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MULTICENTRE AND MULTISPECIES PRECLINICAL TRIAL OF REMOTE ISCHEMIC CONDITIONING IN ANIMAL MODEL OF ACUTE ISCHEMIC STROKE (TRICS–BASIC)

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ABSTRACT

Remote ischemic conditioning (RIC) represents an ideal candidate to enter a multicenter trial for acute ischemic stroke (AIS) treatment, since previous results from single laboratories support its efficacy, but unfortunately phase II–III clinical trials still provided inconclusive results. TRICS–Basic is the preclinical trial in the TRICS project, a multicentre translational Trial of Remote Ischemic Conditioning in Acute Ischemic Stroke from the Italian Stroke Organization (ISO) Basic Science network, which consisted in the collaboration of 7 Italian institution. TRICS–Basic is a robust, translationally oriented, multicentre, randomized preclinical trial, which includes two animal species (rats and mice) and both male and female sexes are equally represented. The aim of this project was to investigate the efficacy of RIC treatment in AIS experimental models. All the animals in the MCAo+ groups were subjected to the same time of occlusion (60 min in mice; 100 min in rats). The treatment was applied by clamping the ipsilateral femoral artery for 10 min in mice and 20 min in rats. Blinded outcomes assessment was performed both for dichotomized functional neuroscore (primary outcome) and for infarct volume (secondary outcome) at 48 hours. Statistical analyses were performed in a blind status and according to an intention–to–treat paradigm. During the initial experimental period, we carried out a harmonization phase, including all the involved centres, in order to reduce the assessment bias during the neurobehavioral test evaluation. After we have reached the target of Inter class correlation (ICC) ≥0.60 imposed a priori by the protocol paper, we started the real experimental phase. The experimental cohort was composed by n=206 animals (n=110 mice and n=96 rats) but only n=168 were allocated in the MCAo+ groups (n=88 mice; n=80 rats) and n=152 animals were included in the study (n=78 mice; n=74 rats). The obtained data showed that RIC improve the good functional outcome (+20% in mice; +18% in rats) and reduce the area of ischemic injury (-4.3% in mice; -26.6% in rats) in both species. Despite the large number of animals used in this study and as compared to previous preclinical studies on RIC treatment, we did not reach the statistical significance in our two major outcomes, if we compare the single species alone. On the contrary, if we combine together all the animals, we obtained a significant result in both the analysed outcomes. This suggest that, similarly to clinical trials, a larger sample size would have resulted in more significant results in the functional and the infarct size outcomes single species analyses.

INTRODUCTION

ACUTE ISCHEMIC STROKE (AIS)

Definition of stroke

The universal definition of stroke by The World Health Organization (WHO) is: "Rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer, or leading to death, with no apparent cause other than of vascular origin" (1). Today, stroke is the first leading cause of disability, second of dementia and death worldwide and resulted to be one of the main causes of functional deterioration of the central nervous system (CNS) (2,3).

Epidemiology of AIS

Ischemic stroke results from a time–dependent and critical reduction of regional cerebral blood flow (CBF) after arterial occlusion (4,5). It accounts nearly 80–85% of overall cases and could be classified as:

- Thrombotic stroke, if the clot formation results in a narrowing of the lumen and blocks the passage of the blood through the artery, typically due to atherothrombosis.
- Embolic stroke if an embolus is a blood clot that occlude an artery within the brain.

Atherothrombosis or embolization led to territorial infarcts of variable size, which are larger if supplied by the middle cerebral artery (MCA) or smaller, if branches of large arteries are occluded or if cerebral collateral is efficient. The neurological outcome mainly depends on the location, extension, and shape of the ischemic lesion (6).

Incidence and risk factors

Globally, one in four people over age 25 will have a stroke during their lifetime. Each year, over 16% of stroke occur in people between 15–49 years old, reaching the maximum peak in patients under 70 years of age (62%). In this range, nearly 57% of stroke patients present a marked limitation in daily lifetime activities related to post–stroke disability (6). Up to 80% of overall cases of stroke is new onset, while the other 20% refers to relapses (2). Although during the last years, various innovative neuroprotective therapies have been developed, prevention strategies still remain the best approaches to reduce the incidence of the disease. In this regard, it is essential to be aware of the main predisposing risk factors i.e. sedentary, obesity, dyslipidemia, diabetes mellitus, hypertension, alcohol, smoking, atrial fibrillation and other associated cardiovascular diseases (7,8).

Pathological classifications of AIS

Every year, approximately 25% of diagnosed strokes cases remain of unknown etiology, therefore both researchers and clinicians have to perform intensive investigations in this setting. One useful approach could be the identification of the possible pathophysiological mechanisms behind the disorder, in order to better classify the different types of ischemic strokes (6). The most used method is the Trial of Org 10172 in Acute Stroke Treatment (TOAST) study, based on stroke's origin, which identify five subtypes of ischemic stroke (9):

- *Large artery atherosclerosis:* The pathological lesion derived from the formation of an atheromatic plaque, which commonly affected the aorta, the coronary arteries, the carotid artery at its bifurcation and the basilar artery. Arteriosclerotic lesions include different types of injures such as the hardening and thickening of the arteries, the calcification within the tunica media and the proliferative and hyaline activity changes. In particular, this latter subtype becomes clinically symptomatic when the affected organ became completely occluded (4,6,9).
- *Cardioembolism/Thromboembolism:* After plaque erosion, procoagulant molecules (such as von Willebrand factor, vWf) and subendothelial collagen are exposed to blood flow. Platelets rapidly adhere and aggregate to the intimal layer of the vessel through the binding between fibrinogen and glycoproteins (GP). Then the thrombus grows and completely obstruct and/or blocks the blood flow. Atherosclerotic thrombi can also represent a possible source of embolisms, responsible for the primary pathophysiological mechanism of ischemic stroke (4,6,9).
- *Small–vessel occlusion:* This kind of occlusion is associated to hypertension and primally affects arterioles. Moreover, this process is caused by abnormal hyaline protein accumulation in subendothelial vessel layer, resulting in two pathological conditions: status lacunaris (lacunar state), characterized by small irregularly shaped infarcts and status cribrosus (état criblé), characterized by small round cavities around affected vessels (4,6,9).
- *Other determined etiology:* In this category are included patients –often young people– with unusual causes of stroke as *arterial dissection, abuse substances, vasculitis, non–inflammatory vasculopathies, hypercoagulability pathologies and genetic pathologies* (4,6,9).
- *Undetermined etiology:* Different situation have been described, including cryptogenic strokes, which are ischemic strokes events of "undetermined" cause, mainly because of an incomplete investigation or because of multiple coexisting factors within the same patient (4,6,9).

Etiopathogenesis of AIS

AIS is due to a series of sub-acute and chronic events which start from the occlusion and continue also beyond the reperfusion. These events are related to vascular, biochemical, and physiological modification of the brain **(Fig.1).**

Perfusion impairment

Suddenly after occlusion, the brain region exposed to the critical reduction of cerebral blood flow (CBF), which is above 18%, is severely impaired due to loss of nutrients within the brain parenchyma. After this initial step, cell's membranes start to depolarize, cytoskeleton is disrupted and neuronal cells begin to die by necrosis. The specific localization, where these latter events occurred is usually identified as "ischemic core". Indeed, it is known that the periphery of the involved tissue is more perfused than the lesion core, thus the critical reduction of CBF developed nearly the occlusion. In this regard, the presence of subsidiary vascular network, the so called "cerebral collaterals circulation", is important as it is able to balance the reduction of blood flow (11). Usually, oligemia occurs at 80% of CBF, when the unbalance of nutrients started to alter the normal protein production and the normal ionic pump activity. The range from 20% to 80% of CBF is defined "ischemic penumbra", condition in which, due to progressive energy deficiency, cells are unable to sustain different biochemical pathways i.e. protein synthesis, glycolysis and ionic balance. Neurons, oligodendrocytes, astrocytes and vascular cells are the most sensitive cells to ischemic damage (6,12).

Energetic and ions pumps failure

A large amount of the total oxygen consumption within the brain is directly related to the aerobic metabolism of glucose, which is the main substrate for ATP production (2). In neuronal cell bodies, glucose is metabolized, in order to maintain and eventually restore the physiological ionic gradient and support the cell functions (13). Ischemia causes failure in ATP syntheses and oxidative phosphorylation. This lack of energy leads to collapse of Na+/K+ electrochemical gradient, increasing pre–synaptic glutamate release through membrane depolarization and the subsequent activation of the voltage–gated Ca^{2+} channel (14,15). Deregulation of post–synaptic ions pumps generate an uncontrolled influx of calcium, sodium and chloride ions and also the efflux of potassium ions in post–synaptic neurons (16). Moreover, the activation of metabotropic glutamate receptors increases the IP3–dependent signal transduction pathway, leading to ER stress response (6).

Acid toxicity

The combination of the oxygen depletion and the activation of anaerobic glycolysis induces an increase of the intracellular lactic acid. Depending on the severity of ischemia, these events result in a decline of intracellular pH from 6.5 to below 6.0. Recent evidences demonstrate that acid-sensing ion channels (ASICs) are glutamate-independent vehicles of calcium flux, and that their blockade

could attenuate the stroke–induced injury. This suggests that acidosis represents the real mechanism of acid toxicity; in fact, the unbalanced intracellular acidification is considered a primary cause of cell death (15,17).

Calcium toxicity

At the onset of ischemia, the association of different events like anoxic depolarization, activation of ionotropic glutamate channels and acid-sensing episodes, causes a sharp rise of cytosolic calcium ions (Ca^{2+}). The unbalance in ions concentration, due to intracellular Ca^{2+} increase, leads to several consequences including mitochondrial dysfunction, activation of Ca^{2+}–dependent effector proteins and enzymes involved in the intrinsic apoptotic pathway, thus ending in cell death. Therefore, all the events beyond the Ca^{2+} overload are highly complex and contribute to other several molecular pathways (18).

Endoplasmatic Reticulum (ER) stress and inhibition of protein synthesis

Dysregulated calcium homoeostasis in ER results in a global inhibition of the protein synthesizing machinery. This is due to the activation of protein kinase–R (PKR), which induces the phosphorylation and inactivation of the alpha subunit of eukaryotic initiation factor–2 (eIF2). The downregulation of eIF2 leds to selective inhibition of polypeptide chain initiation, disaggregation of ribosomes, and stops the translation process. In this regard, the inhibition of protein synthesis persists from the onset of ischemia until the cell death (19). For this reason, ER is considered a robust marker for the progression of ischemic injury. ER function could, indeed, be restored by refolding (due to activation of the unfolded protein response, UPR) or degrading (due to ER–associated degradation, ERAD) of misfolded proteins. However, if UPR and ERAD fail to restore ER function, the cells die by apoptosis (20).

Mitochondrial damage

The increased cytosolic calcium activity combined to the production of ROS, causes an increase in permeability of the inner mitochondrial membrane. The mitochondrial permeability transition (MPT) is associated to the formation of a permeability transition pore (PTP). The PTP is a Ca^{2+}, ROS and voltage-dependent located in the inner mitochondrial membrane (21). The dysregulation of this channel increases the permeability of the inner mitochondrial membrane, which in turn, generates the breakdown of the electrochemical gradient. Therefore, this latter membrane is able to interfere with mitochondrial oxidative phosphorylation, resulting in the activation of anerobic energy production. Furthermore, the unbalance of mitochondrial ion gradients causes both the swelling and the disruption of the outer mitochondrial membrane and finally the release of pro–apoptotic proteins within the extracellular space. To this extent, ischemia–induced mitochondrial disturbances probably

contributes to delay cell death either by ATP impairment and by the activation of pro apoptotic pathways (22).

