Lactate infusion as a neuroprotective agent in a rat focal cerebral infarct model

A dissertation submitted to TN Dr. MGR Medical University in part fulfillment of the MCh Neurosurgery exam (Part III) Feb 2009

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DEPARTMENT OF NEUROLOGICAL SCIENCES
CHRISTIAN MEDICAL COLLEGE
VELLORE

CERTIFICATE

This is to certify that the dissertation titled "Lactate infusion as a neuroprotective agent in a rat focal cerebral infarct model" is the bonafide original work of Dr. Hrishikesh Sarkar submitted in partial fulfillment of the rules and regulations, for Branch-II M.Ch. Neurosurgery, Part-III examination of the Tamil Nadu Dr. M.G.R. Medical University to be held in February 2009.

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CONTENTS

AIM

To evaluate the role of lactate as a neuroprotective agent in a rat model of focal cerebral infarction.

OBJECTIVES

1. To develop a rat model of focal cerebral infarction by temporary middle cerebral artery occlusion (MCAO).
2. To assess the efficacy of lactate in reduction of infarct volume.

INTRODUCTION

Cerebral ischemia is a major pathophysiological factor contributing to neural injury in numerous clinical conditions (Gladden 2004) such as traumatic brain injury, vasospasm following subarachnoid hemorrhage and stroke syndromes. Lactate (La) has traditionally been thought of as a 'dead end' product of anaerobic metabolism, and glucose stands unchallenged as the principle metabolic substrate of the mature brain (Chih et al. 2001). However, the recently proposed astrocyte-neuron lactate shuttle hypothesis (ANLSH) assigns a major metabolic role to brain-derived lactate. According to the ANLSH lactate is produced in an activity dependent manner and is then utilized by the active neurons to produce ATP (Brooks 1985, Pellerin & Magistretti, 1994, 2003, 2008; Magistretti & Pellerin, 1999; Magistretti et al. 1999; Bouzier-Sore et al. 2002; Pellerin, 2003). The active brain may also use lactate when glucose is temporarily unavailable. An animal model of focal cerebral ischemia was developed with indigenously developed modifications of the previously described techniques and then a study was carried out on this model to assess the effect of lactate infusion on infarct volume.

LITERATURE REVIEW

In 1950, von Muralt distinguished four different eras in the understanding of muscle chemistry: pre-lactic acid, lactic acid, phosphorylation, and myosin. The pre-lactic acid era began in 1808 with Berzelius's discovery of an elevated concentration of lactate in ‘the muscles of hunted stags’ (Brooks & Gladden, 2003).

Fletcher & Hopkins (1907) ushered in the lactic acid era during which A. V. Hill's studies suggested that the lactic acid (La) was the immediate energy donor for muscle contractions.

In 1920, Meyerhof demonstrated that glycogen was the precursor of La, and the 1930s marked the beginning of the phosphorylation period of muscle chemistry.

In 1939, the myosin period began with the finding that the enzyme responsible for ATP hydrolysis was associated with the muscle protein, myosin (von Muralt, 1950)

By the early 1940s, the full Embden-Meyerhof (glycolytic) pathway had also been elaborated. If we restrict our considerations to La and its metabolism, we might term the period from the 1930s to approximately the early 1970s the dead-end waste product era, when La was largely considered to be a

dead-end metabolite of glycolysis resulting from muscle hypoxia (Wasserman, 1984).

Lactic acid was also believed to be the primary cause of the slow component of the O2 debt (Margaria et al. 1933) and a major cause of muscle fatigue (Hermansen, 1981).

Since the early 1970s, a ‘lactate revolution’ has occurred, and we are in the midst of the lactate shuttle era that began with the introduction of the lactate shuttle hypothesis by George Brooks (1985). The evidence seems to suggest that lactate is an important intermediary in numerous metabolic processes, a particularly mobile fuel for aerobic metabolism, and perhaps a mediator of redox state among various compartments both within and between cells.

Brain energy pathway - Conventional View

Any increase in nervous system activity increases the energy requirements of the neurons, which is conventionally thought to be provided by glucose oxidation (Chih et al. 2001, Figure 1).

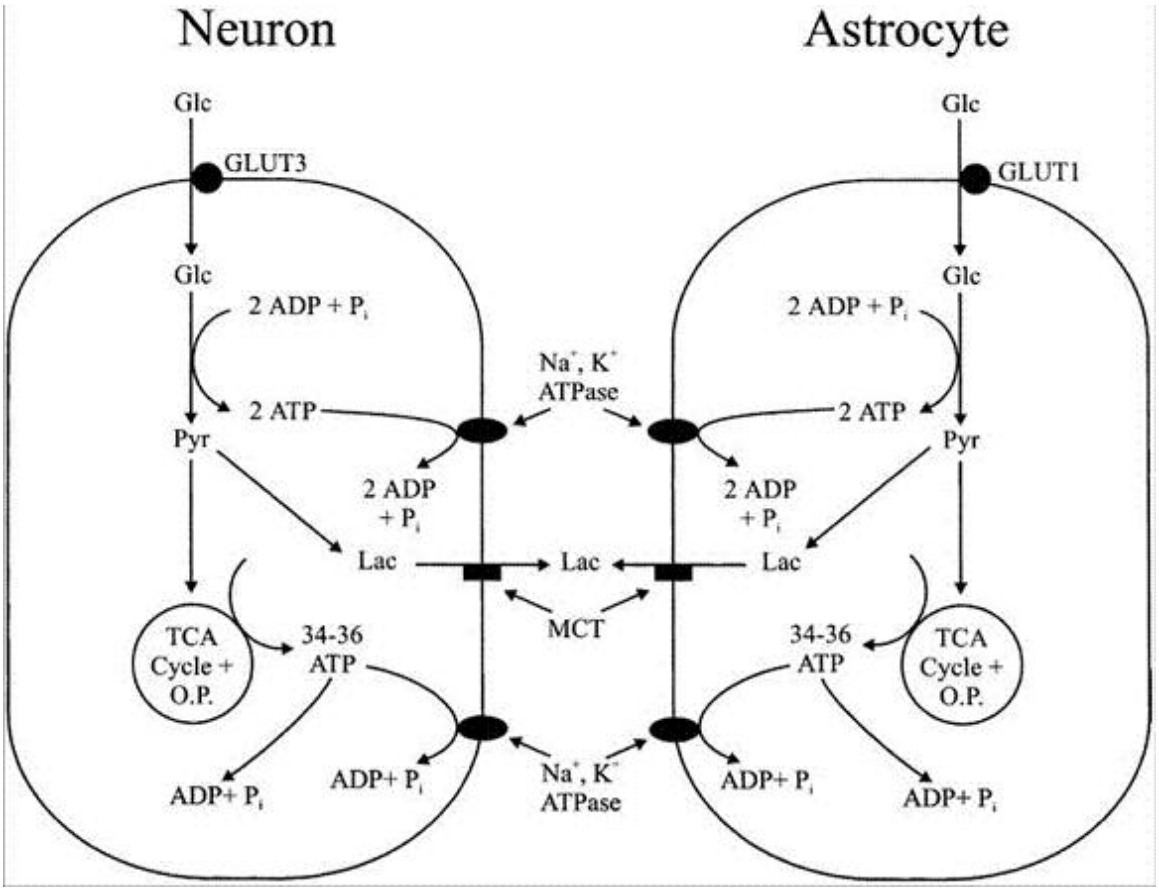

Figure 1: Conventional scheme of neuronal metabolism.

(O.P. - Oxidative Phosphorylation; Glc – Glucose; Pyr – Pyruvate; Lac – Lactate; MCT – Monocarboxylate Transporters)

Glucose use increases in both neurons and astrocytes during activity. Heightened sodium-potassium ATPase activity in both cell types during neural activity increases ATP consumption and stimulates glucose use by activating glycolytic enzymes. Glucose is transported into neurons and astrocytes via the glucose transporters GLUT-3 and GLUT-1 respectively.

Glucose is metabolized to pyruvate (hereafter designated as glycolysis) to produce two ATPs in either cell type. Pyruvate is either converted to lactate (anaerobic glycolysis by LDH) or taken up by the mitochondria where it enters the TCA cycle and is metabolized via oxidative metabolism. Lactate produced by both neurons and astrocytes is transported to the extracellular space via MCT. The increased energy demands resulting from neural activity are met by ATP generated from both glycolysis and oxidative metabolism in this model. The conventional hypothesis does not ascribe any particular fraction of glucose metabolism to either the aerobic or anaerobic pathways.

