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In vivo imaging of experimental stroke : animal models, PET and SPECT radiotracers

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Among the major complications of hypertension, stroke is the third leading cause of death and the leading cause of permanent disability in the developed countries. Pathogenetically, stroke integrates a heterogeneous group of diseases, but, vessel occlusions account for 85% of all strokes. So, this review is focusing on small animal models of ischemic stroke and is discussing their advantages and disadvantages. The goal of experimental ischemic stroke is to reduce oxygen and glucose supply to brain tissue. Understanding the mechanisms of injury and neuroprotection in this disease is critical if we are ever to learn new target sites to treat ischemia. Two main cerebral ischemia experimental models exist, characterized as global (complete or incomplete) and focal (permanent or temporary with reperfusion) ischemia. Global ischemia occurs when Cerebral Blood Flow (CBF) is reduced throughout most part of the brain or the whole brain, whereas focal ischemia is a reduction in blood flow in a very distinct and specific brain region. Focal cerebral ischemia models have been used more extensively because of their purported relevance to human thromboembolic stroke. Most focal cerebral ischemia models involve occlusion of one major cerebral blood vessel such as the middle cerebral artery (MCAO) in small animals. The use of an intraluminal filament is the simple, and most popular and relatively noninvasive method to produce either permanent or transient MCAO in rodents. CBF had been found to decrease by 80% and the animals may survive for days, weeks, and months, which will enable functional outcome measures to be recorded (laser Doppler, neurological outcome, infarction volume, in vivo imaging).In focal cerebral ischemia, there may be absolutely no blood flow in the very central core of the ischemia, but some flow usually reaches the area via collateral circulation. Thus, there is usually a gradient of blood flow from the inner core reaching out to the limits of the ischemic area (penumbra). The penumbra can be visualised by various imaging modalities, including SPECT, PET and MRI. Hypoxic volume combined with the perfusion defect volume from PET or SPECT images could be a predictor of neurological deficit. In humans, PET (^{18}F-fluorodeoxyglucose, ^{15}O-O2) measuring blood flow

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and metabolism is the current gold-standard imaging technique. However, more specific PET and SPECT tracers for neuron viability or cell hypoxia have been tested in animals and humans. ^{11}C-flumazenil binds to central benzodiazepine receptors and, as measured by PET, is an early indicator of preserved cortical neuronal integrity. Nitroimidazole compounds bind preferentially to viable but hypoxic cells in penumbra and, when labelled with ^{18}F or ^{99m}Tc, have been used for TEP or SPECT assessement of myocardial ischemia, tumor hypoxia and more rarely of cerebral ischemia. Radiosynthesis allowing easier radiolabelling protocols has also led to the development of numerous SPECT and PET tracers for apoptosis. The data obtained on tumors from angiogenesis imaging with radiolabelled ligands for alpha v beta 3 integrin receptor are being transferred to stroke. In terms of perspectives, using PET reporter genes can be imagined to image hypoxic cells with and to track stem cells injected in animal models of stroke.

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1. Introduction

A stroke is an acute neurologic injury in which the blood supply to a part of the brain is interrupted. That is, stroke involves sudden loss of neuronal function due to disturbance in cerebral perfusion, commonly arterial. The part of the brain with disturbed perfusion no longer receives adequate oxygen. This initates the ischemic cascade which makes brain cells die or be seriously damaged, impairing local brain function. Among the major complications of hypertension, stroke is the third leading cause of death and the leading cause of permanent disability in the developed countries[1]. Pathogenetically, stroke integrates a heterogeneous group of diseases and can be classified into two major categories[2]: ischemic and hemorrhagic. Ischemia may be due to thrombosis, embolism or systemic hypoperfusion. Hemorraghe can be due to intracerebral hemorraghe or subarachnoid hemorrhage. About 80% of strokes are due to ischemia and about 75% of ischemic strokes are due to intracranial thrombosis (Fig. 1). So, the first part is presenting the different ways to reproduce an ischemic stroke in animals. Then, the second part is focusing on the ischemic cascade which permits to identify the major processes and mediators involved in stroke and the potential targets for stroke imaging. Despite ongoing questions over the choice of parameters to identify the site of injury and their respective clinical usefulness, imaging is gaining widespread use in acute stroke management for the diagnosis and etiological work-up after stroke. The major imaging modalities are radionuclide, magnetic resonance and optical imaging. We are focusing on nuclear medicine techniques including Single Photon Emission Computerized Tomography (SPECT) and Positron Emission Tomography (PET).

