain is the gold standard in this situation. It provides a rapid evaluation of the type of stroke, with a very high sensitivity to detect a haemorrhage[8]. This is really important as the treatment with a thrombolytic agent is contraindicated in haemorrhagic stroke. The CT scan can also reveal a non-vascular process that can mimic stroke and is part of the differential diagnosis[8].

1.3 Treatment Options

Once the diagnosis of ischaemic stroke has been established, one of the only approved therapy is the intravenous injection of tissue plasminogen activator (rTPA). As the lesion severity increases with the duration of the ischemia, the effectiveness of rTPA treatment

depends on the time of its introduction after the first symptoms of the stroke. rTPA acts as a thrombolytic agent by activating the tissue plasminogen factor. This molecule will then catalyse the transformation of plasminogen into plasmin. Plasmin acts by breaking down the fibrin clot formed during intravascular coagulation.

The effectiveness of thrombolysis is time-dependent, a large number of studies have been performed to determine the time-window in which rTPA was useful and did not increase mortality[9-10]. Overall results have shown that intravenous rTPA should be initiated as soon as possible after the first symptoms of a stroke with optimal benefit achieved if given within 90 min. However, rTPA should not be administered after 4.5 hours of symptoms as no beneficial effect has been obtained and an increase in complication rate has been noted.

The use of rTPA improves the overall neurological outcome at 3 months but doesn't have an effect on the mortality of the disease. According to one study patients treated with early thrombolysis (0-90 min) had an odds ratio of 2.11 for favourable outcome at 3 months compared to the placebo group; and an odds ratio of 1.69 if treatment was initiated between 90 to 180 min[11].

As mentioned above, the main complication of rTPA treatment is haemorrhagic transformation of the ischemic lesion. This complication is an important limiting factor that prevents the use of rTPA in every stroke patient. The risk factors of secondary intracerebral bleeding are well known and the eligibility for rTPA treatment is strict. (Table 4, see appendix) Therefore, only a few percent of overall ischemic stroke patients can benefit from treatment with rTPA and one third of the surviving patients still shows signs of important neurological deficits, impairing their autonomy and decreasing their overall quality of life[9].

A new treatment option has been approved since 2015. It has been demonstrated in a series of very recent multicentre trials that mechanical recanalization therapy is efficient and actually achieves better results for stroke patients than drug induced thrombolysis[46]. The benefit of mechanical recanalization appears to extend as late as 7 hours in a very recent metaanalyis[58]. Also a better neurological outcome is achieved if the occlusion is in a proximal and large brain's vessel. The combination of alteplase treatment and mechanical recanalization is currently the best treatment option for patients[12]. However, only a few percentage of patient benefit from this therapy as most of them arrive in a hospital after the

time-window and with an insignificant ischemic penumbra (percentage of salvageable brain) showed on CT scan[46].

All stroke patients should be hospitalized (and if possible transfer in a special stroke unit) as approximately 25% of them can worsen during the first 24 to 48 hours. Stroke patients are at risk of developing brain oedema, secondary cerebral hypertension, seizures and cerebral haemorrhage. Therefore, the patient's vital signs and neurological status should be carefully monitored[13,14,15]. Early mobilisation is important to prevent major complications such as deep vein thrombosis, pneumonia and pulmonary embolism. A careful appreciation of the gag reflex is crucial to prevent pneumonias of aspiration. General support must be provided and early infections must be actively searched and treated[16].

1.4 Secondary prevention

Recurrent strokes are a major problem and account for 25-30% of all preventable strokes[22]. The risk of developing a second stroke is high early after the first event (about 10% at 14 days)[17,18]. It's therefore important to prevent them and to control the major associated risk factors.

The short-term secondary prevention after ischemic stroke is achieved with antiplatelet therapy. All patients treated with rTPA should be given 50-150 mg of aspirin daily, once haemorrhagic transformation has been excluded[19]. This regime should be continued indefinitely or until another antithrombotic agent is instored[20,21]. If the stroke has been provoked by a cardio-embolic thrombus, anticoagulation should be started. Low dose of warfarin is the best option and the aim is an INR between 2-3[22]. New anticoagulants (the factor X inhibitors apixaban, rivaroxaban, edoxaban or the direct thrombin inhibitor dabigatran) are an interesting and promising alternative to warfarin for stroke prevention in the case of atrial fibrillation. A better controlled INR is obtained with the use of these new anticoagulants. However, no antidote is available for these drugs (except for dabigatran), but researches are focusing on developing new antidotes for these drugs[48]. The treatment option should be chosen accordingly to the patient's comorbidities and preferences.

On a long-term basis, the risk of recurrent stroke is about 11.1% at 1 year, 26.4% at 5 years

and 39.2% at 10 years[23]. Long-term secondary prevention is achieved by controlling all cardiovascular risks factors. The medical control of the blood pressure, lipids and diabetes is crucial. Lifestyle changes such as quitting smoking, decreased alcohol consumption, regular physical activity, healthy diet and loss of weight are the cornerstone of an efficient prevention[22].

1.5 TPA: Beneficial vs Noxious effects

Though the beneficial role of rTPA has been well established in dissolving a clot[24], rTPA is also known to cause a number of noxious effects, limiting its use in every patients presenting with ischaemic strokes. A number of studies have shown that rTPA isn't only a proteolytic enzyme acting on the transformation of plasminogen into plasmin, but because of its conformation can act on a large number of cells, especially in the brain. A few mechanisms have been identified in the physiopathology of rTPA.

rTPA has an important noxious effect on the blood brain barrier (BBB), resulting in oedema and bleeding. It induces the synthesis of metalloproteinases from the endothelial cells. Metalloproteinases act on the extracellular matrix and increase the permeability of the BBB. These metalloproteinases also promote the detachment of the perivascular astrocyte end-feet processes, which are capital for maintaining the structure of the BBB. rTPA also weakens the tight junctions of the endothelial cells resulting on a BBB leakage[25-28].