Oxidative stress damage

The primary source of oxygen–derived free radicals (ROS) during the ischemic injury is mitochondria, which normally produce superoxide anion radicals during the electron transport process. However, the adverse effects of ROS are related to the peroxidative injury within the plasma membranes and the intracellular organelles (11,23). Another crucial source of ROS regards the metabolism of arachidonic acid through the cyclooxygenase and lipoxygenase pathways. Indeed, ROS can also be generated by NADPH oxidase enzymes, which are expressed by the activated microglia and infiltrating peripheral leukocytes (11). Nitric oxide (NO), the main ROS involved in the ischemic injury, is generated from L–arginine through one of several NO synthase (NOS) isoforms:

- *nNOS:* the neuronal isoform requires calcium/calmodulin for its activation and is expressed by some neuron subpopulations within the brain;
- *iNOS:* the inducible isoform is expressed by inflammatory cells such as microglia and monocytes;
- *eNOS*: the endothelial isoform has vasodilatory effects and is likely to play a beneficial role, improving local blood flow.

Under the ischemic condition, the uncontrolled activation of nNOS and iNOS isoforms are detrimental to the brain. Indeed, NO freely diffuses across membranes, where can interact with superoxide molecules and generate peroxynitrite ($ONOO^-$), another ROS (11). Both oxygen–derived free radicals and reactive nitrogen species are able to activate several pathways involved in cell death following stroke, such as apoptosis and inflammation (24).

Blood brain barrier disruption

After the ischemic injury, the blood brain barrier (BBB) increases its permeability along with the degradation of the basal lamina of the vessel wall. In this regard, several evidences suggest the interactions of brain endothelium with extravascular CNS cells (astrocytes, microglia, neurons), as well as with intravascular cells (platelets, leukocytes) (19). In addition, BBB disruption is also related to an uncontrolled release and exchange of molecules from the brain to the peripheral system and vice versa. These latter events induce the recruitment of immune cells to the ischemic territory and contribute to the inflammatory response. Finally, the perpetuation of the inflammatory process is related to a secondary damage, the so called “post–ischemic inflammation” (25,26).

Post–ischemic inflammation

After the BBB breakdown and the consequent release of pro–inflammatory mediators from the surrounding ischemic region, leukocytes are facilitated to infiltrate that area; in particular, their role is to clear the debris caused by cell death (25). To this extent, pro–inflammatory cytokines induce an upregulation in the expression of leukocyte adhesion molecules within endothelial cells, thus stimulating the synthesis of chemokines, which in turn, guide leukocytes to the ischemic injury. Nonetheless, growing evidence reports that infiltrating immune cells can worsen the post–ischemic brain damage (27). The reason is that infiltrated leukocytes also produce cytotoxic mediators which protract the stress generated from the inflammatory response, thus causing a positive feedback loop. Indeed, this event increases brain damage, contributes to edema formation and hemorrhagic transformation, that is a secondary complication that commonly influence neurological outcome in patients (25,28).

Edema

Soon after CBF impairment, the failure of Na+/K+ATPasi pumps creates an osmotic gradient, favoring the passage of water from interstitial to the intracellular space. This kind of intracellular swelling is known as oedema. We can distinguish an early cytotoxic oedema type, followed by a late vasogenic one:

- *Cytotoxic oedema* is a CBF threshold dependent event; it starts from 30% of CBF when stimulation of anaerobic metabolism causes an increase of brain tissue osmolality. When CBF reaches the 20%, it was able to cause anoxic depolarization, enhancing intracellular osmolality and cell swelling. This is due to the massive passage of Na+ ions into the post-synaptic neuron, followed by water molecules. Therefore, within this territory, the consequence of cytotoxic oedema is neuronal death by necrosis (29).
- *Vasogenic oedema* is typically caused by BBB break and is able to further enhances the water content of the cerebral tissue. In contrast to the early cytotoxic one, this is an iso–osmotic type of oedema, that accumulates mainly in the extracellular compartment. Vasogenic edema reaches its peak at 1–2 days after the onset of ischemia and increases of tissue water (30).

Cellular death

From the onset of stroke, CNS cells exceed the mechanisms of restoration typically activated during physiological conditions and are triggered into different pathways of cellular death:

- *Necrosis:* Suddenly after the ischemic event, the cells exposed to the greatest reduction of the blood flow are irreversibly damaged and die due to the major energy impairment (31). This kind of cell death is morphologically characterized by cell swelling and subsequently by nuclear, organelle and plasma membranes disruption. The disintegration of the nuclear

structure and of cytoplasmic organelles leads to extrusion of cell content in the extracellular space (11).

- *Apoptosis*: The necrotic tissue is surrounded by peripheral tissue, which is the more perfused one, thus still metabolically active, named ischemic penumbra. In this region, neurons could undergo apoptotic processes if the unbalance of nutrients would not be restored (31). In contrast to necrosis, apoptosis is a programmed energy–dependent process leading to cell death. This process is initiated by two pathways: an extrinsic death receptor–dependent route and an intrinsic pathway, which mainly depend on the mitochondrial release of pro–apoptotic molecules such as apoptosis–inducing factor (AIF) and cytochrome C. During focal ischemia, apoptosis is induced also by activation of toll–like receptors 2 and 4 (TLR2, TLR4), the NOTCH–1 receptor, and the adiponectin receptor 1 (11).

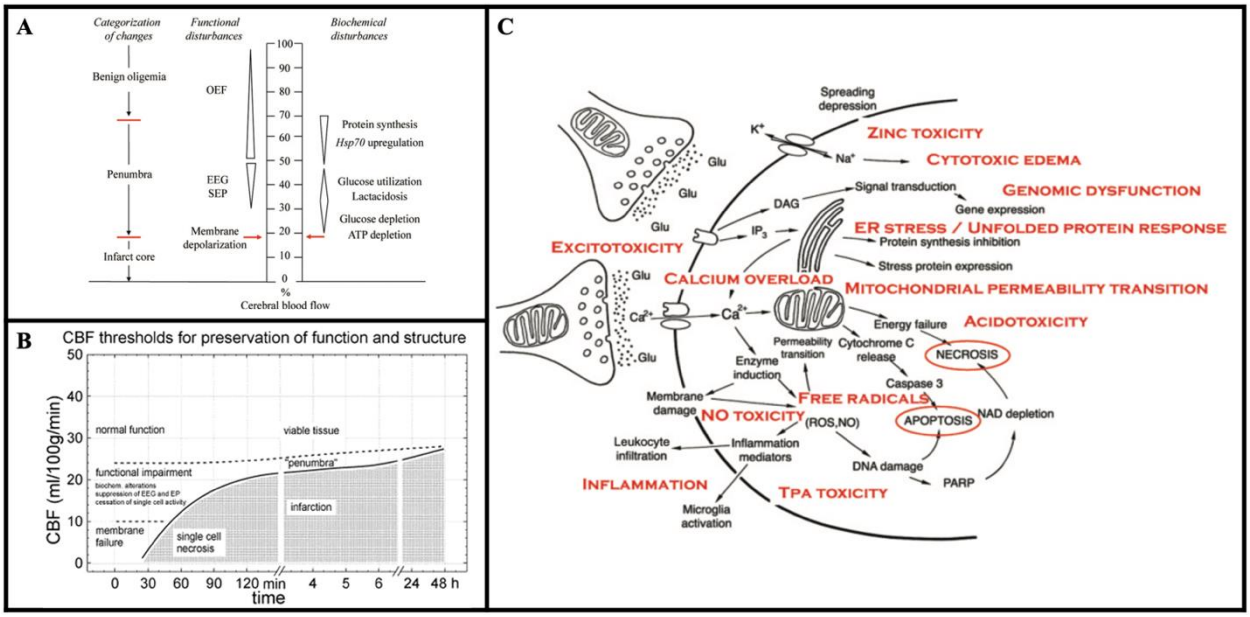


Fig.1 ***A)*** *Graphical representation of viability thresholds of focal brain ischemia. (EEG, electroencephalogram; OEF, oxygen extraction fraction; SEP, somatosensory evoked potential).* ***B)*** *Diagram of CBF thresholds required for the preservation of function and morphology of brain tissue. The activity of individual neurons is blocked when flow decreases below a certain threshold (upper dashed line) and returns when flow is raised again above this threshold. The fate of a single cell depends on the duration for which CBF is impaired below a certain level. The solid line separates structurally damaged from functionally impaired, but morphologically intact tissue, the "penumbra." The upper dashed line distinguishes viable from functionally impaired tissue.* ***C)*** *Schematic representation of molecular injury pathways leading to necrotic or apoptotic brain injury after focal brain ischemia. Injury pathways can be blocked at numerous sites, providing multiple approaches for the amelioration of both necrotic and apoptotic cell death. DAG 1/4 diacylglycerol; IP3 1/4 inositol 1, 4, 5-trisphosphate; PARP 1/4 poly (ADP- ribose) polymerase; TPA 1/4 tissue plasminogen activator. (Brainin M., et al., Textbook of Stroke Medicine (2013) second edition, ISBN-13:9781107047495.)*

DIAGNOSIS

In clinical practice, in order to identify the best therapeutic option for the patient, imaging techniques play a central role in the pathophysiological origin of the occlusion. The major role of imaging in stroke could be summarize in the *identification of ischemic, hemorrhagic or stroke mimics; evaluation of the vasculature and vascular lesion; detection of stenosis, occlusion, aneurysms and/or other vascular malformations; evaluation of ischemic core, penumbra territory and collateral supply; selecting patients for thrombolysis* (32). The most used imaging technique are:

- *Computed Tomography (NCCT)*
  - *Non–Contrast CT (NCCT):* During the hyperacute phase of stroke (< 12 hours), NCCT can give a negative result in about 50% of cases, however, it is essential to identify a cerebral haemorrhage; indeed, the main advantages are the diffuse access and acquisition speed. A cerebral ischemic lesion becomes visible to the CT about 12–24 hours after the onset of the symptomatology, although it may not be recognized reliably for 24–48 hours. Therefore it is essential to repeat a second CT after 48–96 hours to evaluate the progression of the injury (33).
  - *Angiography CT (CTA):* This is a quick and non–invasive scan of cervical and intracranial arteries, intracranial veins and aortic arch. This technique allows the identification of carotid disease, arterial dissection and occlusion or stenosis of intracranial vessels (34). It is usually performed by the intravenous administration of an iodized bolus of contrast. CTA is more accurate in the identification of large proximal arteries occlusions than the small distal ones (35); therefore, CTA images could also be more sensitive than the NCCT for detecting an early cerebral infarction (36).
  - *Perfusion CT (CTP):* This diagnostic method was performed after the intravenous injection of iodized contrast and the contrast's kinetic is analysed through the cerebral parenchyma. In particular, two different hemodynamic maps are generated: cerebral blood flow (CBF), cerebral blood volume (CBV), mean transit time (MTT) and time to peak (TTP) (37), which are useful to evaluate the infarct core size and the penumbra territory.
  - *Digital Subtraction Angiography (DSA):* This represents a still invasive method for the visualization of cerebral vascularization by contrast media injections. Unfortunately, it remains the gold standard to evaluate the severity of arterial stenosis, presence of vascular malformations and it also provides information on collateral flow and perfusion. DSA is rarely performed during triage setting, due to high invasiveness and to the high risk of stroke associated with. On the therapeutic side, angiography is an integral part of

endovascular therapy procedures (mechanical thrombectomy) for patients with an occlusion of a large cerebral artery, where is necessary to confirm the presence of an occlusion and subsequent revascularization (35).