This article is necessarily limited in scope; so the reader is referred to the comprehensive reviews of ischemic cell death[3], models of cerebral ischemia[2] and molecular isotopic imaging[4] elsewhere, for deeper interests.

2. How to reproduce ischemic stroke in animals

About 75% of ischemic strokes are due to intracranial thrombosis which can be reproduced by focal ischemia. In focal ischemia cerebral blood flow is reduced to a very distinct specific brain region. There may be absolutely no blood flow in the very central core of the ischemia, but usually there is some flow that reaches the area via collateral circulation. Thus, there is usually a gradient of blood flow from the inner core reaching out to the limits of the ischemic area. This surrounding zone is called penumbra (Fig.2). In penumbra blood flow is sufficiently reduced to result in hypoxia severe enough to arrest physiological function, but not so complete as to cause irreversible failure of energy metabolism and cellular necrosis. These are the cells that can be rescued and resuscitated by restoration of perfusion and other protective therapies. Most focal cerebral ischemia models involve occlusion of one major cerebral blood vessel such as the middle cerebral artery (MCA). MCA occlusion (MCAO) results in a permanent or temporary (reperfusion) reduction of cerebral blood flow in the cortex (distal

occlusion) or in the cortex and the striatum (proximal occlusion) (Fig.3). Several different types of MCAO models exist[2]. The first group of MCAO models require craniotomy and, thus, are relatively invasive. MCA is surgically dissected and subsequently permanently or transiently occluded, e.g. by electrocutery or ligation. MCAO can be combined with temporal or permanent common carotid artery occlusion. The second group of MCAO models avoid craniotomy and includes embolic MCAO, photochemical MCAO and endovascular filament MCAO. In embolic MCAO the occlusion is achieved either by injecting clots that were formed in vitro[5] or by endovascular instillation of thrombin for in situ clotting[6]. The thrombembolic model is closest to the pathophysiology of human cardioembolic stroke but the quality of MCAO – and thus the volume of brain infarcts – is very variable. The photochemical MCAO model involves irradiation of several branches of the distal MCA with beams from an argon dye laser following intravenous administration of the photosensitizing dye rose bengal. This model requires only a small craniotomy, and the dura remains intact. Temporary common carotid artery occlusion can be added to restrict collateral supply to the area of the MCAO. This model results in a consistent infarction in the frontoparietal neocortex but the disadvantage is that the photochemical reaction can result in microvascular injury. The most used, relatively noninvasive method to produce either permanent or transient MCAO in rodents is with the use of the intraluminal filament[7]. It involves inserting a 4-0 nylon suture into the internal carotid artery of rats (Fig.4) and then advancing the thread cranially to block the MCA. This thread can be passed and is usually advanced 17 to 20 mm from the origin of the internal carotid artery, thus occluding collateral circulation from the anterior communicating arteries. Using the intraluminal filament technique, CBF had been found to decrease by 80% in the cortex and caudoputamen. Reperfusion easily occurs when the thread is withdrawn, and the animals may survive for days, weeks, and months, which will enable functional outcome measures to be recorded. Generally, animals are evaluated by simple neurological scores (Tab. 1) and by motor tests such as the rotarod test. Then, after animals were sacrificed, the volume of stroke is measured by integrating areas of infarction over multiple brain slices colored with 2,3,5-Triphenyltetrazolium chloride or TTC (fresh slices), cresyl violet or hematoxylin/eosin (fixed slices).

Whatever the model used for investigation and imaging, it must be underlined that, although the basic mechanisms of stroke are identical between humans and other mammals, there are differences that may impair the transferability of animal results to human stroke.

3. The ischemic cascade : identification of the processes and mediators involved in stroke and of the potential targets for stroke imaging.

The so called ischemic cascade describes the pathophysiology of focal ischemia in the penumbra[8]. The concept of penumbra has received a great deal of attention because the injury of this area may be essentially reversible (even if it is time-limited) and thus this area could be the target of therapy by pharmacological intervention or reperfusion. During focal ischemia, the injury leads to neuronal death by apoptosis and necrosis. It has been shown[9] that the ratio between apoptotic cells and necrotic cells is much higher in the penumbra than in the core. The

ischemic cascade (Fig.5) and the pathophysiological processes that could be further imaged by SPECT or PET are explained hereatfer.