It enhances excitotoxicity by acting on N-Methyl-D-aspartate-receptor (NMDAR). Hyperactivity of these receptors creates a state of neuronal over-activity resulting in loss of function and apoptosis. Studies have shown that the proteolytic action of rTPA is implicated in the over activation of these receptors[29-31]. On the other hand, it could have a protective effect in neurons. These proprieties are still under investigation but studies suggest that the deleterious or protective effects on neurons depend on the concentration of rTPA as well as the type of receptors on which it binds[29, 32].

Controversial effects on apoptosis have been described. Some studies have shown an increase in apoptotic cells by the way of excotoxicity[33]. Others that rTPA could have a protective role against apoptosis[27, 34, 35].

rTPA can activate microglia, which is an important player of the inflammation. This activation results in neurotoxic effects, BBB injuries, and maintenance of the inflammation by migration of leukocytes and mast cells[36, 37].

Through its fibrinolytic action, it allows a better axonal protection and reparation[38].

It might promote post-stroke recovery via gene activation of growth factor synthesis and activation of specific neurological pathways[39].

Although the principal effect of rTPA in acute ischemic stroke patient is to restore the cerebral blood flow, these properties can translate the clinical effects of rTPA, with an important toxicity during the acute phase (cerebral haemorrhage) but with a beneficial effect on the long term (better neurological outcomes).

1.6 New therapies for Strokes

The technological improvements during the last few years have allowed a better understanding of the neurological field. The introduction of extended laboratory technics and the experiments on mice brains have allowed to better understand the mechanisms of brain ischemia and therefore highlight new mechanisms and factors involved in stroke physiopathology. Those researches focus on phenomena that happen after deprivation of oxygen and nutrients and entail a large number of brain cells (microglia, neurons, vascular cells, matrix components) as well as transcription factors, how to regulate calcium dysregulation during hypoxia, possibilities of blocking oxidative stress and ROS formations, decreasing inflammation and protection against excitotoxicity. These researches try to find what we call a "neuroprotective agent" that could help in the management of stroke patients and improve neurological outcomes.

For example extensive researches have been performed about excitotoxicity: Energy failure in the brain cells during ischemia induced neuronal depolarisation and a massive release of glutamate as well as decreasing its re-uptake by the synapses. Glutamate is a major activating neurotransmitter and during hypoxemia it activates certain pathways that enhance calcium accumulation in the intracellular compartment. This accumulation perturbs cell's physiology and induces inflammation as well as apoptosis.

An interesting trial has been done by treating rodents who underwent brain ischaemia with peritoneal dialysis. The aim of this experiment was to scavenge glutamate from blood after a stroke. It's particularly interesting as this technic does not interfere with normal brain physiology. Currently a clinical trial is conducted on humans[46].

Excess of glutamate leads to the release of secondary messenger systems which induce the formation of free radicals such as superoxide anions, hydrogen peroxide etc. The formation of these free radicals may be enhanced by the presence of oxygen especially during reperfusion. Those molecules are particularly toxic during ischemia as they can induce mitochondrial and DNA damages, lipids peroxidation and destruction of the blood-brain-barrier. Brain tissue is especially susceptible to these molecules as it hasn't an important endogenous antioxidant capacity. A great number of trials have been performed trying to prevent the formation of oxides each of them targeting a specific free radical[46].

Peroxynitrite resulting of the interaction between nitric oxide and superoxide is one of these free radicals. Blocking this molecule is really interesting as it has no physiological use in the brain. Using acid uric (a powerful antioxidant) has been proven to impair Peroxynitrite damage and therefore prevent glutamate induced cell death and decrease infarct volume. Uric acid has already been tested in association with alteplase with positive results and safety on humans. The beneficial effects were only seen on the chronical outcome of patients but not in the acute phase[49]. Reducing the infarct growth, improving neurological outcomes and increasing the number of independent stroke patient, compared to a placebo-group[49].

These are only a few examples of neuroprotective agents that have shown some promising results in rodent models. But currently no neuroprotective agent has shown actual benefits on human. It could be because the trials done in this field often lack robustness. Stroke is an acute condition that cannot be predicted therefore it's hard to have a reproductive sample of patient and it's difficult to gather the same situations for each patient. The treatment option also depends on the time of administration of the drug, which increase the difficulty of collecting reproducible data.

Another reason is that brain physiology is not yet fully understood and a lot of mechanisms still remain theoretical. A better understanding of the physiology as well as the chemical

properties of the different neuroprotective agents must be reached to offer new treatments. However, scientists remain hopeful as a lot of these neuroprotective drugs have shown promising results in animal experiments[40].

1.7 Lactate

Lactate, one of the degradation products of anaerobic glycolysis, has long been overlooked as an energetic substrate during cell metabolic perturbations.

The first time lactate ability to protect brain from hypoxic insult has been shown goes back to 1988 with an experiment of Schurr and al. From there, a large number of studies have been performed to try to better understand the role of lactate in hypoxic cerebral tissues[41]. One study has summarized the neuroprotective action of lactate[41]. Lactate is the aerobic glycolysis end-product in the brain. During brain hypoxia it plays an important role as it decreases glutamate excitotoxicity and reduces the production of ROS (reactive oxygen species) and therefore have a neuroprotective properties[41].

This hypothesis has been demonstrated by a number of experimental studies that show the neuroprotective effect of lactate when administered either through intra-ventricular infusion or systemically at reperfusion in an in vivo animal model of cerebral ischemia with reduced lesion size and milder neurological deficits, in an acute phase. The neurological deficit is also attenuated in the long term (14 days after MCAO) with smaller hemispheric atrophy at this late time-point [42-44].

During ischemia, delivery of energy substrates to the injured brain tissue may be insufficient. Immediately there's an increase in lactate concentration. This is due to anaerobic glycolysis. Due to the lack of energy, it is therefore impossible for brain tissue to use ATP to convert glucose to glucose-6P for aerobic glycolysis, lactate becomes a preferential source of energy. We assist to a second raise of lactate, 1 hour after reperfusion which continues up to 72hours. At reperfusion time, lactate level decrease drastically, this can be explained by the huge energetic demand required during reperfusion. If we inject lactate exactly during the reperfusion time, we can improve energy supply and decrease neuronal death[44].