- *Magnetic Resonance Imaging (MRI):* MRI protocols include different kind of scans like T1–weighted, T2–weighted, fluid-attenuated reversal recovery (FLAIR), T2*–weighted gradient–recalled echo (GRE) and diffusion–weighted (DWI) sequences, which can reliably diagnose both ischemic stroke and acute haemorrhagic.
  - The ischemic lesion appears hypointense on T1–weighted and hyperintense on T2–weighted images.
  - Dynamic Susceptibility Contrast MRI (DSC–MRI) which is based on the loss of signal induced on T2 weighed sequences* after the administration of a gadolinium–based contrast bolus through a capillary; In this way, different parameters can be calculated (for example, rCBV, rCBF, MTT).
  - Diffusion Weighted MRI (DWI–MRI) is more efficient than NCCT for acute infarction diagnosis and visualization, but some stroke mimics can be ruled out. DWI can be positivized within 3–30 minutes of onset, when conventional MRI and CT images are still negative (38,39).
  - Perfusion Weighted MRI (PWI–MRI) evaluates changes in cerebral perfusion in acute cerebrovascular disease and is able to show penumbra territory. The identification of ischemic penumbra areas occurs by mismatch between perfusion (PWI) and diffusion sequences (DWI) (40). This mismatch is used as an imaging marker to select patients who present a life–saving brain tissue and who could then benefit from reperfusion therapies.

PREVENTION

Stroke prevention plays a fundamental role in both new onsets' diagnosis and relapses. In this regard, the aim of researchers is to reduce the high incidence of stroke events. Three types of prevention strategies are currently applied in the clinical practice, which are different according to their action:

- *Primary:* it refers to are a group of strategies, which have been implemented in healthy patients without a previous stroke or TIA event and consist in the control of modifiable risk factors (divided ad *major*–sedentary, obesity, dyslipidemia, diabetes mellitus, hypertension, alcohol, smoking, atrial fibrillation, and other heart diseases– and *minors* –migraine, drug abuse, sleep apnea, hyperhomocysteinemia, hypercoagulability, inflammatory states and infections) (7,8,41).
- *Secondary:* it refers to prophylactic measures in patients who had TIA in order to avoid the occurrence stroke, or in already diagnosed stroke patients to avoid a relapse (41,42).
- *Tertiary:* it refers to the measures, which can be evaluated in patients which already had an ischemic or hemorrhagic cerebral vascular event, in order to reduce the severity and the impact of the current pathology and to avoid a recurrence of disease. This approach includes rehabilitation strategies, which can help reducing the limitations in the everyday life and promoting their independence and reintegration into the society (43).

TREATMENT

The sudden onset of a cognitive impairment, especially in elder subjects with one or more risk factors for cerebrovascular diseases, might be associated to clinical suspicion of a cerebral stroke. The management of a stroke patients during the acute phase of AIS is dramatically important to reduce neurological damage, post–stroke disability and avoid infarction (32).

Acute treatment for AIS

Despite the treatment options for AIS are still limited, the use of recanalization therapy, both mechanical and pharmacological, has a significant impact in patient recovery. To this extent, the main limitations are related to the time window of its application, which is restricted in a specific range of time. In fact, the aim of acute therapies is to improve neurological outcome and reduce post-stroke disability (44).

- *Intravenous thrombolysis:* A lytic agent, recombinant tissue plasminogen activator (rtPA), is administered either intra–venously or intra–arterially to lyse the blood clot. The activated plasminogen, plasmin, has a fibrinolytic effect, which degradates the clot/thrombus. The rtPA dose is weight–dependent and goes from 0.9 mg/kg up to a maximum dose of 90 mg (2,6,44,45). Thrombolysis is contraindicated in patients with:
  - Stroke onset over 4.5/6 hours, neutral efficacy over this application time
  - Seizure at stroke onset, possible of stoke mimics;
  - Severe hypertension, due to the increased risks of hemorrhagic transformation;
  - Age less than 18 years old
- *Mechanical thrombectomy:* It is usually administered when patients are ineligible for rtPA. The most studied and used clot retrieval device is the Mechanical Embolectomy Removal in Cerebral Ischemia (MERCI). It is a catheter, which is inserted into the artery and then corkscrewed into the clot and then pulled out taking the clot with it. However, newer thrombectomy devices are under study (2,6,45,46).
- *Antiaggregant therapy:* Aspirin (ASA) is the most worldwide antiaggregant agent used, that irreversibly inhibits cyclooxygenase–1 (COX1) in platelet. COX1 is a key enzyme in thromboxane (TxA2) synthesis, a platelet activator that induces thrombus formation. Once ICH has been excluded, aspirin should be administered at a dosage of 300 mg either orally or rectally depending on the patient's disability. Subsequent doses can be lower (75–300 mg), with the evidence suggesting that the same benefit can be conferred with 75 mg daily whilst avoiding the potential side-effects which are more commonly observed at higher doses. Although there are only few trials in AIS patients, ASA represents a simple preventive therapy to reduce the number of recurrent vascular events, so stroke events (2,6,47).

- *Surgical decompression:* The highest mortality (80%) is recorded in patients suffering of large MCA territory infarctions. In this case, cerebral edema induces a dangerous increase in the intracranial pressure, which frequently leads to death. Clinically, the intracranial pressure should be maintained at ~70 mmHg; in fact, the surgical decompression needs to be considered in patients with high levels of intracranial pressure, in order to restore safety value (2,4,6).
- *Stroke unit:* There are well–structured departments where patients can be followed by specialized personnel in order to constantly monitor the vital functions and the evolution of the patient's neurological state. The stroke patient is followed from the acute emergency phase to the rehabilitation one (2,4,6).

REMOTE ISCHEMIC CONDITIONING (RIC)

Definition

Remote Ischemic Conditioning (RIC) treatment is based on repetitive and transient mechanical obstruction of blood vessel in a remote organ. This brief sublethal occlusion, in single or both hindlimbs, is able to trigger neuroprotective agents and mechanisms within the brain **(Fig.2)** (48,49). Numerous preclinical and clinical trials observed its safety and efficacy in AIS. RIC treatment could be classified into 3 subtypes based on its time window of application:

- Remote Pre-Conditioning (RIPreC);
- Remote Per-conditioning (RIPerC);
- Remote Post-conditioning (RIPostC).

Preclinical evidence of RIC

Preclinical studies demonstrated that RIC treatment interacts with different pathways in a neuroprotective way (49). In particular, many trials revealed that RIC improves neurological outcome and reduces the infarct size in preclinical rodent models of AIS (50,51).

RIC was demonstrated to significantly improve leptomeningeal anastomoses and increase the vessel diameter of ACA and MCA (52,53,54). In addition, it induces angiogenesis and collaterals formation (55). All these latter morphological and anatomical changes were also able to improve CBF (56). Indeed, the increased CBF levels are strictly related to the rise of nitric oxide synthase–3 (NOS3) activity within the endothelium and in circulating blood cells (57). NO is a well-characterized vasodilator; therefore its upregulation increases CBF after the proposed treatment (58,59). Nitrite also exerts its protective activity preventing oxidative stress damage into mitochondria (60).

Moreover, most recent works showed that RIC can reduce oxidative stress through the release of endothelin–1 and the upregulation of several pathways like Nrf2, HO1, NQO1 (61,62,63,64).

Another protective effect of RIC against ischemic injury is linked to the downregulation of proinflammatory pathways as HIF1α, HIF2α and activating the Notch1, NCCD and NF–KB pathways (65,66,67,68,69). As for immunomodulation, the treatment improves the peripheral immune response decreasing the levels of the major pro-inflammatory cytokines such as IL1β, IL6, and TNFα (70,71). Additionally, RIC induces autophagy by AKT/GSK3β activation, mitophagy by upregulation Parkin/DJ-1 expression and activation of the JAK2/STAT3,mTOR/p70S6K signaling pathway (71,72,73,74,75). Following this line, other studies demonstrated that apoptosis was attenuated after treatment due to the downregulation of Bax expression and upregulation of pBcl2 and heat–shock protein 70 (HSP70) (76,77,78).

At last, conditioning in AIS also acts on reducing edema and BBB permeability by MMP9 and AQP4 downregulation, also by eNOS upregulation (79,80,81,82,83).

As regards safety, the treatment needs to be tested either for AIS and for ICH, as it is usually administered to patients before any imaging assessment. To this insight, a work by Geng X. et al 2012, showed that neither a single acute administration of RIC, nor a more chronic treatment at 24 or 72 hours, got worse the infarct size in ICH rat model (84). Moreover, no significant differences between the analyzed groups were observed in cerebral blood volumes, edematous lesion and MMP9 and AQP4 expression (84). In another work, the authors observed a reduction in the hematoma volume together with the improvement of the functional outcome when RIC was applied 2 hrs after ICH and continued for the following five days (85). Moreover, RIC demonstrated its effectiveness even in an ICH mouse model, when administered for 5 consecutive days. These evidence supports the great applicability of RIC treatment in the prehospital setting for stroke patients (86).

The major advantage of RIC is its multiple mechanisms of action. RIC triggers adaptive, endogenous, neuroprotective responses in the brain as “ischemia tolerant” state through nerve, humoral, and immune-inflammatory pathways. Despite this potentiality and its multiple organ applicability, the molecular mechanism of action is still unclear.

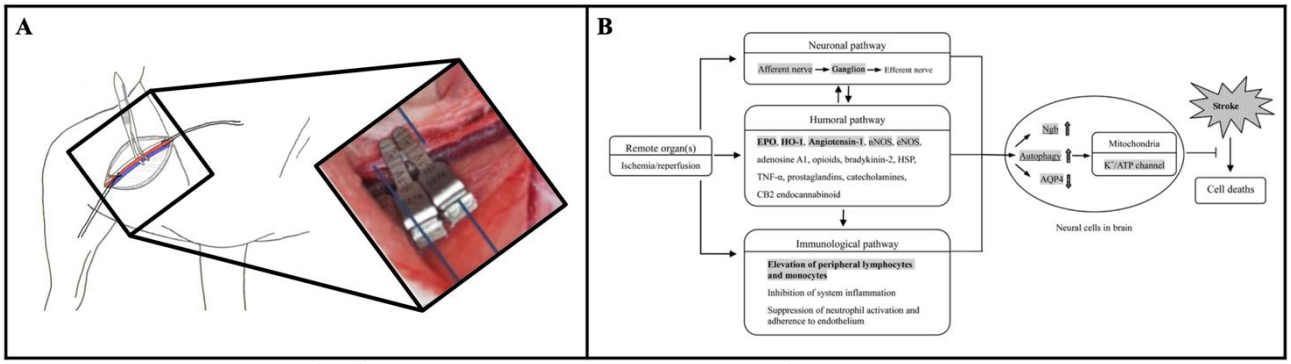


Fig.2 ***A)*** *Graphical representation of the femoral artery occlusion (FAO) in animal model of remote ischemic conditioning. The conditioning treatment is applied by a transient clipping of the femoral artery.* ***B)*** *The real mechanisms beyond the neuroprotection induced by remote ischemic conditioning (RIC) is still unknown. Probably, the protective effect is generated by different pathways as neuronal, humoral and immunological pathways. (Pan J., et al., "Remote ischemic conditioning for acute ischemic stroke: dawn in the darkness" (2016) Neurosciences, vol. 27, no. 5, pp. 501-510. https://doi.org/10.1515/revneuro-2015-0043)(48)*

Clinical evidence of RIC

Beyond preclinical evidence, the neuroprotective effects of RIC in AIS have been observed in several clinical studies **(Fig.3)**.