Astrocyte Lactate Shuttle Hypothesis

Pellerin & Magistretti (1994) challenged this conventional scheme of nervous system energetics with their introduction of the astrocyte–neuron lactate shuttle hypothesis (ANLSH, figure 2). In this model (Pellerin & Magistretti, 1994, 2003; Magistretti & Pellerin, 1999; Magistretti et al. 1999; Bouzier-Sore et al. 2002; Pellerin, 2003), glutamate that is released as a neurotransmitter from the neurons is primarily taken up into astrocytes via a transporter that carries one glutamate, three Na+, and one H+ inward while one K+ is moved out of the cell (Attwell, 2000).

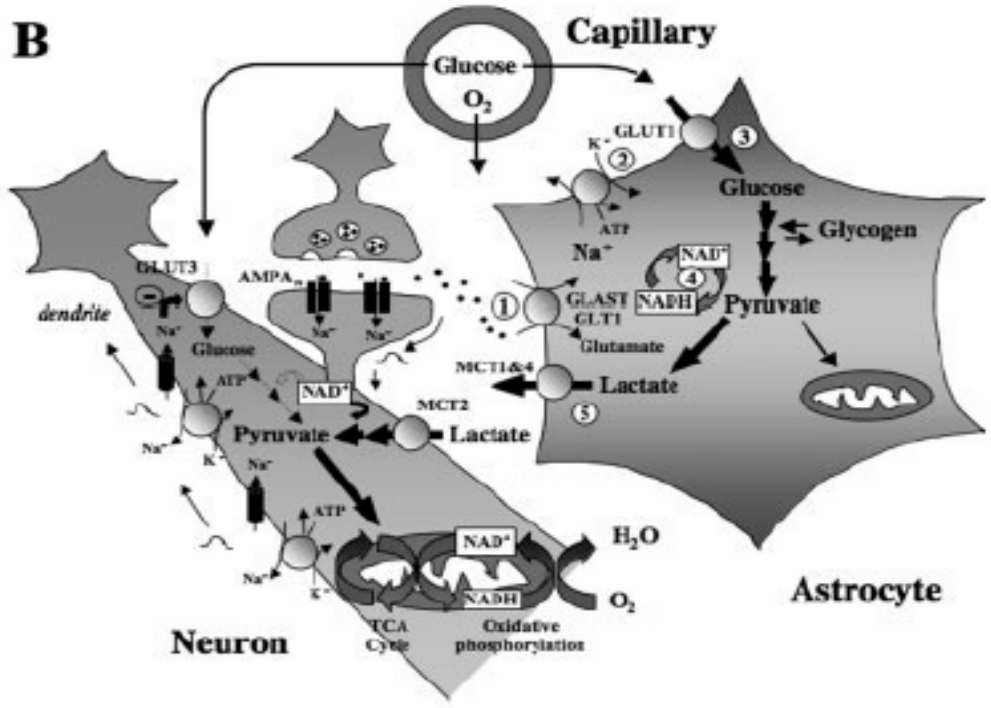

Figure 2: Putative astrocyte neuron lactate shuttle hypothesis (Gluc - glucose; Gly - glycogen; Pyr - pyruvate; La - lactate; Glu - glutamate; Gln - glutamine; GS - glutamine synthetase; LDH1 and LDH5 - specific forms of lactate dehydrogenase in neurons (LDH1) and astrocytes (LDH5); ECF - extracellular fluid; GLT-1 and GLAST - glutamate transporters; 2: glia-specific Na+–K+ ATPase subunit)

This astrocytic transport activity then leads to Na+–K+-ATPase activation (perhaps via an increase in [Na+] to restore ionic balance, and to glutamine

synthesis from the glutamate that has been taken up. The energy costs of the ATPase pump and glutamine synthesis result in [ATP], [ADP], [Pi], and [AMP], which largely stimulate glycolysis in the astrocytes with resultant La production. Here, glycolytic enzymes may be compartmentalized with the ATPase pump and glutamine synthesis pathway, allowing preferential activation of the glycolytic energy system. La moves into the extracellular compartment along its concentration gradient via MCT1 transporters in the astrocyte plasma membrane. Next, extracellular space La rises, driving La into the neighbouring neurons via MCT2 transporters in the neuronal plasma membrane. Inside the neurons La, along with glucose, serves as an oxidative fuel for elevated neuronal energy metabolism that has been triggered by an activated Na+–K+-ATPase to restore ionic balance and resynthesis of glutamate from glutamine, largely derived from astrocytes. While glucose can be taken up by neurons via their GLUT3 transporters, larger amounts of glucose may be used by astrocytes, and taken up into astrocytes via their GLUT1 transporters. In fact, Loaiza et al. (2003) have reported that glutamate stimulates glucose transport into cultured hippocampal astrocytes more rapidly than any stimulation of mammalian glucose transport yet known. In short, in the ANLSH, much of the fuel for increased energy demands of neurons is supplied by La from surrounding astrocytes. As a

result, metabolism of the astrocytes is largely glycolytic while that of the neurons is largely oxidative. The ANLSH has also been proposed for other support cell–neuron/receptor energetic interactions such as those between Schwann cells and peripheral neurons (Véga et al. 1998), and between Müller glial cells and photoreceptors (Poitry-Yamate et al. 1999).

The implications of the ANLSH are extremely far-reaching. For example, apart from its inferences concerning basic mechanisms of nervous system metabolism, the ANLSH offers intriguing explanations for functional brain imaging (Magistretti & Pellerin, 1999; Magistretti et al. 1999). Local brain activity is monitored by visualization of changes in blood flow, glucose usage, and oxygen consumption via positron emission tomography (PET), changes in blood oxygenation via functional magnetic resonance imaging (fMRI), and spatio-temporal patterns of metabolic intermediates such as glucose and La via magnetic resonance spectroscopy (MRS). Magistretti & Pellerin (1999) argue that the ANLSH 'provides a cellular and molecular basis for functional brain imaging techniques'. It is possible that current functional neuroimaging is more a reflection of astrocyte function rather than neuronal function (Meeks & Mennerick, 2003).

Overall, the model suggests that glial cells account for about 6.5% (two ATP from substrate phosphorylation in the glycolytic conversion of one glucose

to two La) of nervous system activity while neuronal energy expenditure accounts for the other 93.5% (29 ATP from oxidation of two La) (Salway, 1999). For the glial cells, it has been proposed that one glycolytic ATP is used to extrude three Na+ ions via the Na+–K+-ATPase pump while the second ATP is spent to convert glutamate to glutamine (Magistretti & Pellerin, 1999). However, it seems unlikely that metabolism is so tightly coupled and compartmentalized as to allow such strict stoichiometry. A more probable scenario may be a mixture of the ANLSH with the conventional view. In this scheme, astrocytes would utilize at least some of their own glycolytic products in oxidative metabolism and neurons would utilize some La from astrocytes in addition to endogenous, neuronal glycolytic products (Mangia et al. 2003b).

<u>Key points of evidence in support of the ANLSH</u>

1. Nuclear magnetic resonance spectroscopy has provided evidence of La utilization as an energy substrate in brain tissue, specifically as a neuronal fuel (Hassel & Brathe, 2000; Qu et al. 2000)
2. There is a cell-specific expression of monocarboxylate transporters (MCTs) in the central nervous system. In cultured mouse cortex preparations, MCT1 and MCT1 RNA are found almost exclusively in

astrocytes while MCT2 and its RNA are exclusive to neurons. (Bröer et al. 1997; Debernardi et al. 2003). Adult rat brain cells show a similar pattern. As explained earlier MCT1 transports lactate out of a cell and MCT2 into the cell, making it likely that this differential expression of the two phenotypes of the transporter functions to move La from within the astrocytes into the neurons. MCT1 is also present in endothelial cells of the blood–brain barrier (Mac & Nalecz, 2003).

3. Specialized astrocytic processes are wrapped around synaptic contacts (Rohlmann & Wolff, 1996), and in most brain regions, the astrocytes : neuron ratio is 10: 1 (Bignami, 1991). It is possible that they play more that a mechanical role in neuronal support.

<u>The most salient criticisms of ANLSH</u>

1. There is no solid explanation as to why neural activity either *in situ* or *in vivo* should activate glial glycolysis but not neuronal glycolysis (Chih *et al.* 2001).
2. Neuronal activity has been reported to increase cytoplasmic pyruvate and NADH levels, making it unclear that increases in La are sufficient to drive the LDH reaction towards pyruvate and NAD^+ formation (see Chih *et al.* 2001).

3. Direct measurements of extracellular lactate and glucose in severe head injury in humans by microdialysis show increased lactate – glucose ratios result in worse outcome (Goodman JC et al, 1999).
4. Whereas the astrocyte–neuron lactate shuttle predicts an initial La overproduction, recent experiments using time-resolved proton magnetic resonance spectroscopy found a significant decrease in La 5 seconds after visual stimulation in humans (Mangia *et al.* 2003).