A comprehensive review, summing up the beneficial effect of lactate has been release in 2014.

In this papers, the authors show that during an acute brain injury the increased glutamate release activates an interaction between astrocytes and neurones. Increase glutamate concentration in the extracellular space activates Na,K-ATPase pumps at the cell membrane of astrocytes . These pumps allow astrocytes to activate glycolysis, and increase the release of lactate in the extracellular space. Lactate can then be used as an alternative source of fuel, thus allowing glucose to be spared. This interaction between astrocytes and neurones could explain the increase in lactate concentration after acute brain ischaemia. Based on this hypothesis, Bouzat & Oddo and al, were able to show that treatment with hypertonic perfusion of lactate could decreased cortical oedema, intracranial pressure and increased cerebral blood flow during acute brain injury.[61]

Most recently a study has been performed by the department of neuroscience of the CHUV, Lausanne, Switzerland. The aim of this study was to understand if the beneficial role of lactate could only be explained by its energy supply or if it can act as an endogenous ligand on neuronal receptors. The expression by neuronal cells of the receptor HCA1, which lactate has been proven to be a ligand[50-52], is increased after an ischemic insult. The authors were able to show that injection of lactate systemically during reperfusion time window increased the number of HCA1 receptors expressed on the cell membrane. They tested in an in vitro model the result of treatment with a HCA1 receptor agonist (3,5-dihydroxybenzoic acid) after OGD on hippocampal cultures. It shows that 3,5-dihydroxybenzoic acid produced significant protection, as it reduced neuronal cell death in the CA1 region of the hippocampus after OGD[47]. Those results allow us to confirm that lactate plays a double role in neuronal protection. First, it acts as an energy substrate when aerobic glycolysis is impossible and second it functions as a signalling molecule on neuronal cells after ischemia.

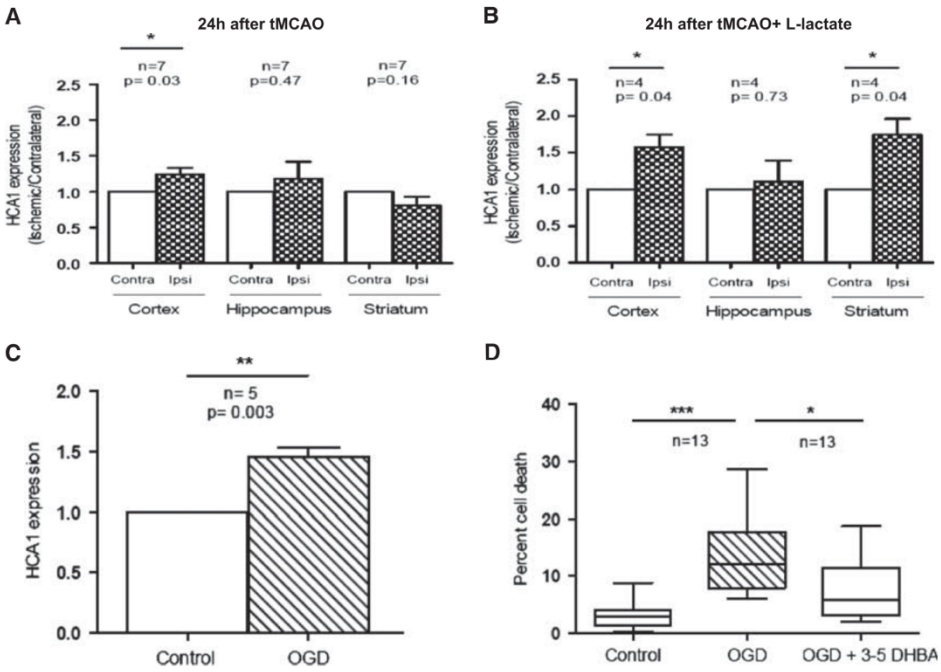


Figure 1. Results of HCA1 receptor experiment[47]. (A) Western blot analysis showing an increase in the HAC1 receptor expression at 24 hours after MCAO, in comparison with the contralateral hemisphere. (B) intravenous L-lactate injection induced expression of HCA1 receptor in the lesion site in comparison with the contralateral hemisphere. (C) Western Blot analysis showed an increase in the HCA1 receptor 48 hours after OGD. (D) Administration of 4 mmol/L 3,5-dihydroxybenzoic acid attenuated cell death when administrated directly to the medium after OGD. Image and text taken from article reference 47

2. Working Hypothesis

In light of these results, lactate appears as an important player of the brain energy metabolism that could help reducing overall morbidity and mortality in stroke patients and should be tested in stroke patients. As rTPA is the standard of care, patients would receive lactate in combination with rTPA. Before attempting to test lactate in stroke patients, it is therefore necessary to first assess the association of lactate and rTPA in vitro and in vivo.

3. Materiel and methods

3.1 Aim of experiments

Previous experiments have shown excellent results when using Lactate as a neuroprotective agent at the time of reperfusion[42-44]. Our experiment was to assess the beneficial or noxious

effect when associating two products (D-Lactate and rTPA) that have already proven their efficacy as neuroprotective agent during focal brain ischaemia. We performed two experiments, one in-vitro and the other in-vivo, all with rodent models. The final result was obtained by comparing size lesion of ischemic brain in four different groups (control, rTPA, rTPA plus lactate and lactate alone).

All experiments were conducted according to the Swiss Federal Veterinary Office and approved by the Service Cantonal des Affaires Vétérinaires.