Within minutes of vascular occlusion, brain tissue is deprived of glucose and oxygen and the acidic byproducts of metabolism accumulate. This loss of substrate and decrease in pH level lead to cessation of the electron transport chain activity within mitochondria, which results in a rapid decline in ATP concentration. Loss of ATP leads to failure of the Na+,K+-ATPase, which results in a marked intracellular increase in intracellular Na+ concentration. Persistent depolarization allows Ca2+ entry, and higher intracellular Na+ levels reduce the efficacy of the 2Na+-Ca2+ symport, which further increases intracellular Ca2+. Because the membrane potential reaches the electrical threshold for discharge, neurons inside the core infarct exhibit ischemic discharges whereby they repetitively fire, releasing their transmitters locally and at distant targets. These ischemic depolarizations further exacerbate energy needs. A high intracellular Ca2+ level initiates several events, including activation of calpain protease activity that effects the structural integrity of both the intra- and extracellular structure and phospholipase activity that degrades cellular membranes. Increased Ca2+ also induces nitric oxide synthase activity and expression, which favors the formation of peroxynitrate, a highly reactive free radical species. The resulting influx of Ca2+ damages the mitochondria, which further exacerbates energy failure and initiates apoptosis. Mitochondria also produce free radicals that are toxic both to the mitochondria and the cell as a whole, especially if sufficient oxygen is present as during reperfusion. Moreover, during ischemic depolarizations, glutamate is released in the synapses from cells damaged by ischemia. Glutamate agonizes the *N*-methyl-d-aspartate (NMDA) receptor, a Ca2+ membrane channel, which increases calcium influx to the cell. Glutamate also agonizes the amino-hydroxy-methylisoxalone propionic acid (AMPA)/ kainate receptor, which allows both Na+ and Ca+ entry, and the metabotropic receptor (quisqualate), which increases intracellular cyclic adenosine monophosphate levels, altering protein kinase activity, proteolysis, and lipolysis. This cascade of events can directly damage penumbral neurons by excitotoxicity. Finally, ischemia also damages the brain capillaries and endothelium, disrupts blood brain barrier and incites an inflammatory response whereby white blood cells, microglial cells and astrocytes infiltrate regions of infarct. Increased activation of matrix metalloproteinases (MMPs) may be involved in the blood brain barrier disruption[10]. On the other hand, ischemia-activated endothelial cells also permit angiogenesis i.e. the growth of new blood vessels from pre-existing vessels which helps restore blood flow to ischemic tissue and likely benefit long-term functional recovery. Among all the processes described in this ischemic cascade, some represent recognized or potential targets for imaging penumbra by SPECT or PET as shown next.

4. Stroke imaging by SPECT or PET.

As indicated in Fig.5, the main processes of the ischemic cascade that could be imaged by SPECT and PET are : the reduction in CBF, apoptosis and free-radicals induced necrosis, cell infiltration, MMPs activation and angiogenesis. Some of the tracers used in this goal have initially been developed to explore tumor hypoxia and myocardial ischemia and have to be tested in animal models of cerebral ischemia.

In humans, PET (^{18}F-fluorodeoxyglucose, ^{15}O-O2) measuring blood flow and metabolism is the current gold-standard imaging technique to image the perfusion defect, during cerebral ischemia. However, more specific PET and SPECT tracers for cell hypoxia have been tested in animals and humans. Nitroimidazole compounds undergo a series of enzymatic reductions in hypoxic cells, mediated by nitroreductase enzymes, followed by ring fragmentation, leading to the formation of reactive radicals, which then irreversibly bind to the cellular components. In normoxic cells, the presence of oxygen prevents the enzymatic reduction of nitroimidazole, and hence no binding is occurring. ^{18}F (18F-fluoromisonidazole or ^{18}F-FMISO, 4-bromo-1-(3[^{18}F]fluoropropyl)-2-nitroimidazole or 4-Br-^{18}F-FPN), ^{68}Ga (^{68}Ga ethylenedicysteine-metronidazole) or ^{99m}Tc (^{99m}Tc ethylene dicysteine-metronidazole or ^{99m}Tc-EC-MN) labelled nitroimidazoles have been used for in vivo assessment of myocardial ischemia, tumor hypoxia and more rarely (Fig. 6) of cerebral ischemia[11, 12, 13]. Other specific PET and SPECT tracers for neuron viability have also been used in animal models of focal cerebral ischemia. These tracers are ligands for membrane receptors which are down regulated during cell hypoxia. The most used of this kind of tracers is the antagonist of cerebral benzodiazepine receptors flumazenil which indicates the intactness of cortical neurons after stroke and differentiates between tissue with and without potential of recovery in the first hours after focal experimental ischemia. In a cat model of MCAO (Fig.7), the reduction of ^{11}C flumazenil binding reflects irreversible neuronal damage[14] early after focal ischemia.