The Remote Ischemic Conditioning Paired with Endovascular Treatment (ET) in Acute Ischemic Stroke (REVISE–1) trial **(NCT03210051)** is a non–randomized, single–arm study. Safety and feasibility were assessed in n=20 subjects with AIS undergoing ET. Moreover, no adverse events were reported after the treatment; indeed, the combination of RIC together with the ET was safe, feasible and well tolerated (87).

RESCUE BRAIN **(NCT02189928)** is a multicenter, randomized, blinded trial. A total of n=188 patients were randomized into usual medical care (n=95) and RIC (n=93) treatment was administered one time with a thigh cuff within 6 hours from AIS symptoms. No significant results were observed between groups in infarct growth at 24 hours and neurological outcome (assessed by modified Rankin scale) at 90 days, which was the only improved in RIC patients (51% vs 41%, p=0.11). Moreover, 87% of the patients were treated with tPA and 34% with ET (88).

RECAST **(NCT86672015)** is a randomized blinded trial of n=26 patients with AIS treated within 24 hours with RIC (4 cycles of 5 minutes). The treatment showed its feasible and safety profile, and also results were well tolerated. Subjects treated with RIC treatment had significantly lower National Institute of Health stroke scale (NIHSS) scores at 90 days suggesting benefit on long term neurological outcome (89).

The single-center, open-label, blinded, randomized trial **(NCT00975962)** on remote ischemic preconditioning (rPerC), tested the effect of the treatment when it was applied (4 cycles x5 minutes) in a prehospital setting in adjunction to tPA in patients with AIS. After neurological examination and MRI, patients which received tPA for a verified stroke were included in the study. The cohort of the study was composed by patients with TIA (n=58), AIS (n=240) or ICH (n=37). Transient ischemic attack was more frequent (P=0.006), and NIHSS score on admission was lower (p=0.016) in the intervention group compared with controls. Any significant effects were highlighted among groups in infarct size, infarct growth at 1 months and functional outcome at 3 months. On the contrary, in voxelwise analysis they observed a reduction of infarction (P=0.0003) (90).

RICAMIS **(NCT03740971)** is a multicenter, open-label, blinded–end point, randomized clinical trial. It included n=1893 AIS patients. RIC was applied to the bilateral upper limbs of eligible patients (5 cycles of 5 minutes). The treatment was administered within 48 hours after the onset of symptoms for 10 to 14 days as an adjunct to guideline–based treatment (n=922) or guideline-based treatment alone (n=971). The primary functional outcome was at 90 days, (modified Rankin Scale score from 0 to 1). Comparing the effect of the treatment between groups they observed a significant increased

neurological outcome at 90 days in RIC patients (91). However, these findings need to be supported by an efficacy investigation for this intervention.

RICH **(NCT03930940)** is a single–centre clinical trial. The cohort of the study was composed by patients with ICH (n=40) which were randomized to RIC within 24–48 hours of the onset and treated daily for 7 days or usual care. There was no significant difference in hematoma volume at 7 days but the perihematomal resolution rate was higher in the RIC group. There was no difference in favorable functional outcomes at 90 days (92).

Repeated remote ischemic postconditioning (REPOST) **(NTR6880)** trial was stopped after the enrollment of 88 out of 180 scheduled patients. Repeated rIPostC was performed by inflating a blood pressure cuff around the upper arm (4 cycles ×5 minutes), which was repeated twice per day during hospitalization and last a maximum of 4 days. In this regard, no significant improvement was found in infarct size or clinical outcome in acute ischemic stroke patients subjected to the treatment. However, due to the lower inclusion rate, no definitive conclusions about the effectiveness of RIC can be addressed (93).

There are several pre–clinical and clinical evidences supporting the possible neuroprotective activity of RIC; indeed, different trials assessed its safety and its feasible profile. Thus, this could be a promising therapeutic approach for AIS patients and it is also under clinical evaluation for the application on ICH and ESA. Moreover, RIC reduced the infarct size and improved the functional outcomes in preclinical models. In addition, because of its plug and play profile, RIC can be applied also in prehospital setting as ambulance or helicopter, or in a clinical centre. Nevertheless, the exact molecular events involved in the neuroprotective activity of RIC are not completely determined, as its definition will be crucial for the understanding its mechanism of action.

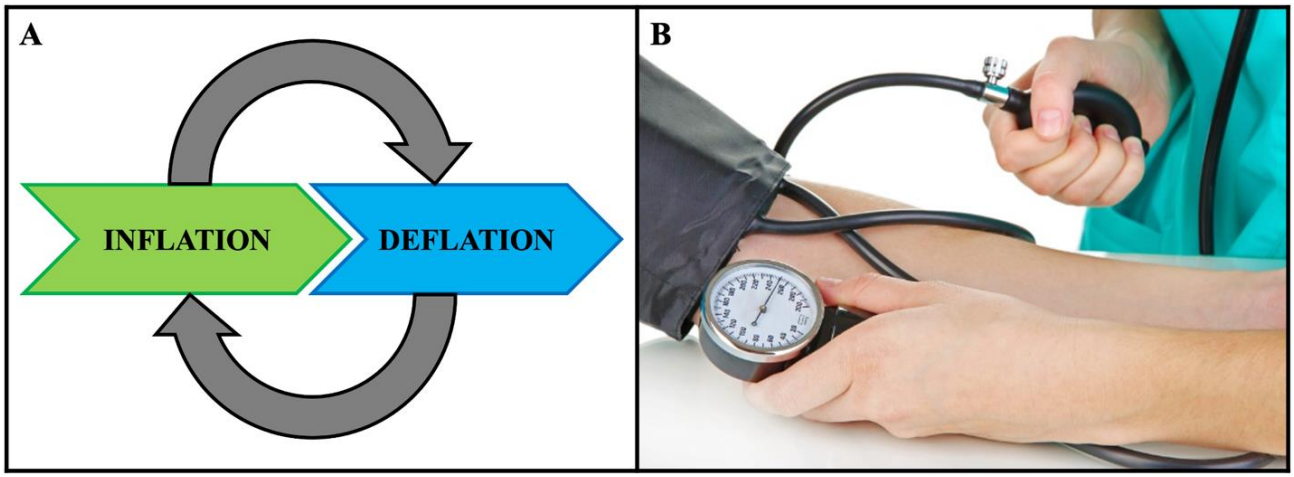


Fig.3 *Graphical representation of remote ischemic conditioning application. In clinical trial RIC treatment is commonly applied by brief inflation/deflation cycles **A)** by using a cuff around the arm **B)** or the limb of the patient.*

AIM

Remote ischemic conditioning (RIC) is an intervention based on blood flow reduction applied in a remote organ, away from the injured one. TRICS Basic study aims to investigate the translational efficiency and safety of remote ischemic post–conditioning in the experimental model of transient middle cerebral artery occlusion (tMCAO), in mice and rats of both sexes. We hypothesized that the hyperacute application of RIC would improve good neurological outcome assessed using a dichotomized neuroscore at 48 hours. Secondary objectives were the comparison of the means of the infarct volumes and comparison of the neuroscore as a continuous value between the two main experimental groups.

MATERIALS AND METHODS

Study design

TRICS–Basic study is a multicentre, multispecies, randomised, controlled, preclinical trials and blinded for primary and secondary outcome. The strength of the study is a well–structured pre-published protocol (94). All experiments were carried out within animal facilities of seven Italian academic and research institutions. The centres involved in the TRICS–Basic project were identified by a code (CentreId), as represented in **Tab. 1**.

Table 1 Centers from ISO Basic science network involved in TRICS Basic

CentreId	Italian academic or research institutions	Animal model used in the study
1	Pharmacological Research Institute Mario Negri (Milano)	Mouse
2	**University of Milano–Bicocca (Milano)**	Rat
3	University of Napoli Federico II (Napoli)	Mouse/Rat
4	University of Milano–Statale (Milano)	Rat
5	University of Firenze (Firenze)	Rat
6	University of Calabria (Arcavacata di Rende)	Mouse
7	San Raffaele Hospital (Milano)	Mouse

In order to limit excessive variability in the interpretation of the neurobehavioral test during the first experimental training, we set a satisfactory agreement between neurobehavioral raters at Intraclass correlation coefficient (ICC) ≥0.60, according to the protocol paper (94).

We planned to use n=192 animals that were equally stratified for species (n=96 mice and n=96 rats) for the experimental phase. Each centre was authorized to perform n=24 experiment equally stratified for sex (n=12 animals per sex; n=5 MCAo+/RIC+; n=5 MCAo+/RIC-; n=1 MCAo-/RIC+; n=1 MCAo-/RIC- pre sex) and a maximum of n=4 surgical procedures per day was authorized. A total of n=6 animals (n=3 per sex) per centre were authorized as replacement animals, as descripted below in **Animal replacement** paragraph.

The coordinator centre performed all the histological analysis.

Randomization

Two randomization lists were produced, separately per species and stratified by centre and sex. Each centre received n=24 sealed envelopes *(or external envelops)* per species, marked with blue label for male animals (n=12) and pink label for female animals (n=12).

Labels contained an animal identification code (IdCode), as shown in:

- the first number represents the centre identification code (CentreId), **Tab. 1**
- the second number for:
  - 1 = mouse animal model;

- 2 = rat animal model;
- the third number for:
  - 1 = male animal;
  - 2 = female animal;
- the last number, from 01 to 12, was the progressive experimental number.

Each envelope *(or external envelope)* contains:

- a label with the surgery randomization (MCAo±)
- an envelope (or inner envelope) containing a label with treatment randomization (RIC±).

The *external envelope* was opened just before the insertion of the occluding filament in the external carotid artery (ECA), while the *inner envelope* was opened just before the administration of the treatment. Randomization lists and envelopes containing randomization treatment allocations were prepared by personnel not involved in the implementation of procedures with the animals.

Animal replacement

If any animal died before RIC application, this latter was replaced. Each centre could replace a total of n=6 animal per species, equally stratified per sex (n=3 male and n=3 female). The replacement was done almost exclusively on animals subjected to MCAo+ surgery.

Blinding

In order to guarantee the blindness of the study, each centre chose two different researchers to perform the surgery and the functional outcome. Inclusion criteria guaranteed blindness since the intra-ischemic clinical assessment score was applied before treatment randomization. Exclusion criteria were applied after surgery in a blinded manner by the same researchers assessing functional outcome. Histological outcomes were centralized to the coordinating center (University of Milano–Bicocca) and performed by a senior researcher blinded to group allocation. Data analysis (RIC+ vs RIC-) were conducted blinded to the group allocation.

Animal Model

The experiments were conducted in accordance with institutional guidelines in accordance with national laws (Decree Law No. 26/2014; authorization No. 1056/2020–PR, prot. FB7CC.43, by the Ministry of Health), with authorizations for personnel conducting the experiments provided by Mario Negri Institutional Regulations and Policies (Quality Management System Certificate–UNI EN ISO9001: 2008–Reg. N° 6121) and with international laws (EEC Council Directive 2010/63/UE; Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council (Eighth Edition 2011). All experiments on animals were approved by the Ethics Committee of the University of Milano Bicocca, the Coordinating body of the project.