Lactate in Animal Behaviour and Clinical studies

Rice et al (2004) examined the usefulness of lactate administration in the attenuation of behavioral deficits following a moderate head injury in rats. A fluid percussion of 2.17atm was administered through the craniotomy onto the intact dura. In the experimental group (n=8), lactate infusion (100 mM@0.65ml/hour))was started 30 minutes after lateral fluid percussion injury and continued for three hours. Control group consisted of eight rats in whom 0.9% saline was infused at the same rate that of the experimental group. They chose 30 minutes as this is the time frame when a moderately brain injured individual may arrive in an emergency room and treatment could be initiated. The cognitive deficits were measured by Water Maze Test, and neurons in CA1 and CA3 region of the hippocampus were

counted. The lactate infused animals demonstrated significantly less deficits than saline infused injured animals. Neuronal loss was not seen in either the CA1 or CA3 areas. They concluded that the lactate therapy amplifies a naturally occurring mechanism in the acutely injured brain.

In another study (Glenn, 2003), a cohort of 49 brain-injured patients were subjected to 179 determinations of lactate metabolism by magnetic resonance spectroscopy over the first five days. It was shown that cerebral uptake occurred in 76% of them, and that patients with a favorable outcome showed a greater brain lactate uptake when compared with those who had an unfavorable outcome.

Our hypothesis is that the initial response of the brain to any injury is to have the astrocytes take up glucose and produce lactate for the neurons to use as an energy source. Exogenous supplements of lactate may enhance this response and minimize neuronal damage and synaptic disruption. This study assessed the effect of lactate infusion on the infarct volume following temporary intraluminal occlusion of MCA in adult male Wistar rats.

Laboratory model of focal cerebral infarction

Desirable animal models are those that replicate features of human cerebrovascular syndromes. Rat models of focal cerebral ischemia have

gained increasing acceptance in recent years owing to their relevance to the human clinical setting. MCAo (Middle cerebral artery occlusion) in the rat has been in use since 1975.

One widely used technique of MCAo involves cauterization of the MCA via a craniotomy (Backhauss 1992); this technique is invasive and does not permit reperfusion. In human ischemic stroke, however, recirculation occurs frequently after focal ischemia, particularly in the case of cerebral embolism. In the rat, mechanical clipping of the MCA (Yanamoto 1998) and photothrombotic occlusion (Markgraf 2003) of the vessel are in common use, but these techniques also involve a craniotomy.

In 1986, Koizumi et al reported a novel, relatively noninvasive method of achieving reversible MCAo by the use of an intraluminal suture. Subsequently, Zea Longa et al (1988) reported a variation of this method and stated that their technique reliably produced regional infarcts. Since then this method and its further modifications have been used extensively in stroke trials (Shimamura 2006, Zao 2008). This study has been conducted on a focal infarct model developed with modification of above mentioned technique.

MATERIAL AND METHODS

A total of 32 male adult Albino Wister rats weighing 250 – 350 grams were acquired from the animal facility house of the institute. 16 rats were allocated for standardization of the procedure another 16 rats were allocated for the main experiment. Financial assistance was obtained from FLUID grants committee and the necessary ethical clearance was obtained from the animal ethics committee, Christian Medical College, Vellore. All rats were fasted overnight and day night cycle was maintained.

Over view of the procedure

Anesthesia was induced with chloroform and maintained with an intra-peritoneal injection of ketamine sulphate in doses of 40 – 80 mg/kg. All rats underwent prophylactic tracheostomy. The femoral vein and the tail artery were cannulated with 24 G intravenous catheter to facilitate and blood sampling for sodium, glucose, lactate and arterial blood gases. Complete loss of righting reflex and loss of corneal reflex was taken as beginning of deep anaesthesia. At this moment, the tail artery was canulated with a heparin flushed PE 50 catheter for blood sampling. A rectal thermometer was used to monitor the temperature.

The color of the eyes, pinna, respiratory rate, temperature were the clinical variables noted for each rat at the end of procedure. Blood was also analyzed at the end of procedure for pH, pO2, Sodium, Lactate and Glucose levels. The duration of the entire procedure and approximate blood loss were also recorded. Once the procedure was standardized, 8 rats were to be assigned to the drug arm (lactate solution) and another eight to be given normal saline as control. The surgeon was blinded to the study groups. Lactate was used as 2ml solution of 60 mM sodium lactate where as 2 ml of 0.9% sodium chloride was used as control. Both these solutions were administered intraperitoneally over one and half hours at the rate of 20 units (0.5 ml) every 30 minutes from the time of induction of anesthesia.

All rats included in the experiment underwent a standard surgical procedure which involved occlusion of right MCA with insertion of 4-0 nylon suture through the right ECA (modified, Zea Longa 1990). This technique has been described in details below. Two hours following the procedure the rats were observed for neurological deficits and were graded by system proposed by Bederson et al. Rats were allowed to come out of anesthesia after completion of the surgery and food and water were given at libitum. As a means of assessing adequacy of occlusion a neurological score was assigned

to each animal 5 minutes before removing the occlusion and at the end of the 24 hours following the occlusion.

Figure 3: Operated rat with a left hind limb weakness. This severity of the neurological deficit was scored as 1.

<u>Neurological scoring (Bederson et al)</u>

0 - No deficit

1 – One Limb weakness

2 - Circling to affected side

3 - Partial paralysis on affected side

4 - No spontaneous motor activity

Rats with Score 1 – 3 were included in the subsequent part of the experiment.

The duration of occlusion was 120 minutes. Rats that did not demonstrate extremity weakness at least five minutes prior to reperfusion were excluded from the study. Reperfusion was performed under mild sedation. The suture was pulled back until a resistance was felt indicating that the tip had cleared the ACA-ICA lumen and then it was trimmed at the skin. Animals were allowed to survive for twenty four hours.

Twenty four hours following the reversible occlusion of right MCA these animals were euthanized with deep chloroform anesthesia. Heads were quickly decapitated and then processed for making brain slices and subsequent image analysis in a standardized way as described below.

A successful model was defined as rat showing 24 hours post surgical neurological deficit (Neurological grade 1-3) and a simultaneous documentation of infarct on TTC staining.

Preparation of suture material

1. 5.0 cm long segments of 4-0 ethilon were prepared.
2. To make the tip of the suture segment blunt; it was heated near a flame. (See figure 4)
3. 20 mm distal part of the suture material was coated with Dr. Mac's solution (commercially available preparation used for insulation of copper wires, contains epoxy resin 20% in ethyl alcohol) and dried in oven at 60 degree for one hour.
4. Visual inspection of the tip to make sure that the blunting was complete. Any sharp segment was discarded.
5. At a distance of 17 mm from the rounded tip a permanent mark was made with print eraser so as to limit excessive insertion of the occluder.