3.2 In vitro experiment

3.2.1 Organotypic hippocampal slice cultures

For this experiment we used P8 to P12 rats (OFA Sprague Dawley, France). Coronal hippocampal sections of 350µm were sliced and cultured on sterile porous membranes (Millicel, Millipore, Billerica, MA, USA) for 10 days. Brains dissection was performed in wells containing 1 mL of dissection medium with D-glucose 36 mmol/L, 88% of ultra-pure water, 10% Medium 199, 1% Tris 1M pH7 and 1% L-Glutamine 2mmol/L. They were switched on wells containing 1 mL of culture medium with 25% horse serum, 2,4% of HBSS (Hank's balanced salt solution) diluted in 21,6 % of water, 50% minimal essential medium (supplemented with HEPES and sodium bicarbonate) and L-glutamine 2mmol/L and then kept in an incubator (33°C, 5% CO_2, 100% humidity) for 10 days. We replaced the medium with fresh identical preparation at day 4. We then changed the medium with culture medium with 15% horse medium, 60% minimal essential medium, 2.4% of HBSS (Hank's balanced salt solution) diluted in 21.6 % of water and L-glutamine 2mmol/L at day 7 and day 10. We conducted the experiments at day 10 of culture[47].

3.2.2 Oxygen-Glucose-Deprivation

After 10 days of culture, we performed OGD on hippocampal cultures. We transferred the hippocampal cultures in a serum-free low glucose medium. The serum contains Dulbecco's modified Eagle's medium supplemented with D-glucose 1 mmol/L and L-Glutamine 2 mmol/L

[47]. This medium was previously prepared and then placed for 1 hour in a hypoxic chamber (5% O_2, 5% CO_2, and completed by N_2 at a temperature of 37°C. Hippocampal cultures were placed in the OGD chamber for 1 hour. They were then placed in fresh culture medium and kept at 33C° for 48 hours. Control culture were placed in a normoxic incubator at a temperature of 37C, in wells containing 1 ml of culture medium 15% horse serum (same as above).
After OGD culture were immediately treated either with lactate 4mmo/L and rTPA 9µg/ml, Lactate 4mmol/L alone or rTPA 9µg/ml alone. Control culture were switched into new wells containing 1 ml of culture medium 15% horse serum[47] (same as above).

3.2.3 Cell Death assessment.

48 hours after OGD and treatment we analysed the CA1 region cell death rate on the hippocampal cultures. We used the ability of Propidium iodide (extremely lipophobic agent that intercalates itself into the DNA) to show cell death. Propidium iodide was added in each wells (50µg/mL) 1 hour before measurement. We measured Propidium iodide fluorescence emission (excitation wavelength 536 nm, emission wavelength 617 nm) with an epifluorescence microscope with a x 5 lens coupled to a camera (Leica). We took the images with normal camera settings and then used ImageJ software to measure the signal intensity. After subtracting the background fluorescence on each slice, the results were expressed as a percentage of maximal cell death obtained by submerging slices in PBS for 24 hours at 4C°. Cell death was averaged for the four slices of each culture well. The experimenter was not masked[47] (see figure 2 for example).

3.3 In Vivo experiment

3.3.1 Transient middle cerebral artery Occlusion (MCAO)

For this experiment we used a total of 30 CD1 mice (*body weight 26 to 35g, Charles River, L'arbresle, France)*. The mice were kept in the animal facility of the Department of fundamental neuroscience of the université of Lausanne. The mice were kept in groups of 5 per cage during one week before surgery with 12:12 light/dark cycle with normal illumination.

The exact step-by-step technic of MCAO can be found in this article[42]. The aim of MCAO is to mimic the environment in which a stroke happens although an actual clot is not present. Mice were anaesthetized using a face mask with isoflurane (1.5% to 2% in nitrous oxide/oxygen 70%/30%). We monitored and recorded the cerebral blood flow (CBF) continuously using a Laser-Doppler flowmetry (Perimed AB, Stockholm, Sweden) coupled with a flexible probed fixed on the skull (1 mm posterior and 6 mm lateral from bregma). Temperature was controlled and kept at 37±0.5 C° over the entire period of surgery[47] using a temperature control unit with a heating pad coupled to a rectal thermometer (FHC, Bowdoinham, ME, USA). We induced transient focal cerebral ischemia during 30 minutes by occluding the left middle cerebral artery with an intra-arterial suture. Surgery begun with a ventral midline neck incision and exposition of the left common carotid artery and the left external carotid artery. They were then ligated with an intraarterial suture. We inserted a silicon-coated nylon monofilament (0.17 mm diameter) through the the left common carotid artery into the internal left carotid artery to induce transient focal cerebral ischemia. CBF was monitored and once 20% of baseline CBF was reached, we left the monofilament for 30 minutes. CBF was monitored and kept under 20% of initial rCBF through the operation. After 30 minutes, the coil was removed and reperfusion was considered successful if the CBF raised above 50% of initial CBF[47].

We only included in the experiment mice that dropped their CBF under 80% and reached over 50% of CBF at reperfusion. A total of six mice did not meet this condition and were excluded from the study.

Immediately after surgery, we randomly treated mice by injecting them intracerebroventricularly (*injected into the right lateral ventricle)* either sodium D-lactate solution (200mmol/L), rTPA solution (9µg/ml), a combination of D-lactate and rTPA or vehicle solution (phosphate-buffered saline, PBS). They received 5µL per gram of body weight of solution[47].

All mice received a subcutaneously injection of 0.025 mg/kg of buprenorphine for post-op analgesia as well as an injection of water (500 µl) to prevent post-op dehydration. Once animals were awake, they were kept in an incubator at 31C°.

The surgeon was not masked during this experiment.

3.3.2 Behavioural Evaluation

After surgery and reperfusion, we assessed neurological deficits using a composite neuroscore. This neuroscore allows to grade the severity of ischemic insults. It has 4 grades. 0: no neurological deficit observable. 1: Failure to extend the right forepaw; 2: circling to contralateral side, and 3: loss of walking or righting reflex. The evaluation was performed at 24 hours and 48 hours after the surgery. The behaviour evaluation was not assessed in a masked manner[47]. When the scoring was ambiguous, we decided to take the smaller number for each subjects.

3.3.3 Determination of ischemic Lesions Volumes

48 hours after surgery, animals were sacrificed. We used a cryostat to obtain coronal sections. Each section was 20-µm thick. For each brain we collected 10 sections 720 µm distant from each other. They were stained with Cresyl Violet. We acquired digitalized images of each stained sections using a light stereomicroscope (Leica MZ16FA, Heerbrugg, Switzerland). Size lesion was measured using ImageJ software (Image J 1.36b, National Institute of Health) by an examiner blinded for the treatment group. Infarct volume was calculated by multiplying the sum of the lesion areas on each section by the distance between sections[53].