Mouse model

Male C57BL/6J wild–type (WT) mice (10–11 weeks old, body weight of 24 g ± 10%; Charles Rivers Laboratories, Italy) were housed in a single cage, within a Specific Pathogen–Free (SPF) enclosure, with a 12 hours, controlled light/dark cycle and room temperature, food and water available *ad libitum*, for at least a week before any intervention. After surgery, the animals were housed under the same conditions for 48 hours.

Rat model

Adult male Sprague–Dawley rats (body weight of 250 g ± 5%; Charles Rivers Laboratories, Italy) were housed in a single cage in a SPF enclosure, with a 12 hours, controlled light/dark cycle and room temperature, food and water available *ad libitum*, for at least a week before any intervention. After surgery, the animals were housed under the same conditions for 48 hours.

***In vivo* surgery model – transient Middle Cerebral Artery occlusion (tMCAo)**

Mouse tMCAo surgery

Anaesthesia was induced by 3% isoflurane in O2/N2O (1:3) and maintained by 1%–1.5% isoflurane in O2/N2O (1:3). Transient MCAo was induced for 60 min followed by a reperfusion period of 48 hours. A silicone-coated monofilament nylon suture (sized 7–0, diameter 0.06–0.09 mm, length 10 ± 1 mm; diameter with coating 0.23 mm; coating length 2 mm, Doccol Corporation, Redlands, California, USA) was introduced into the right external carotid artery (ECA) and pushed through the internal carotid artery (ICA) to occlude the origin of the MCA. During the occlusion time, the mouse was awakened from anaesthesia to assess the **intra–ischemic clinical assessment** which reveals the correct induction of the ischemic injury (see "**Inclusion and Exclusion criteria**"). After 60 minutes, blood flow was restored by carefully removing the filament, under anaesthesia. During the surgery, the animal's body temperature was maintained at 37°C by a heating pad. Treatment (RIC±) was applied 10 minutes after reperfusion and lasted 10 minutes (see "**Treatment application – Remote**

Ischemic post Conditioning (RIC)"). During surgery, body temperature was manteined at 37°C by a heating pad. MCAo- mice received the same anaesthetic regimen and surgery, but no filament was introduced in the ICA. After surgery, all mice returned to single cages.

Rat tMCAo surgery

Anaesthesia was induced by 3% isoflurane in O2/N2O (1:3) and maintained by 1%–1.5% isoflurane in O2/N2O (1:3). Transient MCAo was induced for 100 min followed by a reperfusion period of 48 hours. A silicone-coated filament (size 5–0, diameter with coating 0.33 mm; length with coating 5-6 mm; Doccol Corporation, Redlands, California, USA), was introduced into the right external carotid artery (ECA) and pushed through the internal carotid artery (ICA) to occlude the origin of the MCA. During the occlusion time, the rat was awakened from anaesthesia to assess the **intra–ischemic clinical assessment** which reveals the correct induction of ischemia (see "**Inclusion and Exclusion criteria**"). After 100 minutes, blood flow was restored by carefully removing the filament, under anaesthesia. During the surgery, the animal's body temperature was maintained at 37°C by a heating pad. Treatment (RIC±) was applied 20 minutes after reperfusion and lasted 20 minutes (see "**Treatment application – Remote Ischemic post Conditioning (RIC)**"). After the surgery, all the rats were housed in single cages. During the surgery breath rate (number of breaths per minute) was recorded at three time points as a surrogate marker of anaesthesia tolerability: baseline (before anaesthesia), after 15 minutes of anaesthesia and 15 minutes after withdrawal of anaesthesia.

Treatment application – Remote Ischemic post Conditioning (RIC)

Remote ischemic post conditioning treatment was applied in the right limb of the animal by a surgical transient femoral artery occlusion (FAo). Briefly, at a predefined timing after MCA reperfusion (20 minutes in rats and 10 minutes in mice), femoral artery (FA) was transiently occluded with two microserrafine clips to stop the blood flow for 20 minutes in rats and 10 minutes in mice. The right execution of the femoral artery blockade was verified by visual inspection on the distal femoral artery territory. RIC- animals received the same surgery as that of RIC+ animals, their femoral arteries will be identified and isolated but not occluded.

Inclusion and Exclusion criteria

Rats and mice were selected for the study only if cerebral ischemia was successfully induced. After MCAo, the following **intra–ischemic clinical assessment** score was applied. Animals were judged ischaemic, and included in the trial, if presenting ≥3 of the following deficits during intra-ischemic period:

- The palpebral fissure has an ellipsoidal shape (not the normal circular one);

- One or both ears extend laterally;
- Asymmetric body bending on the ischemic side;
- Limbs extend laterally and do not align to the body.

Animals were excluded in case of:

- Death during MCA surgery;
- Major experimental protocol violations during MCA surgery (errors or surgical complications major arterial or venous haemorrhage, section of the vagus nerve, carotid artery dissection; filament entrapment or displacement, errors in ischemia time)

Animals that were allocated in the MCAo+ groups but did not develop an ischaemic lesion at histological analysis were excluded *ad posteriori*, since they were not representative of the MCAo+ group. Assessment of this exclusion criteria was carried out by a researcher blinded to the randomisation arm and to the functional outcome.

Health monitoring and safety assessment

A predefined Middle Cerebral Artery Occlusion (MCAo) health report prepared on the basis of the *Ischemia Models: Procedural Refinements Of In vivo Experiments (IMPROVE) guidelines*, was filled at baseline, at 24 and 48 hours with information on animal welfare (weight, breath rate, food and water intake…etc.) (95,96). Health report was composed by n=5 parts:

- *Mouse weight (g) or Rat weight (g) at 24/48 hours*
- *Low distress:*
  - Reduced food and water intake;
  - Abnormal behavior upon handling (increased or decreased reaction to being handled);
  - Lethargy and reduced motility Piloerection/staring coat;
  - Discharge from the eyes and nose
- *Moderate distress:*
  - Animal not drinking;
  - Animal not eating;
  - Severe surgical wound complication (infection, bleeding, opening);
  - Absence of faeces;
  - Audible respiratory noises (rasping, wheezing), intermittent, without respiratory effort;
  - Weight loss exceeding 10%;
- *High distress:*
  - Presence of barrel rolling Presence of tonic clonic seizures;
  - Continuous laboured respiration with increased respiratory effort;

- Animal not moving, unresponsive to stimulation, or in a lateral recumbent position;
- Weight loss exceeding 20%;

- *Death:*
  - Dead animal at 24/48 hours
  - Analgesic drug and dose (first day after intervention)
  - Euthanasia performed
  - Comments

Animals that showed signs of moderate distress were treated subcutaneously with 0.05–0.1 mg/kg buprenorphine every 8–12. Those animals with at least one sign of severe distress, were evaluated by the centre's veterinarians, were euthanized before the end of the experiment. Those animals, if sacrificed after RIC/sham application, were retained in the intention–to–treat (ITT) analysis, and given the highest score, n=56.

Primary outcome – Functional outcome

Difference in the proportion of animals (rats or, separately, mice) with a good functional outcome was measured by the dichotomised De Simoni composite neuroscore (13 items, range 0–56 points) at 48 hours after MCA occlusion (99). A dichotomized functional outcome was chosen (0–20, good outcome; 21–56, poor outcome), in accordance with the translational approach of this study (94). All the clinical trials on stroke use the modified Rankin dichotomized scale (0–2 vs 3–6) as a measure of the primary outcome since the quantification of the patient's disability is a very important parameter (100). Inspired by clinical research, we set the cut–off of score=20 for dichotomization using the De Simoni neuroscore according to both animal behavior and statistical issues. This neurobehavioral test highly correlated with the histological assessment of infarct volume (Pearson r=0.884, two-tailed $p<0.0001$, n=36, unpublished observations).

The neuroscore ranging from 0 (absence of deficits) to 56 (worst neurological result) and was composed in two parts:

- *General deficits:* describe the general well-being of the animal with a score between 0 and 28. This score included information on the physical appearance, i.e: fur (0–2), ears (0–2), eyes (0–4), posture (0–4), spontaneous activity (0–4) and presence of epileptic seizures (0–12);
- *Focal deficits:* describe neurological damage with a score between 0 and 28 and were evaluated through observations: body symmetry (0–4), gait (0–4), ability to climb a 45° inclined plane (0–4), circling behaviour (0–4), forelimb symmetry (0–4), compulsory circling (0–4) and whisker response (0–4).

We observed that MCA occluded mice scoring less than 20, at 48 hours after the ischemic onset, had usually a reduced weight drop, index of preserved feeding and motility and increased chance to survive for long periods after the induced experimental stroke. Moreover, when stratifying De Simoni neuroscores of MCA occluded mice into quartiles, a score of 21 represented the 75 percentile (101). Unpublished results by our network suggested that the same cut–off is applicable also for MCA occluded rats. In our view, a De Simoni neuroscore <20 corresponds, translationally, to a modified Rankin scale <2.

Secondary outcome – Histological analyses

After the neurobehavioral test, rats were sacrificed by deep narcosis with CO_2; brains were extracted and fixed in 10% formalin. Coronal sections (100 um) were obtain using Vibratome1000Plus (Leica) and stained using Cresyl Violet 0.1% (Bioptica, Milano, Italy). Infarct areas were measured in 19 consecutive sections with 200 um distance (bregma +3.0 mm to -2.0 mm). Each section was mounted on a positively charged slide (SuperFrost Plus, Thermo Scientific) and rinsed in a saline solution (Dulbecco's Phosphate Solution w/Magnesium w/Calcium; Euroclone): only after 48 hours sections were stained with Cresyl Violet (Cresilvioletto Kluver Barrera 05–B16001; Bioptica) according to manufacturer's instructions. The staining protocol required that the sections needed to be rehydrated by immersion in ethanol solutions with decreasing concentration (EtOH 95%, EtOH 70%, EtOH 50%; Sigma–Aldrich) and finally in demineralized water (dH_2O). Subsequently the sections were placed in Cresyl Violet for 5 minutes. After this, sections were immersed again in demineralized water, then in alcohol solutions with increasing concentration (EtOH 50%, EtOH 70%+Glacial Acetic Acid 3%, EtOH 95%, EtOH 100%; Sigma–Aldrich) and finally in xylene (Sigma-Aldrich) to wash off the excess dye. Then the samples were mounted with non–aqueous mounting medium (dibutyl–phthalate–xylene, DPX, CL04.0401.0500; Chem_Lab NV). Histological analysis was performed by the coordinator unit. Infarct volume was calculated using ImageJ image processing software (National Institute of Health, Bethesda, MD, USA), corrected for interhemispheric asymmetries due to cerebral edema with the following equation: (ischemic area) = (direct lesion volume) − ((ipsilateral hemisphere) − (contralateral hemisphere)) and expressed in mm^3.

Correlation between ischemic lesion and continuous neuroscore

Original composite neuroscore was assessed combining the ischemic lesion with the De Simoni neuroscore, as a continuous variable; moreover animals were divided by species.