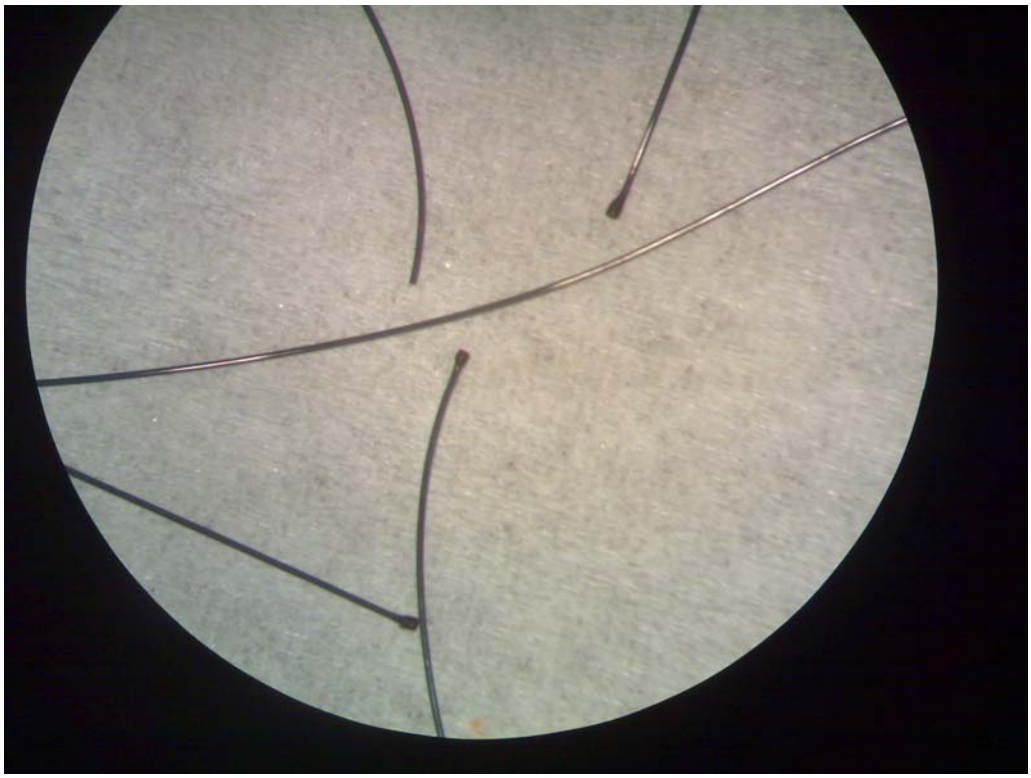

Figure 4: Bits of 4-0 ethilon used for MCAO viewed under microscope

Surgical procedure

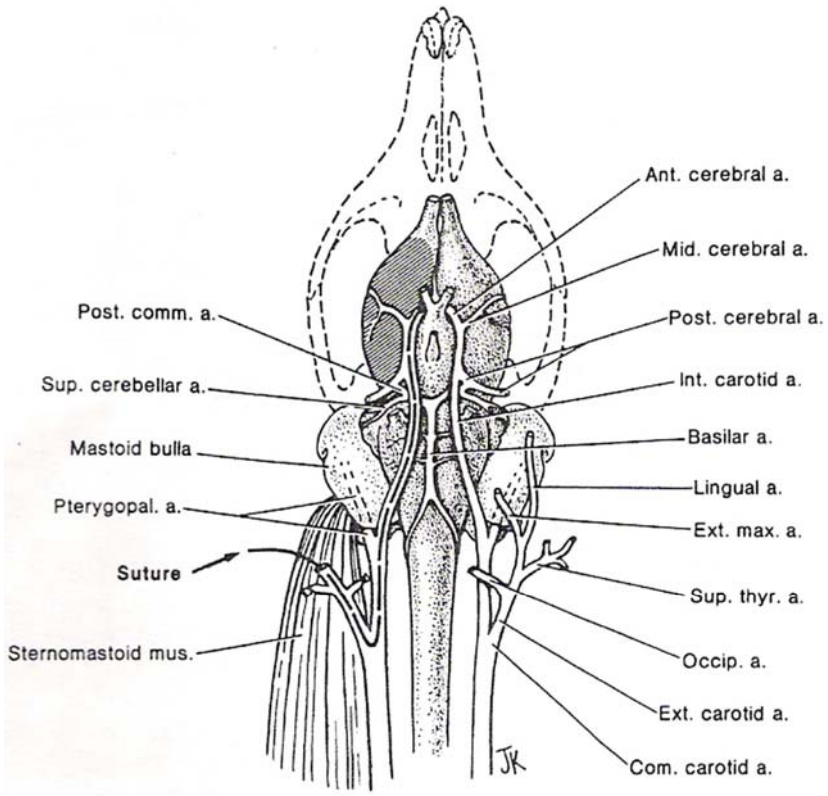

Figure 5: Representative diagram showing rat cerebrovascular anatomy.

Rats were placed on a wooden slab measuring 12 x 7 cm. and covered with warm towel sheets to maintain the body temperature (See Figure 6). All four limbs were extended and secured to the slab with an adhesive. A thread looping around the upper incisors was fixed to the slab to maintain the extension of neck.

Figure 6: Anaesthetized rat placed on a wooden block and limbs secured. Note that the linen covering the rat maintains the temperature.

The operating microscope was now brought in and a midline vertical skin incision was made starting from the hyoid up to the manubrium sterni. (Figure 7)

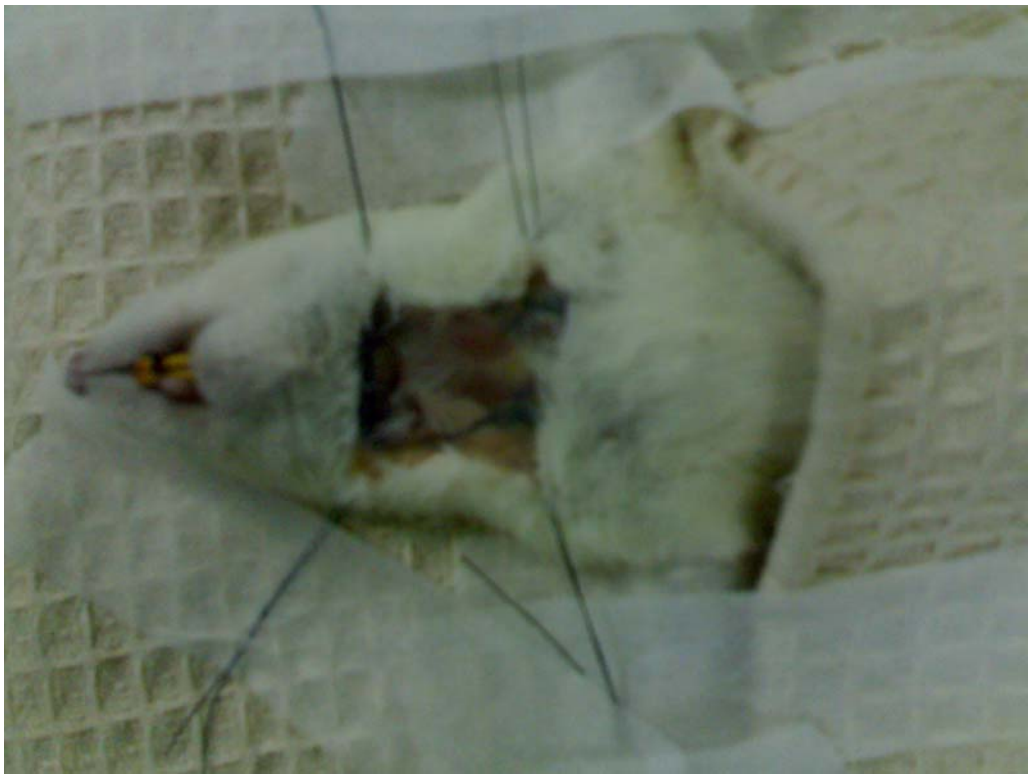

Figure 7: Cervical region after retracting the skin flaps with stay sutures.

The trachea was exposed in the midline and a square window made in two tracheal cartilages. The patency of the tracheostomy was checked with a wisp of cotton. The air eggressing from the trachea would cause movement of the cotton bristle, which confirmed the tracheostomy to be adequate. The

secretions could also be cleared with the help of cotton. With a combination of blunt and sharp dissection, the right common carotid artery (CCA) was exposed through the same incision. The CCA was identified in the carotid sheath lateral to the strap muscles near the tracheo esophageal groove. The trachea and the strap muscles served as initial landmarks for the identification of CCA. It is important to note that CCA becomes superficial as it emerges into the neck and it is at this point where it can get damaged during dissection. Self-retaining retractors were applied with the lateral arm on the sternocleidomastoid and the medial arm on the strap muscles. The carotid sheath was held up with the help of micro forceps proximally and a small temporary occluder is placed on CCA. This temporary occluder served as a micro vascular clip that would prevent excessive ooze from the CCA if it was damaged distally.

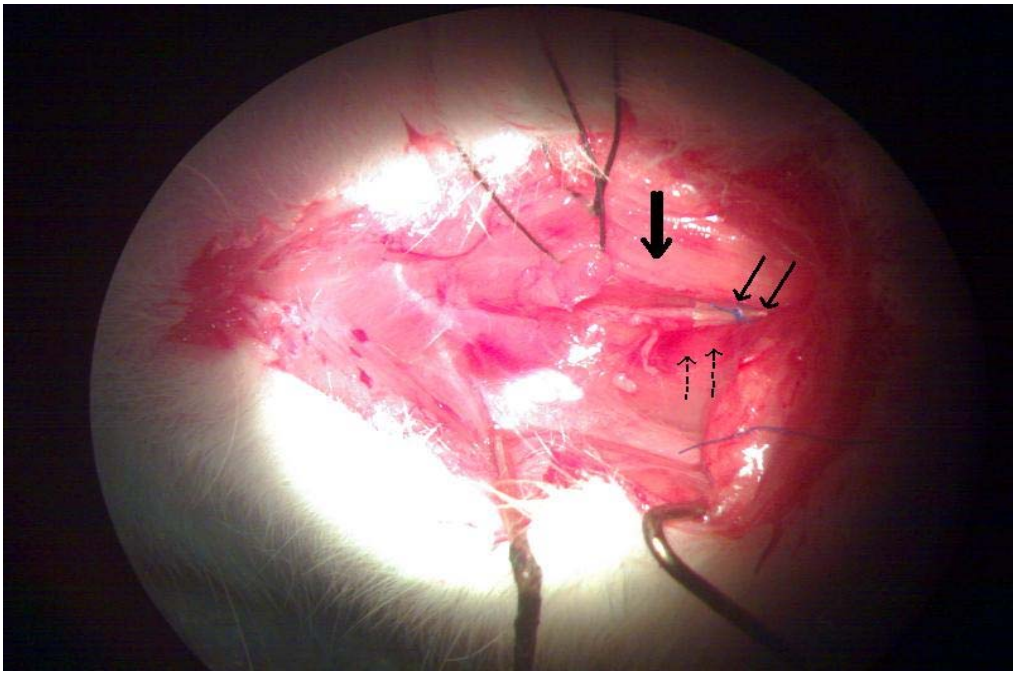

Figure 8 (low magnification): Right common carotid artery clamped with a temporary occluder (fine arrows). CCA is identified between the strap muscles (bold arrows) in the vicinity of the tracheo esophageal groove medially and the sternocleidomastoid laterally (broken arrows)