Other processes of the ischemic cascade that has been imaged by SPECT and PET are apoptosis and necrosis. Imaging of apoptotic cells is based on their surface expression of phosphatidylserine which binds annexin V with high affinity. ^{99m}Tc-HYNIC-annexin V has been used to detect apoptotic areas by SPECT, in a rat model of MCAO[15] and in a rabbit model of neonatal hypoxia[16] whereas ^{18}F and ^{124}I labelled annexin V have only been tested on various models of liver apotposis in mice[17, 18]. Cell necrosis has only been imaged in streptozotocin-treated rats with the ^{11}C labelled phenanthridinone derivative, 2-(dimethylamino)-N-(5,6-dihydro-6-oxophenanthridin-2-yl)acetamide (PJ34) a radioligand for poly(ADP-ribose) polymerase-1 (PARP-1) which activity is increased in necrotic cells[19].

Among the cells that infiltrate in the ischemied area, activated microglial cells and astrocytes can be respectively detected by radiolabelled ligands for peripheral benzodiazepine receptors and by ^{11}C octanoate. During ischemia, peripheral benzodiazepine receptors are expressed at the cell surface of activated microglial cells. Peripheral benzodiazepine receptor labelling by ^{11}C PK11195 in a model of MCAO in baboons showed a delayed (30 days after MCAO)and transient increase in specific uptake[20]. The newly developed high-affinity peripheral benzodiazepine receptor ligand 6-chloro-2-(4'-iodophenyl)-3-(N,N-diethyl)imidazo[1,2-a]pyridine-3-acetamide, known as CLINDE, was radiolabelled with ^{123}I to detect in vivo neuroinflammatory changes in a model of autoimmune encephalomyelitis[21] but has never been tested in models of cerebral ischemia. Octanoate is taken up into the brain and is converted in astrocytes to glutamine through the tricarboxylic acid cycle after β-oxidation. So, ^{11}C octanoate has been used as a tracer for astroglial function in a canine model of MCAO; the uptake of ^{11}C octanoate increase in ischemic regions[22].

Finally, the blood brain barrier disruption and compensatory angiogenesis can be respectively imaged using radiolabeled MMPs inhibitors and ligands for the αvß3 integrin receptor. Activation of MMPs is associated with numerous deseases including stroke[10]. Non invasive visualisation of MMP activity in vivo can be achieved using peptidyl MMP inhibitors such as the peptide CTT (CTTHWGFTLC) conjugated with the bifunctional chelator DOTA (1,4,7,10-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid) for radiolabeling with the PET isotope ^{64}Cu[23]. Currently, several research groups are working on the development of ^{123}I-, ^{11}C-, and ^{18}F-labeled ligands based on nonpeptidyl MMPIs with carboxylate or hydroxamate moieties for zinc complexation that could function as in vivo MMP imaging agents[24]. These MMP inhibitors have been used to detect hypoxia-induced MMP hyperactivity in experimental metastatic tumor models. The αvß3 integrin is a receptor for the extracellular matrix proteins (vitronectin, fibronectin, fibrinogen, lamin, collagen, Von Willibrand's factor, osteoponin) with the exposed arginine-glycine-aspartic (RGD) tripeptide sequence which is expressed in angiogenic vessels and therefore represents a potential novel target for imaging angiogenesis[25]. ^{18}F or ^{111}In radiolabeled cyclic RGD peptide dimers and tetramers have been proposed as new radiotracers to image angiogenesis in tumors[26] and during myocardial ischemia[27] (Fig. 8) or inflammatory diseases. Finally, VEGF, which is an angiogenic protein secreted in response to hypoxia and overexpressed by ischemic microvasculature has been used, after ^{111}In labeling, to image angiogenesis in a rabbit model of hindlimb ischemia[28]. ^{124}I radiolabeled mAb against VEGF[29] also permitted to localize angiogenic areas in tumor-bearing mice.