Statistical analysis

The following statistical analyses (***Primary outcome – Functional outcome; Secondary outcome – Histological analyses; Correlation between ischemic lesion and continuous neuroscore; Correlation between ischemic lesion and continuous neuroscore***) were performed only for MCAO+/RIC+ and MCAo+/RIC- groups, because only MCAO+ animals are the subject of this thesis. Cut–off for statistical significance was set at 0.05, two–tailed. Analyses were performed in a blinded status. Analyses were performed using Stata/IC V.15 or higher (Statacorp). Data preprocessing and descriptive analyses were performed using JMP Pro V.14 or higher (SAS Institute).

Health monitoring and safety assessment

Health report was descriptively reported at all available times (Baseline characteristics, +24 and +48 hours) presenting numbers and percentages.

Primary outcome – Functional outcome

The proportion of animals with good neurological outcome at 48 hours after MCAo was compared between RIC+ and RIC- groups by means of a logistic regression. Descriptive analyses with means, medians, SD and quartiles, and graphical methods were carried out on the raw values of the neuroscore by treatment in order to investigate the effect of the treatment in reducing the percentage of animals with bad functional outcome.

Another analysis was performed using the original neuroscore value (not dichotomised) as an outcome, using a linear regression. In these latter analyses, dead animals were not included.

Secondary outcome – Histological analyses

The secondary analyses were performed looking at the infarct volume, expressed as a continuous variable (mm^3). The effect of the treatment between groups was studied using a linear regression model. Standardization of infarct volume was carried out in order to mix the two different scales, and was obtained by subtracting from each raw value the respective (mouse/rat) mean and then dividing by the respective (mouse/rat) standard deviation.

Correlation between ischemic lesion and continuous neuroscore

The correlation analyse was performed using the continuous variable of the primary and the secondary outcome. The correlation was studied using a linear regression model and expressed by Pearson r factor and by t–test.

Subgroup/adjusted analyses

The primary and secondary outcomes was reanalysed in the subgroup of animals reaching alive the 48 hours after MCA occlusion.

Population analysis and missing data

Primary analyses were conducted according to an ITT paradigm, including all RIC+ randomised animals (with the exception of animals without sign of ischaemia at neuropathological inspection, since these cannot be considered a model of ischaemic stroke). In case an animal was died (after RIC) before being evaluated at 48 hours, it was given the worst attainable score (score=56) and therefore it was counted as a negative outcome. Considering that 25–30% of the animals would not be available for the outcomes analysis, we would need n=120 total animals per species.

Auditing

Quality assessments was carried out by the coordinating unit using onsite visits and remote form checking. A manual check was performed by a researcher from the coordinating unit in charge of monitoring (CUCP: coordinating unit checking person). The CUCP accessed in read mode to all records, while the PRIs could only input/modify data in the database, for their specific centre. The CUCP was in charge of visiting the centres to check the consistent application of the procedures and to resolve pending problems. The CUCP was present in person in each local centre at the time of execution of the first experiment in order to verify the correct application of the trial rules. A report was compiled for each visit. A final visit to each centre was carried out after the end of the experiments to clear the pending queries and collect as much as possible data. On–site visits checked inclusion and exclusion criteria, correspondence between handwritten and web form imputed data, presence of violations of protocol, presence of sacrificed animals before the end of the study, envelopes conditions and participation to the centrally coordinated meetings. A specific data monitoring plan was created before the beginning of the study. No external auditing was planned.

Access to data

During the course of the trial, the database was curated by a subunit based at the Istituto di Ricerche Farmacologiche Mario Negri IRCCS, and the access was available only for input and verification. On completion of the trial, a complete cleaned dataset was prepared, together with its related dictionaries. This dataset was made available on the publication of the scientific papers using online free data repository like Fig.share, under the CC BY 4.0 license.

Ethics approval

Approved by the Animal Welfare Regulatory Body (OPBA) of the University of Milano Bicocca (01/2020, FB7CC.43), under project license from the Italian Ministry of Health.

Preregistration

Registered at https://preclinicaltrials.eu, identifier PCTE0000177.

Published on 6 January 2020: Tettamanti M., et al., Multicentre translational Trial of Remote Ischaemic Conditioning in Acute Ischaemic Stroke (TRICS): protocol of multicentre, parallel group, randomised, preclinical trial in female and male rat and mouse from the Italian Stroke Organization (ISO) Basic Science network. (2020); BMJ Open Science. 4(1):e100063. DOI: 10.1136/bmjos-2020-100063. PMID: 35047692.

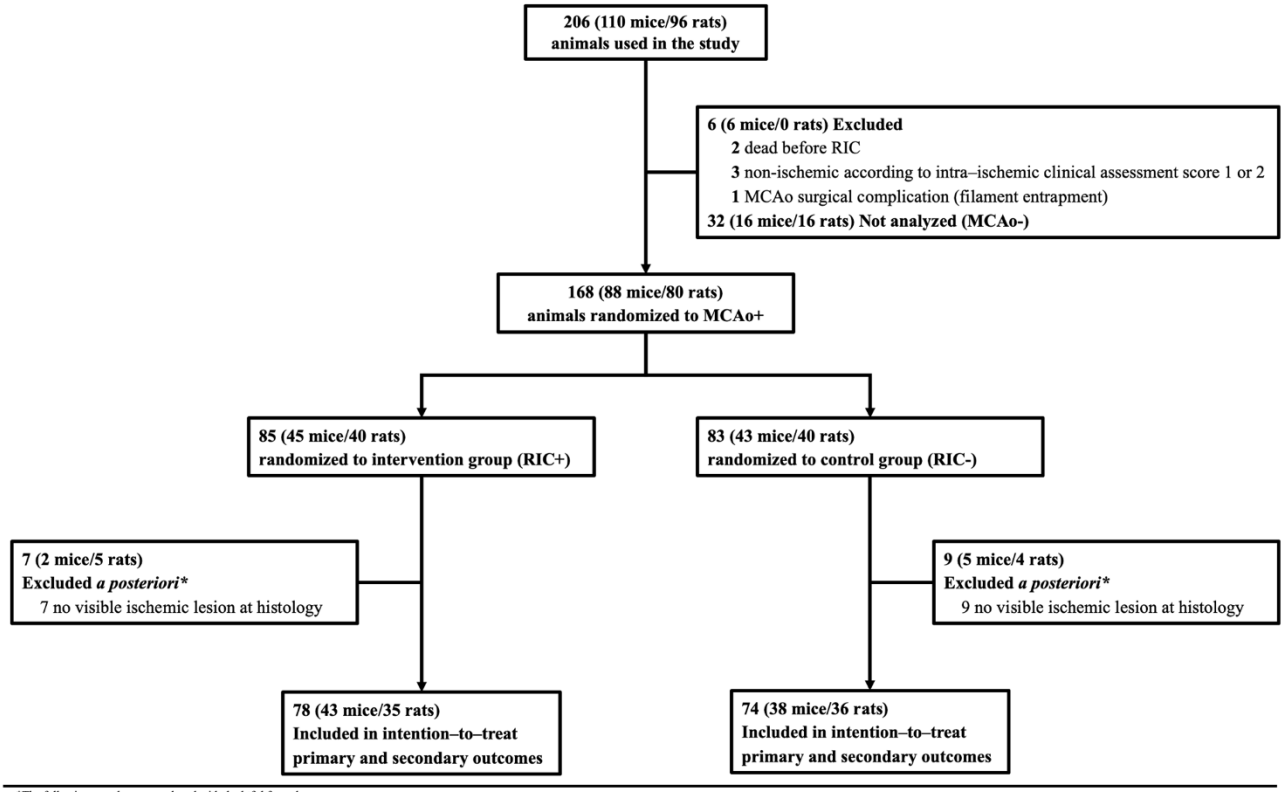
RESULTS

According to the protocol paper (94), once we have reached the threshold of ICC >0.60 De Simoni neuroscore assessment among all the raters, we have started the experimental phase. This preliminary phase, or harmonization phase, was directed by centres 1 and 2; however, these latter results were not analyzed in this thesis.

TRICS–Basic trial flow diagram

A total of n=206 animals were used in the experimental phase. As described in **Tab.2**, once exclusion criteria were applied we obtain a sample size of n=152 animals (n=81 mice; n=71 rats).

Table 2 TRICS–Basic experimental cohort flowchart



*The following sample were analyzed with doubtful formula.
n=14 samples were not used in the analyses (n=3 samples was damaged; n=11 samples were not collected)

Baseline characteristics

In order to evaluate the consistency among the experimental groups, we recorded all the baseline characteristics during the surgery. Data from n=152 animals subjected to MCAo for 60min in mice and 100min in rats were reported in **Tab.3** divided by species, sex and treatment allocation.

Table 3 Baselines characteristics of the study population

		Mice			Rats		
	Treatment group	Intervention group (RIC+) median (IQR) or n (%)	Control group (RIC-) median (IQR) or n (%)	p value	Intervention group (RIC+) median (IQR) or n (%)	Control group (RIC-) median (IQR) or n (%)	p value
	number	43	38		35	36	
	unit						
Male/female sex*	n (%)	19/24	18/20	0.774	16/19	19/17	0.552
Weight 24 hours before surgery*	gr	23 (22,2; 24) [43]	22,85 (22,1; 24) [38]	0.959	247 (230; 272) [35]	255,5 (229; 268) [36]	0.995
Weight at the time of surgery*	gr	23,1 (22,2; 24) [43]	23 (22,4; 24) [38]	0.883	250 (230; 284) [35]	261 (230; 275,5) [36]	0.778
Isoflurane exposure time during MCAO* surgery*	min	18 (17; 23) [43]	18 (13; 22) [38]	0.812	34 (10; 80) [35]	46,5 (27,5; 93,5) [36]	0.394
body temperature during MCAO surgery (min)*	°C	36 (35,9; 36,4) [42]	36,25 (36; 36,6) [38]	0.307	36,8 (36,4; 37,2) [35]	36,8 (36,3; 37,1) [36]	0.751
body temperature during MCAO surgery (max)*	°C	37,1 (36,7; 37,4) [42]	37,2 (36,8; 37,5) [38]	0.373	37,6 (37,1; 37,7) [35]	37,2 (37,1; 37,5) [36]	0.027
Breath rate at baseline*	breaths/min	212 (188; 254) [42]	204 (76; 248) [37]	0.572	76 (68; 104) [35]	76 (68; 100) [35]	0.809
Breath rate 15 min after starting anesthesia*	breaths/min	108 (78; 120) [42]	112 (72; 132) [37]	0.844	60 (56; 72) [35]	60 (50; 72) [36]	0.707
Breath rate 15 min after withdrawing anesthesia*	breaths/min	158 (106; 188) [42]	164 (74; 182) [37]	0.961	76 (64; 88) [35]	70 (63; 86) [36]	0.416
Isoflurane exposure time during RIC surgery*	min	27 (24; 32) [41]	25 (21; 31) [29]	0.413	48 (30; 55) [34]	30 (27; 56) [27]	0.301
Body temperature during RIC surgery (min)*	°C	36,9 (36,4; 37,4) [41]	37,2 (36,7; 37,4) [28]	0.615	37,45 (37,2; 37,8) [34]	37,4 (37,2; 37,8) [26]	0.952
Body temperature during RIC surgery (max)*	°C	36,2 (35,6; 36,4) [41]	36,15 (35,5; 36,5) [28]	0.170	37 (36,2; 37,3) [34]	37 (36,1; 37,2) [26]	0.487
Surgical complications during RIC*	n (%)	1 (2%)	1 (3%)	0.845	0	0	-
Post-operative analgesia*	n (%)	4 (9%)	10 (26%)	0.043	0	0	-
Death < 24h from surgery*	n (%)	7 (16%)	5 (13%)	0.693	2	0	0.146

*chi-squared for categorical variables, Wilcoxon-Mann-Whitney for numerical variables

Health monitoring and safety assessment

We monitored the animal's welfare at 24 and 48 hours after the surgery day. As reported in **Tab.4**, it was found that mice had higher risk of severe distress than rats (absolute difference +50%: 63% male mice RIC+ vs 13% male rats RIC+; absolute difference +41%: 46% female mice RIC+ vs 5% female rats RIC+). Moreover, we observed that the treatment positive affected female mice RIC+ and male rats RIC+ by an absolute difference of -14% and -24% respectively, as compared to RIC- groups.