The occluder was a small bit of PE- 50 catheter that was placed parallel to the CCA and in close proximity to it. A loop of 4-0 silk was tied around the CCA and PE-50 to occlude the artery. This set-up was used to facilitate removal of the occlusion by cutting the 4-0 silk with a knife directly onto the PE-50 without damaging the artery. Once the occluder was placed on the CCA, the carotid sheath was further dissected distally to expose the bifurcation and the external carotid artery. The various branches of the

external carotid artery (ECA) were then finely dissected, exposed, ligated with 6 - 0 prolene and then cut. The prominent branches requiring ligation were superior thyroid artery, maxillary artery and the occipital artery. The latter branch arises proximally near the bifurcation and extreme care has to be taken while dissecting it off the internal carotid artery (ICA). A lymph node partially covering the carotid bifurcation needed to be carefully separated from the CCA and the ICA, a point in the dissection where these vessels were prone for damage. Once the lymph node was cleared out, the ECA was cut between the ligatures and then transposed inferiorly until it was parallel with the CCA.

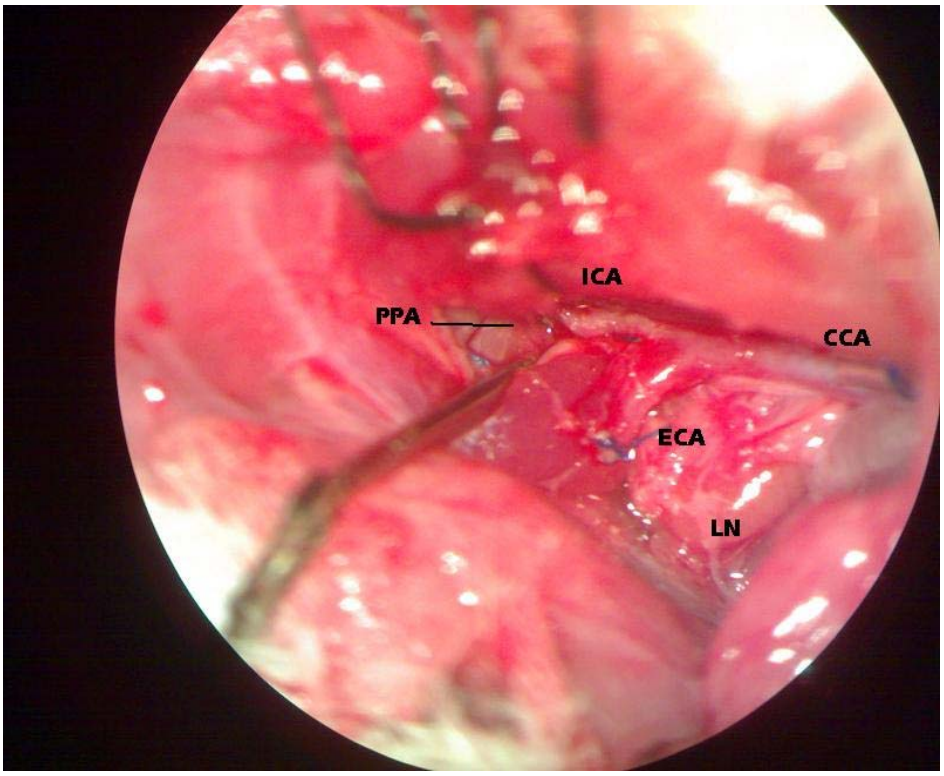

Figure 9: Isolated and transposed right ECA. Note that the common carotid artery is still clamped.

Now the proximal part of the internal carotid artery (ICA) was isolated and the pterygopalytine artery (PPA) which is lateral to ICA was ligated with 6-0 prolene. This bifurcation was quite deep and required good visualization. The ligature over the PPA should not be too far proximal as this would narrow the ostium of the ICA, and it should not be too distal as in that case

the tip of the inserted occluder would lodge in PPA. This could happen as the origin of the PPA is straighter. At this point ICA was the only remaining extra cranial branch of the CCA. Next, a 4-0 silk suture was tied loosely around the mobilized ECA stump, and a temporary micro vascular clip was placed across ICA adjacent to PPA origin.

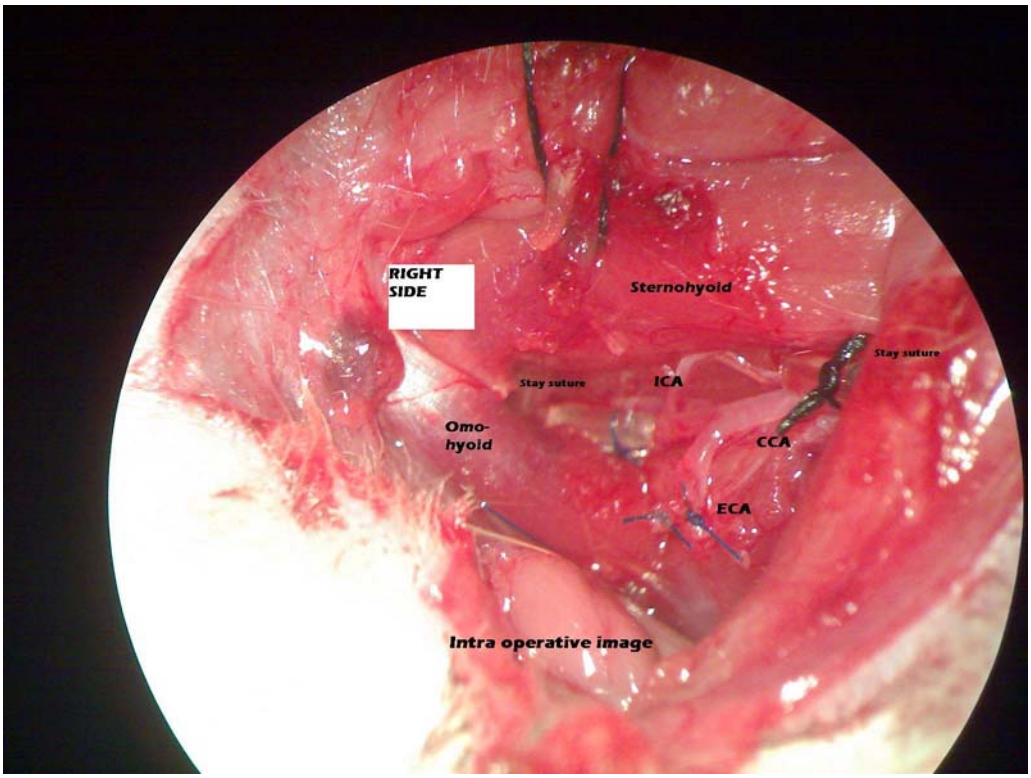

Figure 10: After dissecting the internal carotid artery and its branches, a temporary occluder has been placed to prevent the retrograde blood flow while doing arteriotomy.