3.3.4. Statistics

Numeric data were presented as mean +/- standard deviation. Multiple comparisons were performed using ANOVA online calculator. The Kruskal-Wallis test was applied to compare multiple groups of non-parametric data *(neurological scores)*.

4. Results

4.1 In vitro

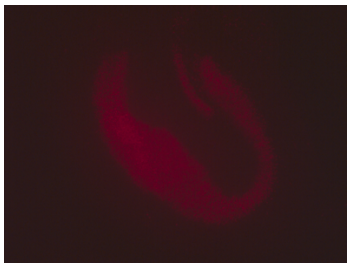
In our experiment we performed OGD on rat Organotypic hippocampal slices, immediately after OGD cultures were randomly treated either with PBS, lactate 4 mmol/L rTPA 9µg/L or

both lactate and rTPA. It appears that lactate decreased neuronal cell death in the CA1 hippocampal region 48 hours after OGD. Lactate decreased cell death from 60.4 ± 12.6% to 48.1±13.3% (P=0,03). Whereas treatment with rTPA increased cell death comparing to vehicle treated group from 50±14% from 58±21% (P= 0,33). When combining lactate and rTPA, we assisted to a slight increase in cell death compared to vehicle-treated group from 60±10% to 65±19% (P=0,54). *(Figure 3)*

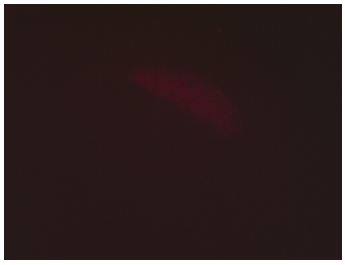
At the term of this experiment we were able to once again prove the neuroprotective effect of lactate, with a net decrease in cell death % compared with non-treated cultures. However, the combination of lactate + rTPA did not show any neuroprotective effects. This suggests that rTPA counteracts the beneficial effect of lactate.

In each of our experiment control cultures have been performed to assess if incubation period was performed correctly and did not induce important cell death. In all our culture plates, one well contained control cultures. P value for control was 0,26.

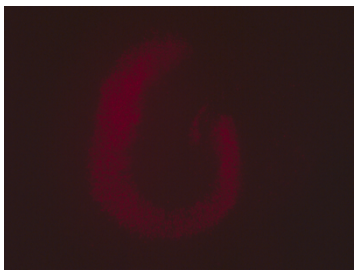
Control



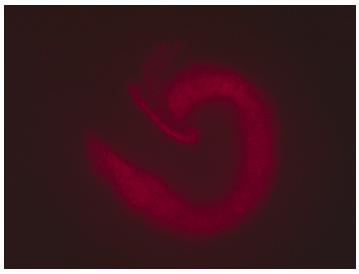
OGD



Lactate

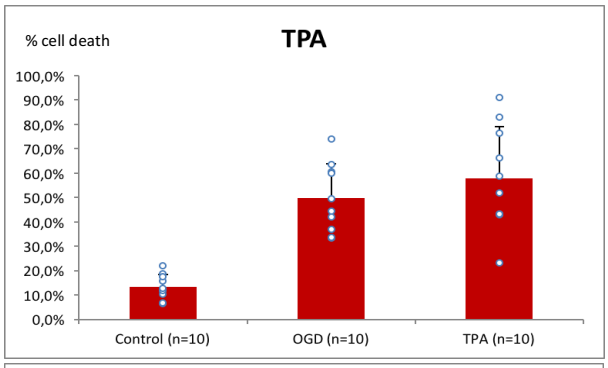


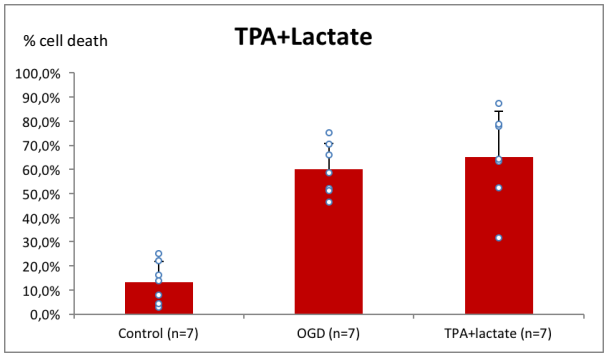
TPA



TPA + Lactate

Figure 2: Examples of images obtained after OGD and Propidium Ioded incubation. We measured Propidium iodided fluorescence emission (excitation wavelength 536 nm, emission wavelength 617 nm) with an epifluorescence microscope with a x 5 lens coupled to a camera (Leica) to assess cell death %.





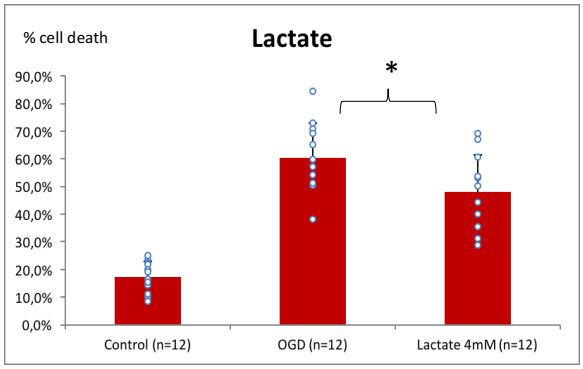


Figure 3: Results of hippocampal culture who underwent 1 hour OGD. Each table shows the comparison between control group (did not undergo OGD), OGD group (which received vehicle treatment) and treated group.
*Final concentrations were 200 mmol/L lactate in the case of lactate treatment and 9µg/L in the case of rTPA treatment. * indicates p<0,05*