Table 4 Health report and safety assessment

		Mice					
	Sex	Males			Females		
	Treatment group	Intervention group (RIC+) median (IQR) or n (%)	Control group (RIC-) median (IQR) or n (%)	p value	Intervention group (RIC+) median (IQR) or n (%)	Control group (RIC-) median (IQR) or n (%)	p value
	number*	17	16		16	19	
	unit						
Weight at 24 hours**	gr	21.6 (20; 22.2)	20.9 (19.7; 22.6)	0.641	19.9 (19; 22)	20.5 (19.1; 21)	0.817
Weight at 48 hours	gr	19.7 (17.3; 21.2)	18.9 (17.4; 21.1)	0.836	18 (17.2; 21.8)	19.9 (19; 22)	0.669
Weight loss less 10% < 48h	n (%)	5 (31%)	5 (31%)	0.592	6 (32%)	6 (35%)	0.229
Weight loss exceeding 10% < 48 hours	n (%)	11 (69%)	11 (69%)		13 (68%)	11 (65%)	
Weight loss exceeding 20% < 48 hours	n (%)	11 (69%)	10 (63%)		10 (53%)	11 (65%)	
No distress < 48h**	n(%)	0	0	0.117	0	0	0.515
Low distress <48 hours**	n (%)	3 (16%)	0		2 (8%)	0	
Moderate distress <48 hours**	n (%)	1 (5%)	5 (28%)		6 (25%)	5 (25%)	
Severe distress <48 hours**	n (%)	12 (63%)	11 (61%)		11(46%)	12 (60%)	
Death <48 hours**	n (%)	3 (16%)	2 (11%)		5 (21%)	3 (15%)	
Analgesic drug administered**	n (%)	6 (32%)	11 (61%)	0.072	7 (29%)	13 (65%)	0.017
		Rats					
	Sex	Males			Females		
	Treatment group	Intervention group (RIC+) median (IQR) or n (%)	Control group (RIC-) median (IQR) or n (%)	p value	Intervention group (RIC+) median (IQR) or n (%)	Control group (RIC-) median (IQR) or n (%)	p value
	number*	19	17		17	17	
	unit						
Weight at 24 hours**	gr	242 (223; 252)	238 (220; 245)	0.378	223 (212; 240)	220 (210; 243)	0.947
Weight at 48 hours	gr	237 (222; 245)	230 (205; 245)	0.174	220 (210; 238)	210 (200; 235)	0.593
Weight loss less 10% < 48h	n (%)	10 (63%)	8 (42%)	0.470	8 (47%)	7 (41%)	0.525
Weight loss exceeding 10% < 48 hours	n (%)	6 (37%)	11 (58%)		9 (53%)	10 (59%)	
Weight loss exceeding 20% < 48 hours	n (%)	2 (13%)	3 (16%)		1 (6%)	0	
No distress < 48 hours**	n (%)	1 (6%)	0	0.307	0	0	0.595
Low distress <48 hours**	gr	9 (56%)	8 (42%)		7 (37%)	7 (41%)	
Moderate distress <48 hours**	n (%)	4 (25%)	4 (21%)		9 (47%)	9 (53%)	
Severe distress <48 hours**	gr	2 (13%)	7 (37%)		1 (5%)	1 (6%)	
Death <48 hours**	n (%)	0	0		2 (11%)	0	
Analgesic drug administered**	n (%)	0	0	-	0	0	-

*excluding animals dead within 24h
**all animals

Primary outcome – Functional outcome

We considered a good functional outcome a scored value lower or equal to 20 out of 56 points; therefore, the reported differences are related to the chance of the animal to have a good outcome, whereas positive differences imply lower score at neurobehavioral test, then a better performance at functional outcome.

If we analysed the primary outcome as dichotomous variable, we found a major prevalence of animals with a good functional outcome among RIC+, as the treated group, in both species (absolute difference +20% in mice, p=0.068; absolute difference +18% in rats, p=0.122) compared to RIC-, as untreated group (**Tab.5**) **(Fig.4A and B)**. Despite these absolute differences between groups, no significant improvement was recorded. In addition, we analysed mice and rats as a combined group (total of n=152 animals), we observed a significant absolute difference of +19% (p=0.021) in treated group compared to untreated one.

If we analysed the neuroscore as a continuous variable, we found a major prevalence of animals with a significant reduction in the functional deficit among RIC+, in both species (score reduction -4.15, 20.1±1.4 vs 24.3±1.2, p=0.027, in mice; score reduction -4.66, 19.3±1.3 vs 24.0±1.5, p=0.022, in rats) compared to RIC-, as untreated group (**Tab.5**) **(Fig.4C and D)**. In addition, if we analysed mice and rats as a combined group (n=137 animals, dead animals excluded), we observed a significant reduction score of -4.41 (19.7±0.9 vs 24.1±1.0, p=0.001) in treated group compared to untreated one.

Table 5 Primary efficacy outcomes

Neuroscore as a dichotomuous variable; good outcome at neuroscore ≤20	unit	Intervention group (RIC+)	Control group (RIC-)	Odds Ratio	95% IC	p value
Mouse (N=81)	n (%)	21/43 (49%)	11/38 (29%)	2.34	0.94; 5.82	0.068
Rat (N=71)	n (%)	21/35 (60%)	15/36 (42%)	2.1	0.82; 5.37	0.122
combined Mouse + Rat (N=152)	n (%)	42/78 (54%)	26/74 (35%)	2.15	1.12; 4.13	**0.021**
Neuroscore as a continuous variable; neuroscore from 0 to 56 points	**unit**	**Intervention group (RIC+) mean (SE)**	**Control group (RIC-) mean (SE)**	**Mean difference**	**95% CI**	**p value**
Mouse (N=67)	score	20.1 (1.4)	24.3 (1.2)	-4.15	-7.82; -0.49	**0.027**
Rat (N=69)	score	19.3 (1.3)	24.0 (1.5)	-4.66	-8.64; -0.68	**0.022**
combined Mouse + Rat (N=136)	score	19.7 (0.9)	24.1 (1.0)	-4.41	-7.08; -1.74	**0.001**

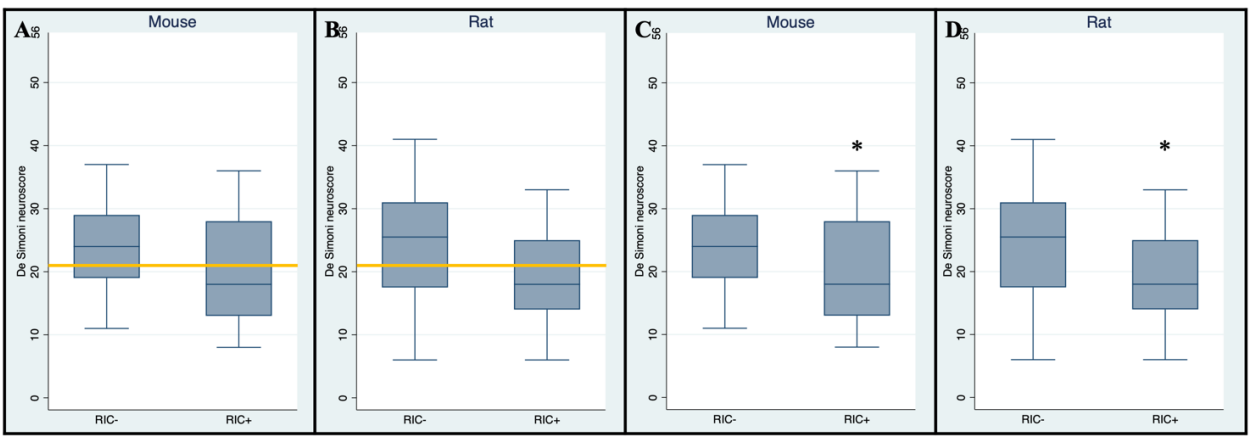


Fig.4 *Quantification of De Simoni neuroscore analysed as a dichotomous variable (good outcome at score ≤20) in mice* ***(A)*** *and rats* ***(B)****. Data shown the odd ratio and the 95% CI. The threshold of 21 point is marked with a yellow line. Quantification of De Simoni neuroscore analysed as a continuous variable (total score range from 0 to 56) in mice* ***(C)*** *and rats* ***(D)****. Data shown the mean differences and the 95% CI.*****(p<0.05)***

Secondary outcome – Histological analyses

No significant differences in infarct size at 48 hours were reported analysing mice treated with RIC+ compared to RIC- (absolute reduction 4.6 mm^3, 27.8±2.2 vs 32.1±1.7 mm^3, respectively; p=0.132) **(Tab.6) (Fig.5A)**. On the contrary, in rats we observed a modest reduction in infarct size in treated group compared to the untreated one (absolute reduction 26.6 mm^3, 85.0±10.9 vs 111.6±12.0 mm^3, respectively; p=0.107) **(Fig. 5D)**. In addition, if we analysed mice and rats as a combined group (total of n=136 animals), we obtained that RIC+ animals had a mean reduction of 0.38 (p=0.013) compared to RIC-, in terms of standard deviation (SD).

Table 6 Secondary efficacy outcome

Infarct size	unit	Intervention group (RIC+) mean (SE)	Control group (RIC-) mean (SE)	Mean difference	95% CI	p value
Mouse (N=67)	**mm3**	27.8 (2.2)	32.1 (1.7)	-4.3	-9.8; 1.3	0.132
Rat (N=69)	**mm3**	85.0 (10.9)	111.6 (12.0)	-26.6	-59.1; 6.0	0.107
combined Mouse + Rat (N=136)	**score**	standardized 0–1	standardized 0–1	-0.38	-0.71; -0.05	**0.013**

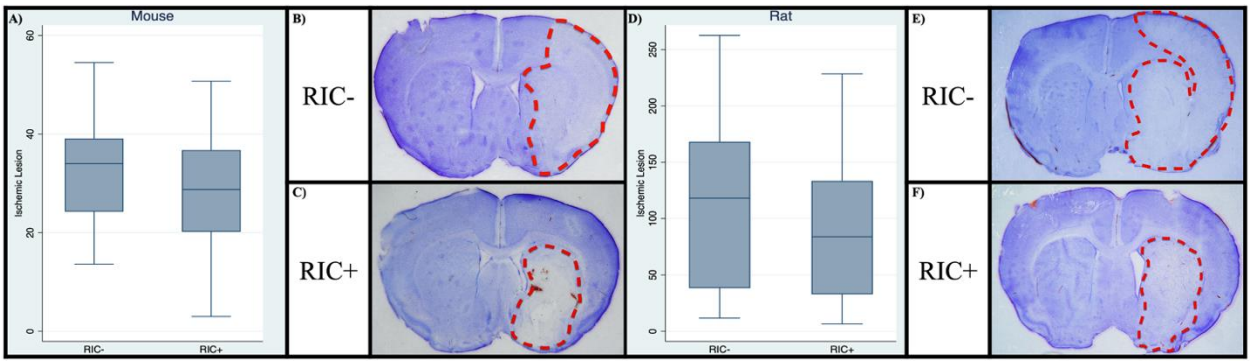


Fig.5 *Histological analysis based on Cresyl Violet staining.* ***A and D)*** *Quantification of ischemic lesion at 48 hours in mice and rats treated with remote ischemic conditioning (RIC) compared to untreated one.* ***B and C)*** *Representative brain slices of a RIC- and RIC+ treated mice showing ischemic lesion at 48 hours.* ***E and F)*** *Representative brain slices of a RIC- and RIC+ treated rats showing ischemic lesion at 48 hours. RIC treatment was applied for 10min in mice and 20min in rats.*

Correlation between ischemic lesion and continuous neuroscore

Linear regression between continuous functional outcome and ischemic lesion, was calculated for n=67 mice (RIC+ n=34; RIC- n=38; Pearson's r = 0.38; p=0.001) **(Fig.6A)** and n=69 rats (RIC+ n=33; RIC- n=36; Pearson's r = 0.60; p<0.001) **(Fig.6B)**.