A 5 cm length of 4-0 monofilament nylon sutures, its tip rounded by heating near the flame, was introduced into the ECA lumen through a puncture or through one of its terminal branches. The silk suture around the ECA stump was tightened around the intraluminal nylon to prevent bleeding, and the micro vascular clip removed. The nylon suture was advanced from the ECA to ICA until a resistance was felt to a maximum of 17 mm indicating that the blunted tip of the suture has passed the origin of MCA and reached the proximal segment of the anterior cerebral artery ACA

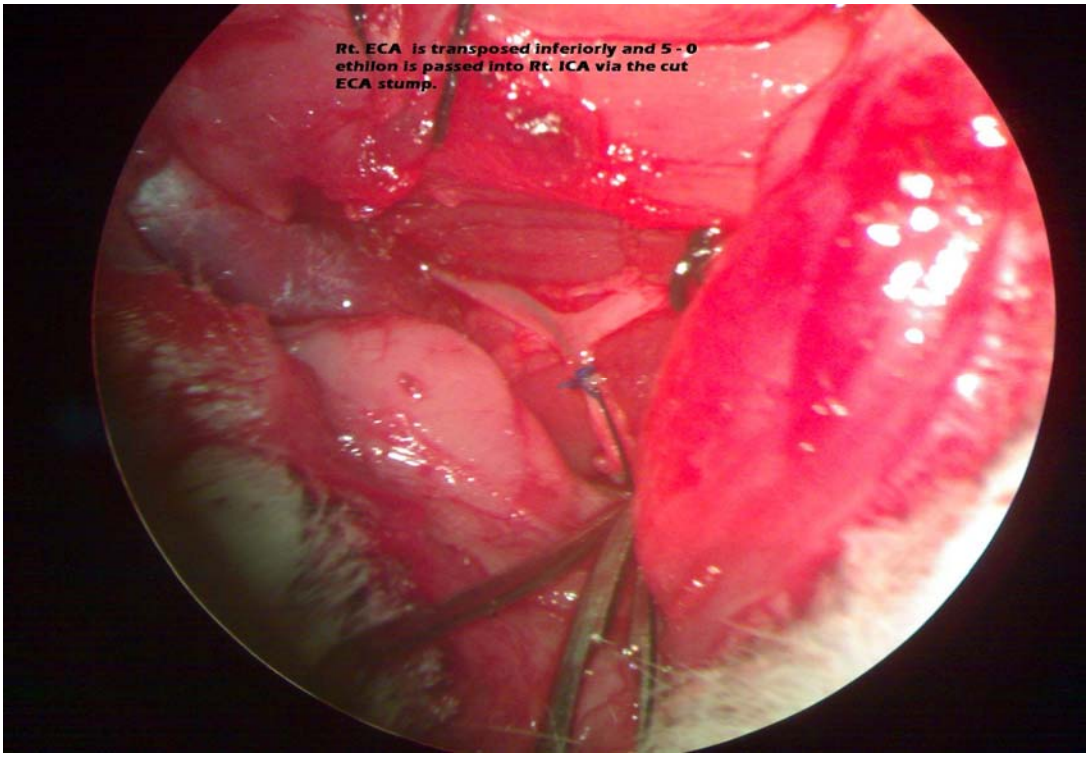

Figure 11: 4-0 Ethilon has been passed into the transposed ECA stump and introduced further into the ICA to block the origin of MCA.

Now, the intra luminal suture has blocked the origin of MCA, occluding all sources of retrograde blood flow from ICA, ACA, and posterior cerebral artery. The incision was closed leaving behind 1 cm of nylon suture protruding beyond the skin so that it could be withdrawn later to allow reperfusion. The wound was closed keeping the tracheostomy site open.

Preparation of Slices

TTC as a staining agent:

Different methods have been used to detect the morphological features of cerebral tissue after ischemic injury. The oxidation-reduction indicator 2,3,5-triphenyltetrazolium chloride (TTC) has been used successfully for early histochemical diagnosis of myocardial infarction. The staining action of tetrazolium salts is based on the presence of a dehydrogenase. In normal tissue, dehydrogenase reduces TTC to formazan, which stains red. In ischemic or infarcted tissue dehydrogenase activity is reduced or eliminated and those areas remain unstained.

Procedure:

Rats were euthanised with deep chloroform. Decapitation was carried out with sharp scissors at the level of craniovertebral junction. The brain was carefully dissected en bloc within ten minutes. (figure 12)

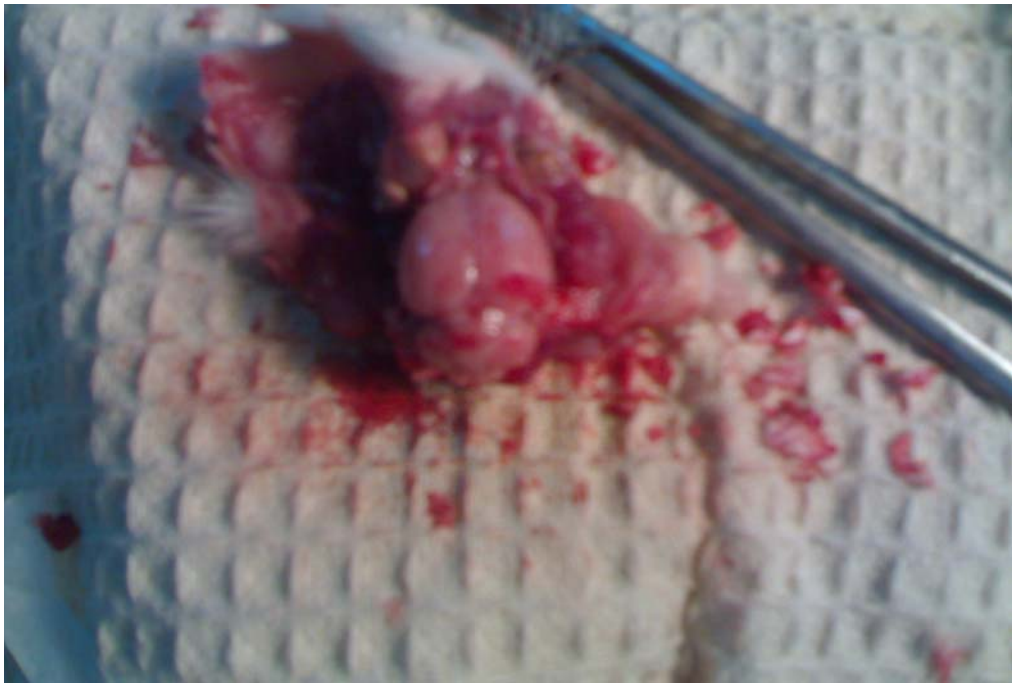

Figure 12: Rat brain being isolated from the skull.

The brain was then quickly transferred and cooled on a bed of ice for about a minute. It was then placed on the brain matrix (Plastics One ^{TM}, USA, see figure 13) and sliced coronally at 2 mm intervals starting from the frontal tip. Four slices were made out of one brain.

Figure 13: Brain matrix that was used to make 2 mm slices of rat brain.

These slices were freed from the dura and the vascular tissue. Then these were soaked for 10 minutes in a solution of 0.1% TTC in 0.1mol/Litre PBS (pH 7.4), warmed to 37 degrees in a water bath. Excess TTC was drained and slices refrigerated at 4 degrees in 10 % formalin.

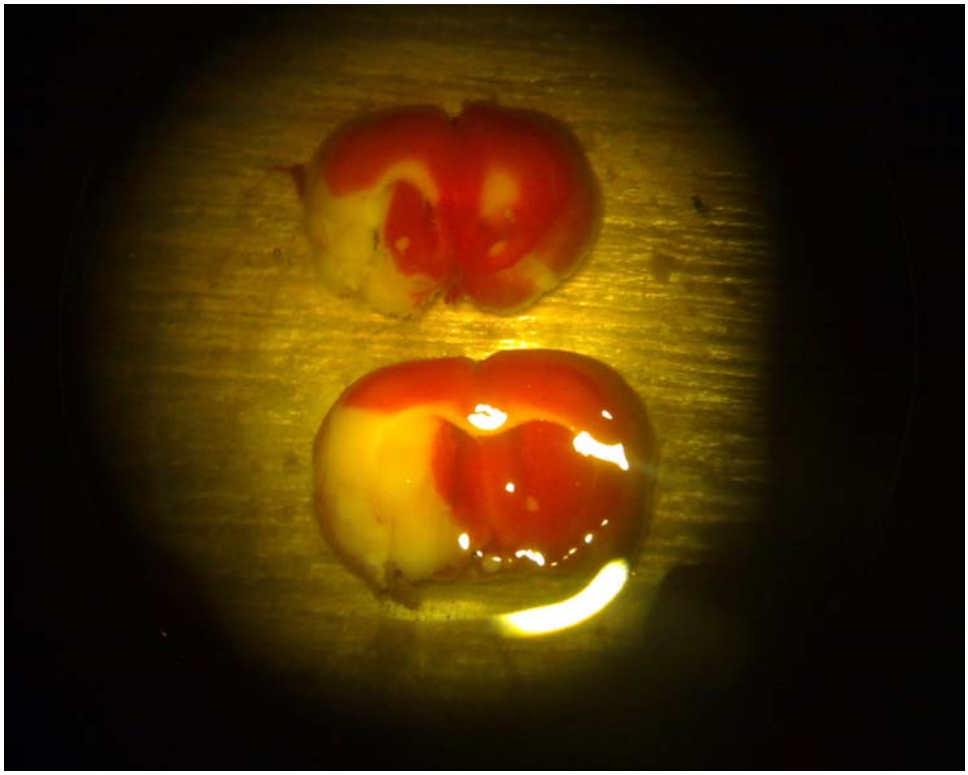

Figure 14: A typical stained rat brain slice. Red areas are the viable area and the yellow area is the infarcted area.