4.2 In vivo

To complete previous experiments which have shown the neuroprotective effect of lactate[42-44,47] when injecting it intracerebroventricularly in mice after MCAO, the next step was to assess if the combination of lactate with rTPA was safe and efficient. We subjected CD1 mice to 30 min cerebral transient ischemia under isoflurane anaesthesia. After surgery we injected rTPA alone or combined with lactate to assess if neuroprotection with lactate was still achieved when rTPA was added or if on the contrary, the combination has noxious effects. Results of experiments have shown no major differences between the 4 groups. Behavioural evaluation at 24 hours after surgery has a mean of 0.833 point for control and lactate group. TPA group and TPA + lactate has a scoring mean of 1 point *(figure 5)*. Results are different from the latest studies that had previously shown an improvement in the behavioural scoring when comparing control with lactate treated mice. Treatment with rTPA or combined with lactate shows a slight increase in the behavioural scoring. This slight increase appears not to be significant, and the experiment shows rather little differences between the 4 groups. *(P value can be found on figure 5)*

	PBS		Lactate		TPA		TPA + Lactate		
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	P value
Initial weight (g)	31,8	2,8	30,8	2,9	32,2	2,4	31,2	2,6	0,81
Sacrifice Weight (g)	25,7	2,6	24,2	1,9	25,8	2,9	26	3,2	0,63
CBF ischemia (%)	11,7	4,3	12,3	5,6	10,7	2,2	13,6	3,9	0,68
CBF reperfusion (%)	55,4	13,3	59,6	11,5	54,1	8,7	55	30,8	0,49
Initial Temperature (C°)	36,8	0,2	36,9	0,1	36,9	0,1	36,8	0,1	0,34
Temperature at ischaemia (C°)	36,9	0,2	36,8	0,3	36,9	0,3	36,8	0,2	0,60
Temperature at reperfusion (C°)	36,7	0,2	37,2	0,8	36,9	0	36,9	0,2	0,26

Figure 4 : Table showing mean and standard deviation for each sample of experiment and for each variables. In all of our experiment P value was greater than 0.05.

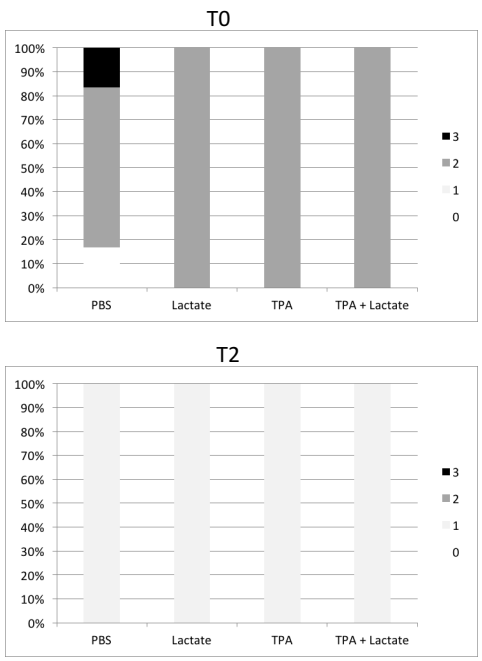


Figure 5 : Neuroscore : comparison of neuroscore at T0 (after surgery) T1 (24h hours after surgery) and T2 (48 hours after surgery) between control group, TPA group (9µg/l), Lactate group (200 mmol) and Lactate + TPA. The P-value using the Kruskal-Wallis test was P=1, P=0,589 and P=1 for T0, T1 and T2 respectively. 0: no neurological deficit observable. 1: Failure to extend the right forepaw; 2: circling to contralateral side, and 3: loss of walking or righting reflex

A slight difference is also found when analysing the total infarct lesion in the 4 different groups. We obtained a volume of infarction of 71.8 mm^3 ± 46.4mm^3 for control group, 70.14mm^3 ± 33.5 for TPA group, 79.4mm^3 ± 31.34mm^3 for lactate group and 77.14mm^3 ± 10.64mm^3 (p = 0,95. *(Figure 6)*

It appears that neuroprotection with lactate has not been achieved in our experiment, indeed lactate group shows the biggest infarct lesion size. TPA group shows the smallest lesion size, although neuroprotective effect of TPA has never been demonstrated in an in vivo model.

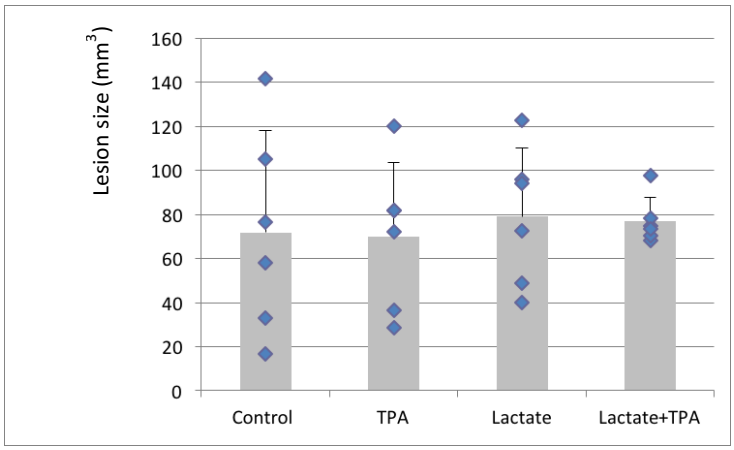
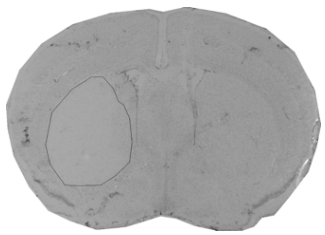
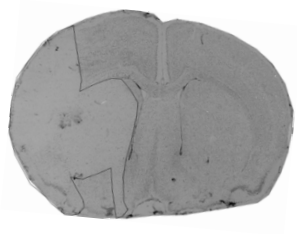


Figure 6: Results of MCAO of 30 minutes in male CD1 mice. A total of 24 mice were included in the study. Comparison of the total infarcted lesion size (mm^3) between control group who did not receive any treatment, TPA group who receive rTPA 9µg/l intracerebroventricular after surgery, Lactate group which receive lactate 200 mmol/l intracerebroventricular after surgery and Lactate + TPA group which receive both products intracerebroventricular. Bars represent the mean infarcted lesion in mm^3. Each points represent one subject of the experiment.