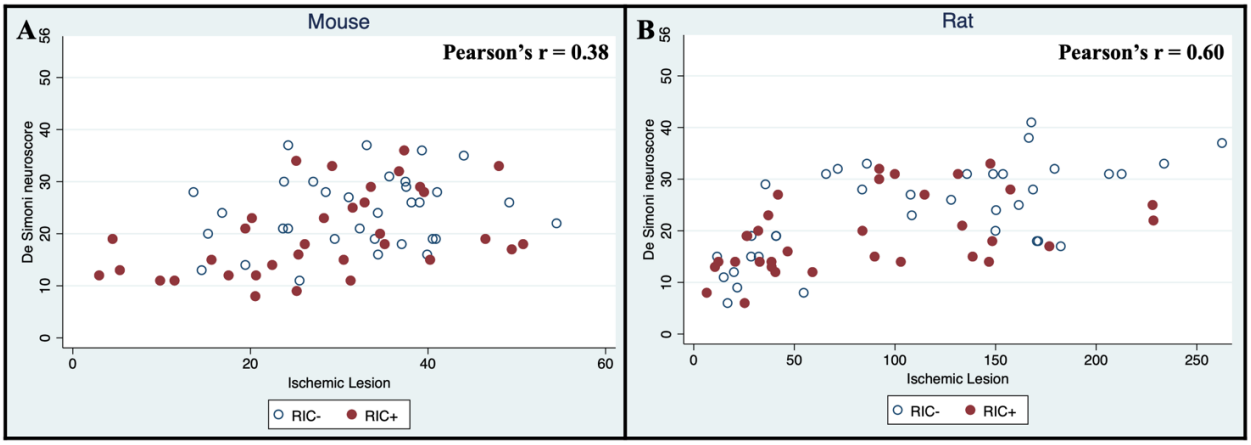


Fig.6 Pearson correlation analyses in mice **(A)** and rats (**B)** between De Simoni neuroscore (as a continuous variable) and infarct volume (express in mm^3). The respective correlation's coefficient (r) is shown for each association.

Mortality associated to RIC treatment

Among the total experimental cohort of animals used in the study (n=206 animals: n=110 mice; n=96 rats), n=13 mice (MCAo+/RIC+ n=8; MCAo+/RIC- =5) and n=2 rats (MCAo+/RIC+ n=2) died before 48h and received the worst value at functional outcome (score=56) according to protocol paper (94). Any animals of the external control group (n=16 MCAo-/RIC+) died cause of the treatment.

DISCUSSION

In the present study we investigated the efficacy and the possible translational role of remote ischemic conditioning in animal models subjected to acute ischemic stroke (AIS). Remote ischemic conditioning (RIC) represents a novel potential neuroprotective strategy in stroke treatment. This procedure is able to trigger the activation of endogenous tolerance mechanisms by the delivery of a sublethal ischemic injury in the limbs, leading to a protective systemic response (48,49). Therefore, RIC represents the ideal candidate for a multicentre pRCT with a rigorous study design, since previous results from single centre trials supported its efficacy, despite the mechanism of action is still unknown (50–86). Several clinical trials in phase II or III provided the safe and feasible profile of the treatment, but still limited efficacy resulted when the treatment was applied after stroke onset (87–93). Usually, clinical trials are conducted following detailed and pre–registered protocols, which describe the study design, the randomization procedure, the outcomes, and the estimated sample size (102). However, a relevant number of pRCTs have shown some weaknesses, especially as regards the study design phase and the execution of the experimental procedures (103–105). When an *in vivo* preclinical trial, especially a multicentre one, aims to translate a promising therapeutic approach to patients' application, it should be as similar as possible to human clinical trials (106,107). Indeed, preclinical researchers are expected to implement the disposable guidelines to reach the standardization and to avoid the common limits of pRCT –the lack of blinding randomization and treatment application, the absence of a priori inclusion/exclusion criteria, the loss of information about the power test study, the animal details and the definition of a quality check strategy– (95,96,108,109).

From this point of view, the Italian Stroke Organization (ISO) Basic Science set up a nationwide collaboration to perform multicentre translational research projects on highly encouraging non–pharmacological treatment in experimental ischemic stroke, to overcome the limitations of single–centre trials. The "Multicentre translational Trial of Remote Ischaemic Conditioning in Acute Ischaemic Stroke (TRICS)" project is composed by two different studies, TRICS–Basic, the concluded preclinical trial, and TRICS–9, the ongoing phase II pilot clinical trial. TRICS–9 is a pilot clinical trial of RIC in patients with acute ischemic stroke who are not eligible for recanalization therapies, such as intravenous recombinant tissue plasminogen activator and/or endovascular mechanical thrombectomy, in selected ISO–associated Stroke Units. Notably, this clinical trial is considered a part of a fully integrated translational research program together with the preclinical one. When TRICS–Basic project was designed, we planned to implement practices as similar as those applied in clinical trials. As a multi–centre trial, an important topic is the grade of agreement among the researchers that evaluate sensimotor deficits, here referred as inter–class correlation (ICC). ICC

can fluctuate based on the individuals' different expertise with the specific test assessments (110–114). This is the reason why we decided to implement a training phase for data collectors (raters) before starting with the experimental one, in order to reduce the variability in the way the raters interpreted and assessed the neurobehavioral data. Although the perfect agreement is difficult to achieve, a substantial agreement (ICC ≥0.60) was required before starting animal randomization, considering the translational aim of multicentre preclinical trials. As described in the protocol paper (94), we set the threshold of ICC ≥0.60 *a priori* in order to standardize the neurological evaluation.

Once we reached the preliminary target of ICC >0.60, we started the experimental phase. The safety of the treatment was performed by the monitoring of heath parameters 48 hours after the surgery. Looking at the severe distress parameter in **Tab.4**, our results showed that RIC + partially prevent the suffering in female mice and in male rats. In general, mice were subjected to a worse distress than rats, suggesting that MCAo surgery procedure has a higher impact on mice than on rats. Regarding gender variation, no difference was reported in the mouse model, in which severe distress condition was mainly represented; on the contrary, male rats had less distress than female ones. From these results, it emerged that these differences could be reconducted to the application of RIC treatment in two different animal models, characterized either from a gender point of view.

The functional outcome of this study was assessed by De Simoni composite neuroscore analyzed as a dichotomous and a continuous variable. RIC treatment improved neuroscore in both mice and rats, which confirmed the positive effect of RIC treatment on ischemic brain injury. However, this effect resulted to be significant in single species only if neuroscore was considered as a continuous variable, while a not significant effect was found when neuroscore was dichotomized. Notably, dichotomized analyses of neuroscore showed a significant benefit of RIC when both species were combined, reaching a larger sample size. Overall, the TRICS–Basic multicenter trial showed a small–to–moderate beneficial effect of RIC treatment of both rats and mice, which is visible only if the most sensitive analysis is used (continuous versus dichotomous; combined population versus single species). On the other hand, our findings disagree with previous single-center experimental studies showing a large therapeutic effect of RIC.

The results obtained for infarct volume are consistent with the primary outcome. In both mice and rats, RIC treatment induced only a small reduction infarct size. This treatment effect was not significant as a single species level but reached statistical significance when both species were combined in a larger sample. Notably, correlation between De Simoni neuroscore and infarct size proved to be moderate in both mice and rats. This finding is translationally important and fundamentally mirrors the human condition when infarct size is only partly predictive of functional neurological outcome.

As previously demonstrated by Cheng X. et al. (115) in mice, RIC treatment is a safe treatment that induce a reduction in infarct size and improve the neurological outcome. Moreover, they observed an inhibitory effect on STAT3 phosphorylation, generated by the treatment. On the same line, in a rat model of post conditioning, Pignataro G. et al. (73), studied a new mechanism by which this therapy can induce a neuroprotective effect after 100 min of MCA occlusion. They demonstrated that p–ERK and NO contribute to the neuroprotection induced by RIC; in fact, it also induced a significant reduction in infarct volume of the treated group compared to the control ones. In this study, it was also underlined the long–term effect of RIC, which can be found even after 7 days from the reperfusion. These findings have a major impact at translational level, since the treatment application could be carried out more times later, to improve its effectiveness. Despite several preclinical studies proved the efficacy and safety of remote ischemic conditioning, in clinical trials there are conflicting evidence on its efficacy; positive rather than neutral effects were reported, without affecting the safety and feasibility profile. In RICAMIS clinical trial **(NCT03740971)** (91), the authors analysed a large number of patients (more n=1800) with acute moderate ischemic stroke, in which RIC treatment consisted in 5 cycles of cuff inflation and deflation for 5 minutes to the bilateral upper limbs to 200 mmHg. Comparing the standard usual care to the association between RIC and guideline–based treatment, a significant effect in neurological outcome at 90 days was demonstrated. On the other hand, in REPOST clinical trial **(NTR6880)** (93) no significant improvement was found in infarct size or clinical outcome in acute ischemic stroke patients, to which they applied 4 cycles of 5 min inflation to the cuff around the upper arm (reaching the pressure of 200 mmHg).

Major advantages of our study were the large number of animals used, compared to previous preclinical studies on this topic, and the rigorous methodology. Similarly to clinical trials, also in our pre-clinical study, it would be useful to reach a larger sample size, which would give more significant results.

A major limitation of our study is that outcome assessment was limited to 48 hours after reperfusion, since a longer follow–up may have revealed a higher potential of this treatment. A second important limitation is the application of RIC as a single treatment, since we can’t exclude that a repeated application could have resulted in larger benefit.

CONCLUSION

TRICS–Basic resulted to be an innovative, multicentres, multispecies, randomized and double–blinded preclinical trial. As previous preclinical and clinical trials demonstrated, our findings proved the safety and feasibility profile of RIC treatment after the reperfusion phase. Despite the lack of a significant effect observed in both species at primary and secondary outcome level, we demonstrated the efficacy of RIC with the combined analysis of mice and rats. This suggest that our findings support the neuroprotective effect of the treatment. However, a larger population is required to reach significant results also in the single species analyses.

To conclude, this non–pharmacological treatment could be useful to those patients with particular drug restriction or even as a combination therapy together with standard medical care.

Further studies will be also necessary to investigate the real molecular event behind the neuroprotection induced by remote ischemic conditioning.

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