Imaging of Slices

The stained slices were photographed using a 2.0 mega pixel digital camera attached to the operating microscope. The resulting image was then transferred and then saved as an uncompressed tagged image file format file on a personal computer. Imaging software was acquired from NIH (National Institute of Health, USA) as free trial software. The software was also loaded in the same computer. An independent assessor blinded to the study,

manually delineated the infarct and total areas with a computer mouse. Two readings were averaged for each section. If the difference of more than 10% was noted between these two readings, then the third reading was taken and the value that was closer was finalised. The infarcted volume was calculated by multiplying this by the slice thickness.

Statistical Methodology

All values, except for neurological score, are presented as mean +/- SD. Infarct size, body weight, and physiological variables were analyzed with the unpaired *t* test. Neurological scores are reported as the median (quartile deviation). A value of $p < 0.05$ was considered significant.

RESULTS

Out of 32 rats allotted, the initial plan was to use 16 rats to establish the model and the other 16 for the actual experiment. However 24 rats were needed to develop and standardize the surgical technique as a satisfactory infarct with clinical deficit was not obtained in the first 19 rats. The 20^{th} to 24^{th} rats (n=5) showed consistent neurological deficits correlating with the focal cerebral infarct as documented by TTC staining. Table 1 shows the probable causes of failure in the first 19 rats.

Table 1: Reasons for failure while developing and standardizing the model

Rat number	Reason for failure
1	Death due to anesthetic complications
2	Death due to anesthetic complications
3	Death due to anesthetic complications
4	Death due to anesthetic complications
5	Death - intra op hemorrhage (pre cannulation)
6	Death - intra op hemorrhage (pre cannulation)
7	Death - subarachnoid hemorrhage due to perforation at

	ICA bifurcation
8	Death - subarachnoid hemorrhage due to perforation at ICA bifurcation
9	Same as above
10	Death due to airway compromise
11	Death due to airway compromise
12	Death due to airway compromise
13	Death - intra op hemorrhage (during cannulation)
14	Death - intra op hemorrhage (during cannulation)
15	No deficit – failure of occlusion by 4-0 ethilon coated with poly L Lysine
16	No deficit – failure of occlusion by 3-0 ethilon coated with super glue
17	No deficit – failure of occlusion by 5-0 ethilon coated with super glue
18	No deficit – 5-0 ethilon coated with Mac’s solution, no occlusion
19	No deficit – 4-0 ethilon coated with Mac’s solution did not reach origin of MCA

As a result only eight rats were available for the final experiment, and were allocated equally into the lactate and control groups (lactate group = 4, control group = 4).

Table 2: Intraoperative parameters monitored

Variable	Lactate	Control	p value
Body weight (gms)	313.75 ± 22.87	310±18.26	Not significant
Temperature (degrees C)	37.1±0.35	37.4±0.8	Not significant
Sodium (meq/L)	140.0±5.94	140.75±4.57	Not significant
Glucose (meq/L)	175.25±8.18	177.51±4.18	Not significant
Serum lactate(meq/L)	2.92±0.22	1.05±0.26	<0.0001
pH	7.30±0.03	7.45±0.05	Not significant
Surgery duration (min)	41.25±2.50	51.25±10.31	Not significant
Anaesthesia duration (min)	81.25±4.79	76.25±4.75	Not significant
Blood loss (no. of pledgets)	2.0	2.5	Not significant
pO2(mm Hg)	104.25±4.57	102.75±6.08	Not significant

Neurological Score

The median neurological score prior to sacrifice was 1.0 in the lactate group (range 1- 2) while it was 2.0 in the control group (range 1-2). (p=0.6) (see table 3)

Table 3: Comparison of neurological scores in the study groups

Rat number	Lactate group	Control group
1	2	2
2	1	2
3	1	2
4	1	1

Infarct volume

The mean infarct volume was 119 ± 34.6 mm3 in the lactate group while it was 180.61 ± 37.26 mm^3 in the saline group. (p = 0.05). The detailed values of infarct volume in all rats are entered in Table 4.

Table 4(a): Infarcted area and total area per brain slice (in pixels) in the lactate group as seen in figure 14. Each rat brain was sectioned into four slices rostrocaudally (a-d).

Rat (Lactate)	Infarct area in pixels	Total area in pixels	Ratio
1a	224	4854	0.05
1b	1556.5	5203	0.30
1c	1496	5649	0.26
1d	1060.5	5569	0.19
2a	277.5	5029	0.06
2b	958	5464	0.18
2c	1436.5	5541	0.26
2d	1335.5	5741	0.23
3a	277.5	5537	0.05
3b	877	5691	0.15
3c	1030.5	5945	0.17
3d	545.5	5765	0.09
4a	269.5	5015	0.05
4b	1287.5	5662	0.23
4c	387.5	5987	0.06
4d	401.5	5839	0.07

Figure 14: TTC stained axial sections of the rat brain that were infused with sodium lactate (experimental group).

Table 4(b): Infarcted area and total area per brain slice (in pixels) in the control group as seen in figure 15.

Rat (Control)	Infarct area in pixels	Total area in pixels	Ratio
1a	1036.5	5003	0.21
1b	1869.5	5334	0.35
1c	1770.5	5665	0.31
1d	1434	5608	0.26
2a	890	5382	0.17
2b	1381	5666	0.24
2c	986.5	5883	0.17
2d	1306	5721	0.23
3a	1127	5084	0.22
3b	1709	5214	0.33
3c	1479.5	5558	0.27
3d	1394.5	5721	0.24
4a	344.5	5035	0.07
4b	962	5503	0.17
4c	1434.5	5805	0.25
4d	1102.5	5687	0.19

Figure 15: TTC stained axial sections of the rat brain that were infused with normal saline (control group).

DISCUSSION

Development of the model

Rat models of focal cerebral ischemia have gained increasing acceptance in recent years owing to their similarity to human clinical syndromes and the difficulty of experimenting with primate models. Various techniques to produce a reliable infarct models such as cauterization of the MCA via a craniotomy (Backhauss 1992), mechanical clipping of the MCA (Yanamoto1998) and photothrombotic occlusion (Markgraf 1994) have been described. Koizumi et al (1986) and Zea Longa et al (1988) reported a novel, relatively noninvasive method of achieving reversible MCAo by the use of an intraluminal suture which has come to be a widely used stroke model. However reproduction of their technique requires specialized equipment such as ventilators, microclips, microelectrocoagulation and sophisticated imaging techniques. This study, despite many initial setbacks, demonstrates that the model can be reproduced with easily available equipment and techniques. The major problems encountered and the way they were solved will be discussed.

Rat Handling

The investigator had no previous experience in handling rats. The tasks of picking up the animal from the cage, identification and marking, recognition of behavioural traits, examination of physical signs like colour of the eyes, pinna and the respiratory rate were all learnt during the development of the model. It is essential to get accustomed to animal characteristics and behaviour for conducting a successful experiment.

Rat anaesthesia

Drugs and Dosages

An ideal rat anaesthetic agent is the one that has good sedative and anaesthetic properties that last for a prolonged period without compromising breathing and airway. Chloroform was used initially, but proved to have an ill-sustained effect (only 1 minute), and on increasing the dose it caused severe respiratory depression resulting in death of the rats. However, 0.5ml of 2% chloroform soaked in cotton was found to be an ideal inducing agent. The rats were placed in the ether glass jar with the cotton impregnated with chloroform. By careful and repeated observation, it was noticed that the drop in the respiratory rate to 50 per minute, loss of postural reflex and fading of the red colour of the eye marked the onset of anaesthesia. This effect lasted

only for a minute, providing a window period for administering the long duration intraperitoneal anaesthetic.

Intraperitoneal ketamine (60-80 mg/kg) and injection atropine (0.1 mg/kg) were administered intraperitoneally with an insulin syringe. It is important to pinch and lift up the skin over the abdomen to avoid injection into the subcutaneous space or into the bowel. The dose has to be fractionated into three parts; one third is administered after induction, one third prior to skin incision and the rest mid-way during the surgical procedure. A single bolus administration resulted in severe haemodynamic compromise and death. This protocol of fractionation developed by trial and error provided a smooth and sustained anaesthesia for approximately two and a half hours.