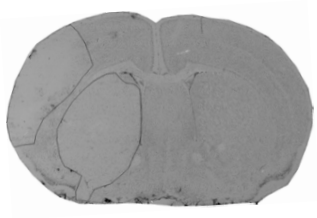
The results in each group were very variable with high standard variations translating a weak robustness of our experiment. We have brought in light a number of reasons that can explain the important variability in our results, they'll be listed and developed in the discussion.



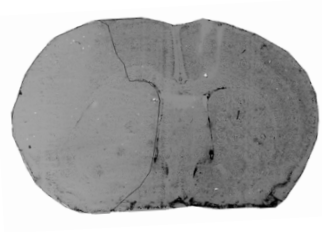
Control



TPA



Lactate



Lactate + TPA

Figure 6: Examples of images obtained after MCAO of 30 minutes in male CD1 mice. The infarcted lesion is highlight using image J software. Control group did not receive any treatment after MCAO. TPA group received 9µg/l of rTPA. Lactate group received lactate 200 mmol/l and Lactate + TPA received a combination of both products. Evaluation of the infarcted lesion was performed in a blinded approach.

5. Discussion

Regarding in vitro experiments, it seems that neuroprotection by lactate is counteracted by rTPA when they are associated. We assist to a very small variation in the percentage of neuronal cell death when comparing TPA and Lactate + TPA group and to an increase in the number of cell death compared to non-treated group, thus letting us believe that combining Lactate and TPA has an important noxious effect on brain cells after transient focal ischemia and that the beneficial effect of lactate is lost in the presence of TPA. These data reflect what happens in an in vitro model and can be different from reality. During our experiments all the conditions were controlled and none has been put aside. We were not able to identify factors that could be incriminated in a major difference in regards to previous experiments. Moreover, we had an important sample of culture and standard deviations were unimportant.

Results of in vivo experiments were not as clear as the one in the case of in vitro experiments. The important lesion size as well as the great variability of the data tend to make us believe that several factors influencing transient focal ischemia were not adequately controlled, thus making our results inconclusive compared to previous experiments.

Although rectal temperature of subjects was monitored closely during the all set of experiments, we realised at the end of the experiment that the thermometer of our rectal monitoring and temperature control system was defective. It indicated several degrees' inferior to what actually was the true temperature. A large number of researches have proved the marked influence of temperature on size lesion and neurological deficits in stroke patients. Hyperthermia increased the infarcted zone after permanent or transient MCAO in mice and increase the rapidity at which penumbral areas became infarcted[53-54]. On the other hand hypothermia has been proven to be neuroprotective and could reduce the infarcted lesion size of 75% if body temperature is kept under 36° before and after transient focal ischemia[55]. Reflecting the dysfunction of our thermometer, our experiments were carried out in a hyper-thermic environment which we believe rendered the treatment either by lactate or lactate + TPA ineffective to achieve neuroprotection. This could be an explanation for the important size lesion that we found, and for the lack of neuroprotection induced by lactate.

Another element in cause could be the anaesthesia with Isofluorane which was different from previous experiments done in our laboratory. Anaesthesia was previously conducted by using an association of xylazine and ketamine. It has been demonstrated that Isofluorane has neuroprotective effect[56-57]. It could be possible that neuroprotective effect of lactate cannot enter in synergism with Isofluorane. However, this hypothesis doesn't look plausible as the infarcted size lesion were very important in all groups suggesting rather the absence of any neuroprotection.

Compared to previous experiments, we used the same mouse strain, from the same provider but from a different breeding facility. As we used a strain of outbred, non-identical mice, a different breeding facility may have played a role and therefore could influence the reaction to transient focal ischemia.

Previous experiments regarding lactate have been performed using the L-lactate enantiomer. In our experiment we used D-lactate. In a recent study it has been demonstrated that D-

lactate has the same neuroprotective effect than L-lactate. This observation has been confirmed in our experiment as neuroprotection was achieved with D-lactate in the In Vitro part[47]. This study was performed in the same laboratory as our, and we wanted to corroborate this result.

In our experiment, we used a dosage of lactate that has been shown to be neuroprotective. As previously described, a lactate concentration under 4 mmol/l did not have have any neuroprotective effect, whereas a concentration over 4 mmol/l was shown to be toxic, as it increased neuronal cell death in an In Vitro model. Those observations are also valid in In Vivo models. We used a concentration of 200 mmol/l which when injected intracerebroventriculary increased the cerebrospinal fluid lactate concentration by ~4 mmol/L in a mouse[42].

The number of sample in our experiment was small, with only six subjects in each group. It could be interesting to perform the experiment once again with a more important sample of subject to increase the validity of our experiment.

At the term of this experiment, it's impossible for us to say if the association of rTPA and lactate is noxious, beneficial or trivial in regards of neuroprotection. The standard deviations were too important to draw conclusion on our experiment and too many uncontrolled variables were identified.

6. Conclusion

It is difficult at the end of our experiments to answer to our working hypothesis. It is true that in our set of in vitro experiment we were able to show a loss of the neuroprotective effect of lactate and an increase in the percentage of cell death when associated it with rTPA. However, it looks important to reiterate the experiment to increase the robustness of our test and to confirm that the combination has noxious effect on cerebral cells.

Unfortunately, the in vivo experiment didn't allow us to confirm the observations obtained in the vitro experiment. There were too many uncontrolled variables which made us think that our experiment failed, and therefore did not allow us to confirm or deny the compatibility of lactate and rTPA. These experiments need to be repeated with a better control of the different

variables to confirm the observation done in the in vitro experiment. It is also important to remember that results can be different from an in vitro model to an in vivo model.