In terms of perspectives, it is possible to image tumor hypoxic cells with a reporter gene construct that would be transactivated by the upregulation of the hypoxia inducible factor 1 (HIF-1). Wen et al.[30] used the herpes simplex virus 1-thymidine kinase (HSV1-tk) as reporter gene construct under the regulation of an artificial hypoxia-responsive enhancer/promoter. In this model, tumor hypoxia would up-regulate HIF-1, and would transactivate the HSV1-tk gene through the hypoxia-responsive promoter. The expression of this reporter gene can be assessed with the ^{124}I-labeled reporter substrate 2'-fluoro-2'-deoxy-1-beta-D-arabinofuranosyl-5-iodouracil (^{124}I-FIAU), which is phosphorylated by the HSV1-tk enzyme and trapped in the hypoxic cells. The reporter gene approach could also be applied to the follow up of stem cell therapy on animal models of stroke by SPECT or PET as Bengel et al.[31] had done with cardiomyoblasts transduced with the human sodium iodine transporter gene (hNIS), injected in the myocardium of nude rats and imaged by ^{124}I.

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5. LEGENDS FOR FIGURES

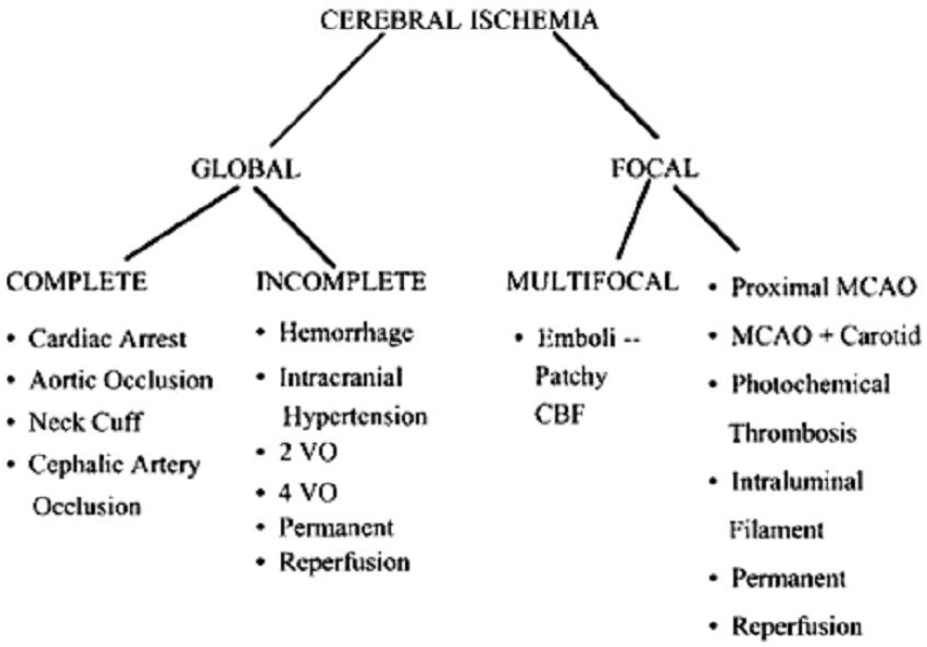

Fig. 1 : Classification of strokes; from Traystman et al.[2]