Airway and ventilation

Administration of ketamine caused excessive parasympathetic activity resulting in salivation and tachycardia. Anaesthesia for prolonged duration required protection of airway as intraoperative deaths due to asphyxia were noted in the first few rats. Since no animal ventilator, endotracheal intubation equipment or monitoring devices were available, several different methods to secure the airway were tried. Initial attempts to cannulate the trachea with the help of an intravenous 16 gauge plastic cannula were unsuccessful. However an elective tracheostomy was found to be extremely

effective in safeguarding the airway. A small window was created in the midline of the trachea by cutting the cartilage with microscissors. This not only facilitated the patency of the airway but also provided access for periodic aspiration of the mucoid secretions that accumulated in the trachea. The skin edges were sutured to the cartilaginous edge with the help of 5-O Prolene (Polypropylene). Development of tracheostomy was an important landmark in preventing rat deaths due to asphyxia. Following regular use of tracheostomy, none of the rats died primarily due to airway compromise.

Blood sampling

The tail artery was cannulated with a PE 50 catheter for sampling. The blood samples were analysed with the help of automated Blood Gas analyzer that provided information regarding pH, PaO2, PaCO2, lactate, glucose, sodium and potassium. Due to financial constraints, these investigations were done during only after the model was successfully developed, and at the end of the surgical procedure.

Equipment

The investigator required help initially with the handling of microinstruments and the microscope, as well as in the initial surgical approach and identification of various anatomical structures. The first few

surgeries were performed with a senior guide (AGC). Various other tools such as microvascular clips, self-retaining retractors were indigenously designed and put into the use gradually. With increasing experience the surgical and anaesthesia time decreased significantly.

Appropriate suture material

Despite gaining expertise in the anaesthesia, microsurgical skills, and keeping the rat alive till the recovery phase, no neurological deficits or infarct formation were obtained. Four factors, all related to the suture material which was used to occlude the origin of MCA, were found to affect the results. The first thing the thickness of the suture material - out of 3-O, 4-O and 5-O Ethilon, 4-O Ethilon was found to be the best. The second requirement was that the tip had to be blunted with heat. Non-blunting of the tip resulted in perforation of the internal carotid bifurcation causing fatal subarachnoid haemorrhage.

Dr. Mac's solution

The third important factor was material used to coat the suture material. Initially, poly L-lysine was used as described in literature but proved unsuitable. After experimentation the solution used to insulate copper wire in electrical equipment was found to be effective.

Marking on the suture

Many authors have suggested that the firm resistance felt after inserting a certain length of the suture marks the placement of the tip into the ACA thereby blocking all blood flow into the MCA. However in this experiment it was found that this was causing perforation of the blood vessel before the pressure could be appreciated. If the suture material was placed short of the occlusion only subcortical infarcts with no demonstrable neurological deficits were obtained, as seen in figure 16.

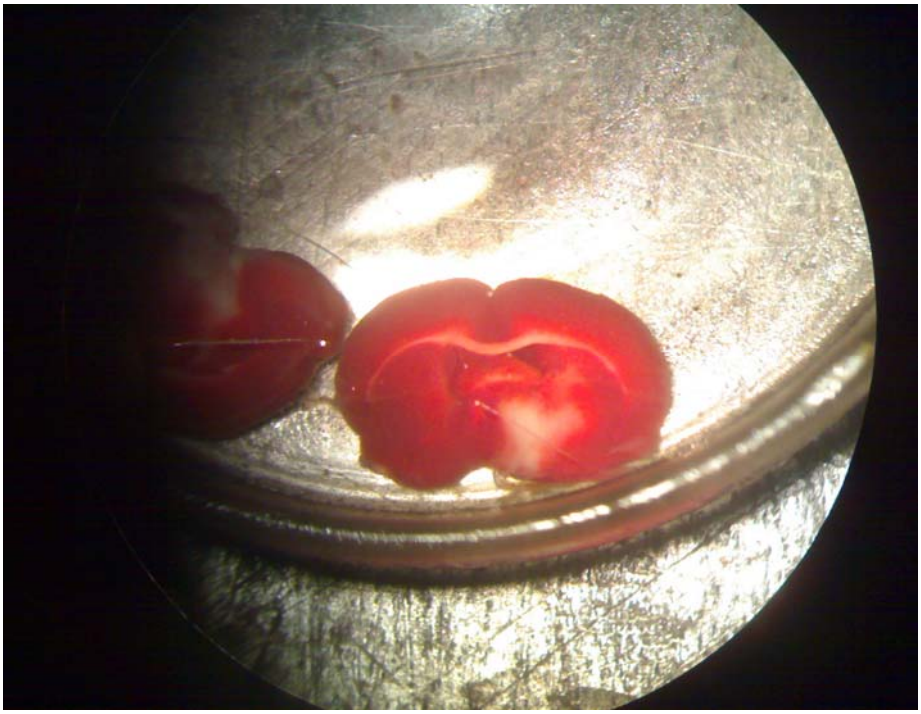

Figure 16: Left sided subcortical infarct (yellow unstained section)

From the cadaveric measurements done on the rats a length of 17-18 mm of the suture was found to almost always result in proper placement with occlusion of the origin of MCA. Hence the strategy was changed and the sutures were marked at a distance of 17 mm from the tip and inserted up to this point. This marking prevented excessive insertion of the suture and still provided effective blockade of the MCA. Marking a thin suture also proved difficult, and finally regular alcohol based white print eraser was used to mark the distance. This was the fourth important factor in preparing the suture material, and was finally achieved with the twentieth rat.

Slice making - Brain matrix

The brain matrix is a device to make evenly spaced slices out of the brain. This was not available in India and was essential to perform the infarct analysis. Hence a plastic version was imported from USA and used for the experiment.

Cost effective way of staining slices

Staining technique described in Western literature used a 2% solution of TTC. Based on a paper published by Murthy et al (2004) from CDRI

Lucknow a 0.5% solution was used and found to be effective. This reduced the cost of the staining technique by half.

Imaging of slices

The operating microscope in the animal house did not have an attached digital camera port. Images taken directly with a digital camera through the eyepiece were not of good resolution, but images were taken with a 2.0 mega pixel cell phone CCD camera were found to be of good quality, probably because of the smaller lens.

Imaging software

Image analysis software was required for an accurate assessment of the infarct size. A demo version of Image J was acquired from NIH (National Institute of Health, Bethesda, USA) and then was used to delineate and quantify the infarct and the area of the brain section.

Limitations of the model

In this experiment a recognised technique of producing infarctions in rats has been considerably modified for it to be performed with minimal

equipment and locally available materials, which are important factors in a resource constrained environment.

The rats in this study were not ventilated and did not have continuous invasive blood pressure monitoring or saturation monitors. Surrogate clinical markers like respiratory rate, color of the pinna, color of the eyes, posturing reflex were used as a guide to assess various physiological parameters. Even blood gas analysis was performed only once at the end of each surgery. Though there was no difference between the two groups it is entirely possible that there was some variation in physiological parameters during the procedure, which in turn could affect infarct size independent of experimental solutions.

All vessels that were sacrificed in this study were individually isolated and divided between two ligatures. This was time consuming and difficult, especially for deeper structures like pterygopalatine artery. A bipolar coagulator should be used in future experiments.

A number of similar experiments performed used various imaging modalities to assess ischemia and infarction including MRA, doppler and perfusion studies. Only clinical evaluation for deficits was used in this study, and no imaging was performed.

Even minimal subarachnoid hemorrhage can be a confounding factor in this experiment, and previous reports indicated that laser Doppler-guided placement of the suture might reduce the incidence of SAH. We could not do this, but in our final series of eight rats none had any evidence of SAH.

Lactate as an alternative fuel

For much of the 20th century, lactate was largely considered a dead-end waste product of glycolysis due to hypoxia, the primary cause of the O2 debt following exercise, a major cause of muscle fatigue, and a key factor in acidosis-induced tissue damage. Since the 1970s, a 'lactate revolution' has occurred. There is now significant experimental support for a cell-to-cell lactate shuttle, along with mounting evidence for astrocyte–neuron, lactate–alanine, peroxisomal and spermatogenic lactate shuttles.

In our study we tried to assess the role of lactate in limiting infarct size. To our knowledge this is the first time lactate has been assessed in a ischemic stroke like clinical paradigm. The lactate levels were found to be significantly higher in the experimental group than the control group. Though the infarct was smaller in the group infused with lactate (119 mm^3 with lactate, 181 mm^3 in controls), the difference was barely statistically significant. The sample size was smaller than initially planned, due to the fact that standardizing the model proved to be much more difficult than

anticipated. However lactate does seem to decrease infarct size, possibly via the ANLSH. A larger trial is needed to further elucidate the role of La in preventing secondary brain injury.

CONCLUSIONS

The experiment describes a simple technique to make a reliable animal model of focal cerebral infarct that can be performed with minimal equipment and materials. Lactate appears to decrease infarct size, supporting the astrocyte neuron lactate shuttle hypothesis.

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