Although the results of our experiments are inconclusive, neuroprotective effects of lactate have been proven and it is important to keep exploring its properties. Researches have been continued in the laboratory of Professor Lorentz Hirt and his team. They were able to perform the same experiments as we did, but with a different strain of mice. Using this strain, they were able to collect more valid data, with more robust results. They were able to show also in the in vivo model that in the case of a joint application of lactate and TPA there was no neuroprotective effect either. It would be now interesting to make researches about the association of treatment with lactate when using mechanical thromboembolectomy as its neuroprotective effect seems to be counteract by rTPA.

Ischemic strokes are a major public health issue and a more efficient treatment must be found. The great variability of the different presentations of stroke makes it a difficult pathology to simulate and to reproduce, this variability brings a difficulty to obtain robust tests and it is here that lies all the challenge for researchers. Research for a neuroprotective agent must be continued to finally allow clinical trials in humans, but before it could be possible, preclinical results should demonstrate robustness by replication in animal model and ideally under conditions resembling multicentre controlled clinical trials.

7. Acknowledgments

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Appendix

Table 1: Common patterns of neurological impairments among patients with acute ischemic stroke [45]

Left (dominant) hemisphere—major or branch cortical infarction:
Aphasia
Right hemiparesis
Right-sided sensory loss
Right-sided spatial neglect
Right homonymous hemianopia
Impaired right conjugate gaze
Right (nondominant) hemisphere—major or branch cortical infarction:
Left hemiparesis
Left-sided sensory loss
Left-sided spatial neglect
Left homonymous hemianopia
Impaired left conjugate gaze
Deep (subcortical) hemisphere or brain stem
Hemiparesis (pure motor stroke) or sensory loss (pure sensory stroke)
Dysarthria, including dysarthria-clumsy hand
Ataxic-hemiparesis
No abnormalities of cognition, language, or vision
Brain stem
Motor or sensory loss in all 4 limbs
Crossed signs (signs on same side of face and other side of body)
Dysconjugate gaze
Nystagmus
Ataxia
Dysarthria
Dysphagia
Cerebellum
Ipsilateral limb ataxia
Gait ataxia

Table 2: Evaluation of a Patient with Suspected Acute Ischemic Stroke[45]

All patients:
Brain CT (brain MRI could be considered at qualified centers)
Electrocardiogram
Blood glucose
Serum electrolytes
Renal function tests
Complete blood count, including platelet count
Prothrombin time/international normalized ratio
Activated partial thromboplastin time
Selected patients:
Hepatic function tests
Toxicology screen
Blood alcohol determination
Pregnancy test
Oxygen saturation or arterial blood gas tests (if hypoxia is suspected)
Chest radiography (if lung disease is suspected)
Lumbar puncture (if subarachnoid hemorrhage is suspected and CT is negative for blood)
Electroencephalogram (if seizures are suspected)
CT indicates computed tomography; MRI, magnetic resonance imaging.

Table 3: National Institutes of Health Stroke Scale[45]

Tested Item	Title	Responses and Scores
1A	Level of consciousness	0—alert
		1—drowsy
		2—obtunded
		3—coma/unresponsive
1B	Orientation questions (two)	0—answers both correctly
		1—answers one correctly
		2—answers neither correctly
1C	Response to commands (two)	0—performs both tasks correctly
		1—performs one task correctly
		2—performs neither
2	Gaze	0—normal horizontal movements
		1—partial gaze palsy
		2—complete gaze palsy
3	Visual fields	0—no visual field defect
		1—partial hemianopia
		2—complete hemianopia
		3—bilateral hemianopia
4	Facial movement	0—normal
		1—minor facial weakness
		2—partial facial weakness
		3—complete unilateral palsy
5	Motor function (arm)	0—no drift
	a. left	1—drift before 5 seconds
	b. right	2—falls before 10 seconds
		3—no effort against gravity
		4—no movement
6	Motor function (leg)	0—no drift
	a. left	1—drift before 5 seconds
	b. right	2—falls before 5 seconds
		3—no effort against gravity
		4—no movement
7	Limb ataxia	0—no ataxia
		1—ataxia in one limb
		2—ataxia in two limbs
8	Sensory	0—no sensory loss
		1—mild sensory loss
		2—severe sensory loss
9	Language	0—normal
		1—mild aphasia
		2—severe aphasia
		3—mute or global aphasia
10	Articulation	0—normal
		1—mild dysarthria
		2—severe dysarthria
11	Extinction or inattention	0—absent
		1—mild (loss 1 sensory modality)
		2—severe (loss 2 modalities)

Table 4: Characteristics of Patients with Ischemic Stroke Who Could Be Treated With rTPA[60] and/or with mechanical thromboembolectomy

Critères thrombolyse intraveineuse (TIV)

AVC ischémique aigu selon neurologue
NIHSS ≥ 4 selon neurologue au moment de la thrombolyse
- ou 1-3 et déficit potentiellement handicapant
- ou 1-3 et occlusion intracrânienne proximale

Traitable ≤ 4.5h depuis dernière preuve de bonne santé
Patient indépendant avec bonne qualité de vie (Rankin ≤ 3)
<16 ans : contacter urgemment neuropédiatre et superviseur cérébrovasc.

→ *Si critères cliniques pour TEV aussi remplis: préavertir ALG (**65114**) pour év. bridging (sera confirmé après angio-CT)*
→ *Si contre-indication à la TIV: viser TEV mécanique direct*

Critères ttt endovasculaire (TEV) mécanique et bridging

NIHSS ≥ 4 selon neurologue au moment de l'initiation TEV
Traitable par TEV ≤ 6h depuis dernière preuve de bonne santé
Rankin ≤ 2. Patient < 16 ans : cf. commentaire TIV.
Supratentoriel : CT natif ou DWI : ASPECTS ≥ 5 (considérer résultats CTP pour situations limites, i.e. pénombre>infarctus, ou ++collatérales)
Angio-CT ou IRM : occlusion accessible (ou signe hyperdense) de ≥1 artères suivantes : siphon carotidien, MCA1, MCA2, ACA1, V4, basilaire, PCA1
Occlusion basilaire traitable < 6h : Ø signes radiologiques d'infarctus étendu du tronc (préférer DWI si pas perte de temps).