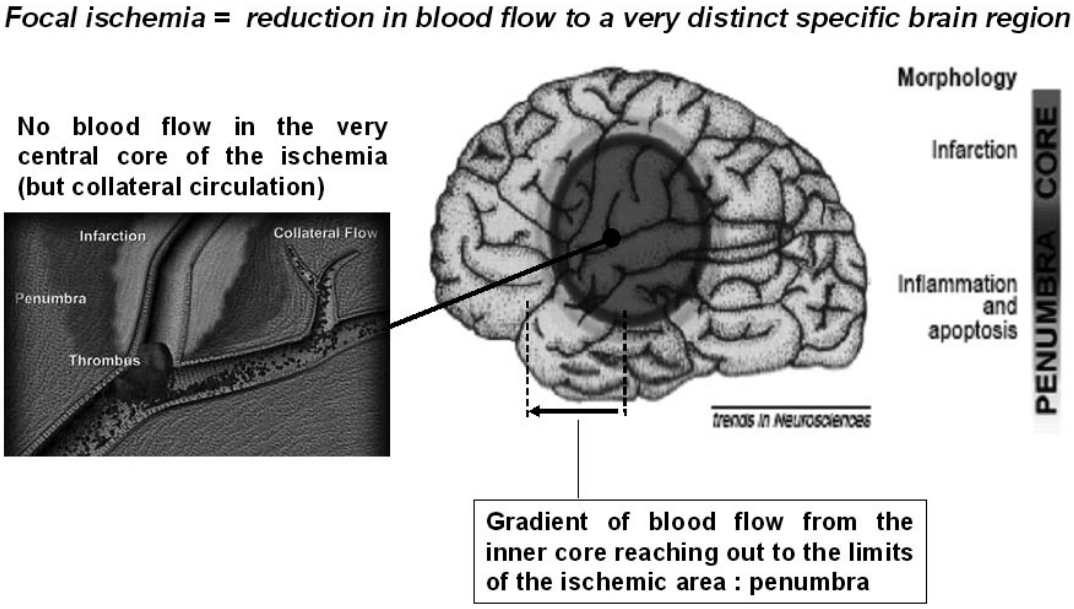

Fig. 2 : The concept of penumbra in focal ischemia from Dirnagl et al.[32]

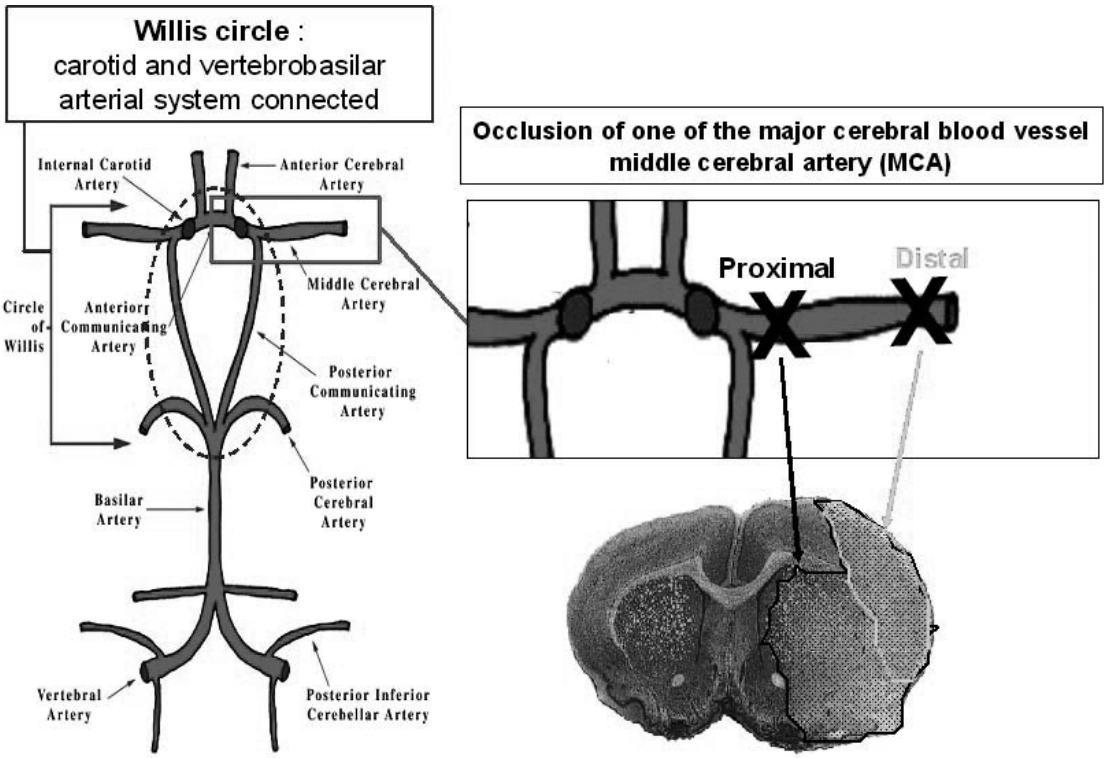

Fig. 3 : Proximal and distal occlusion of middle cerebral artery in rats

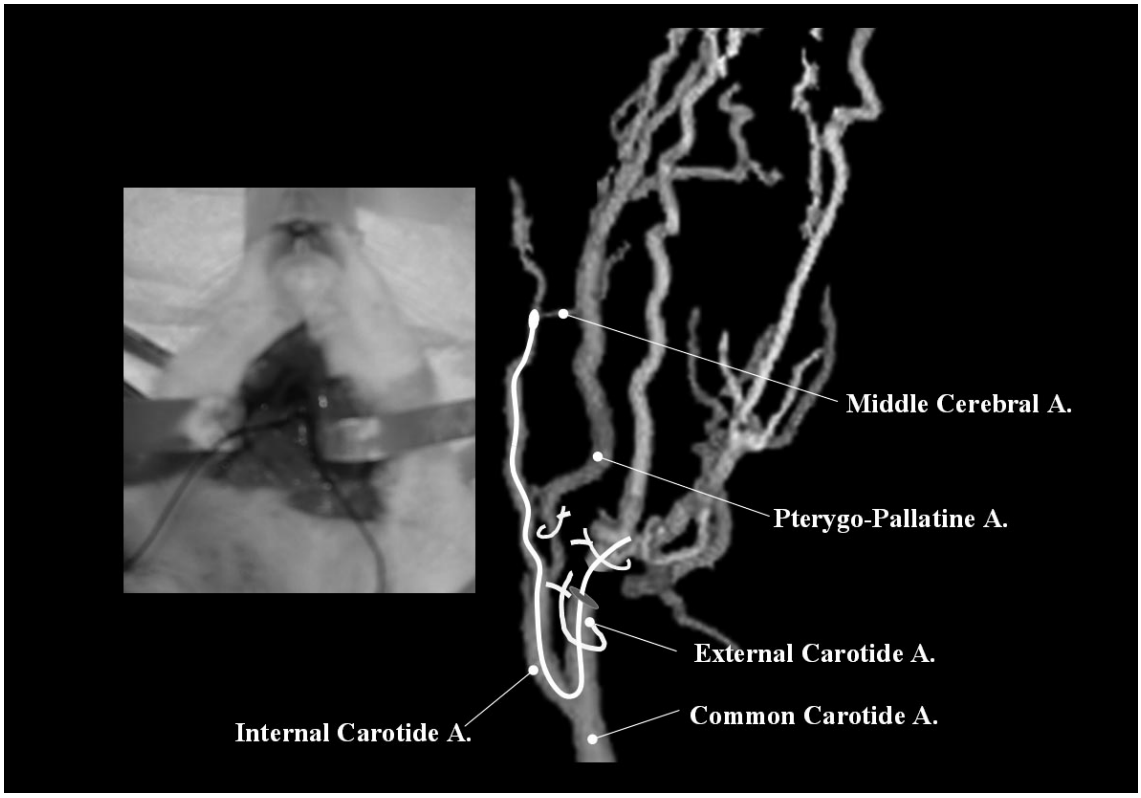

Fig. 4 : Proximal occlusion of the middle cerebral artery in rats by a nylon thread

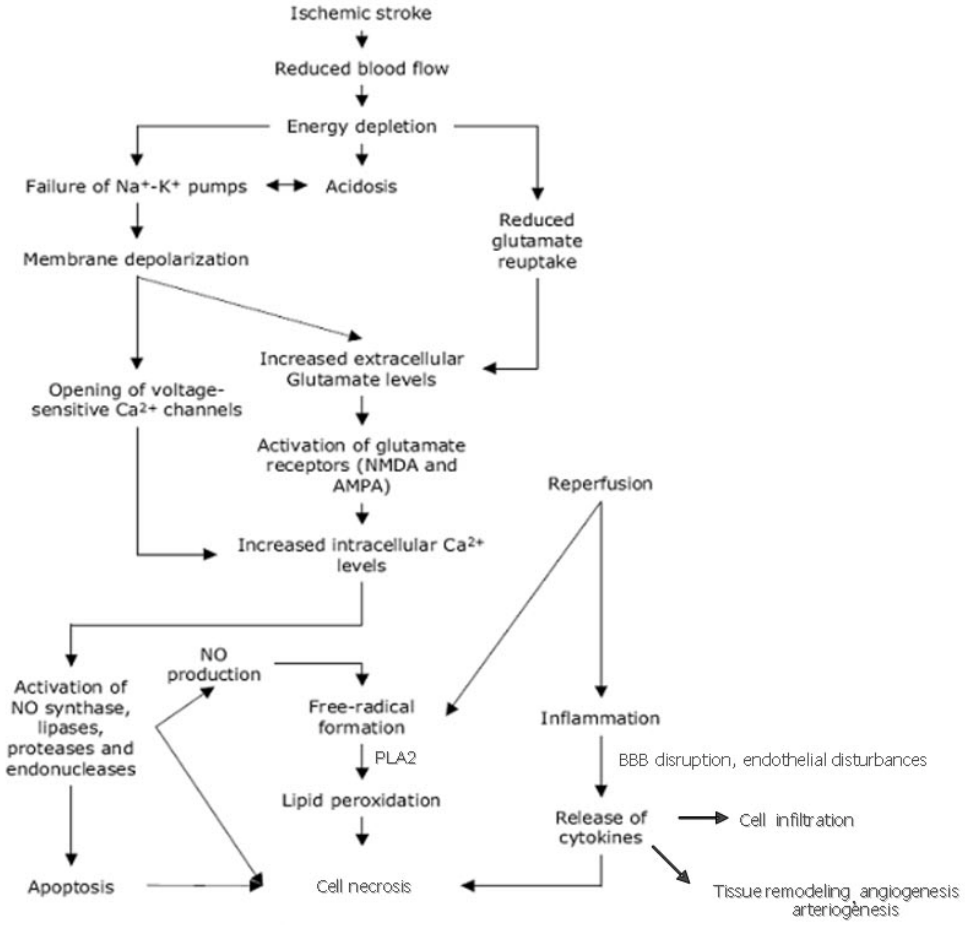

Fig. 5 : Ischemic cascade; from Smith[8]

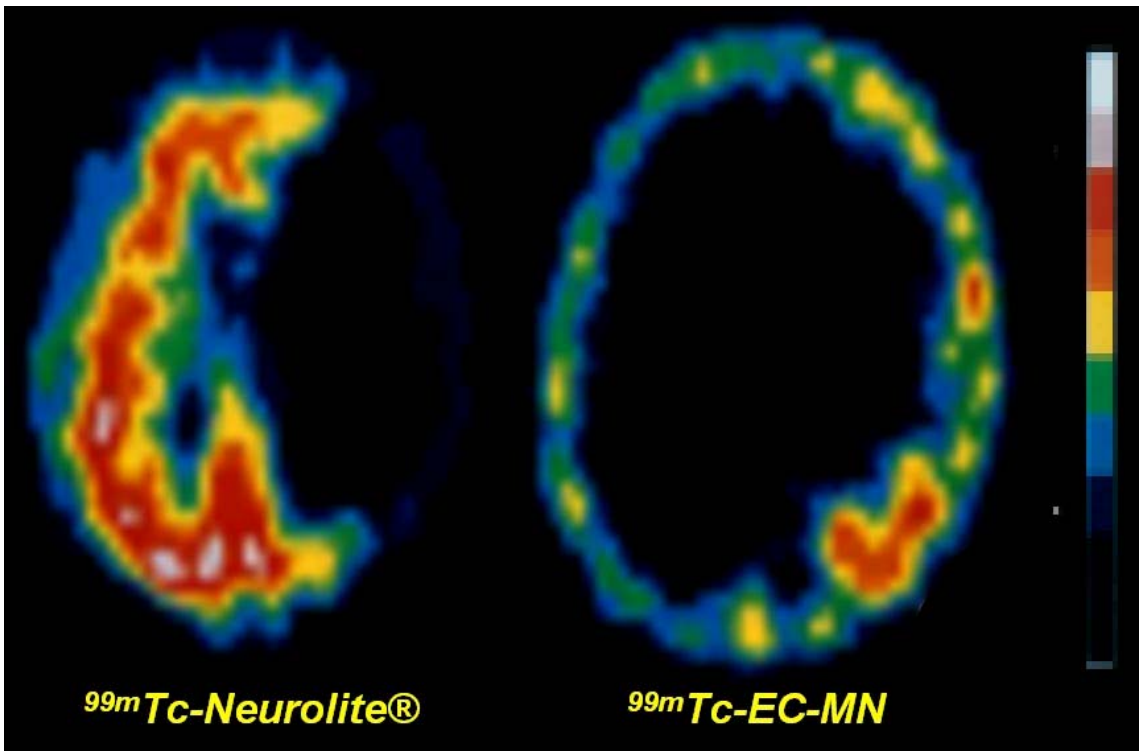

Fig. 6 : ^{99m}Tc-ECD (Neurolite®) and ^{99m}Tc-EC-MN (metronidazole) human brain SPECT showing of good uptake of ^{99m}Tc-EC-MN in the area of poor ^{99m}Tc-ECD uptake; from Song et al.[13]

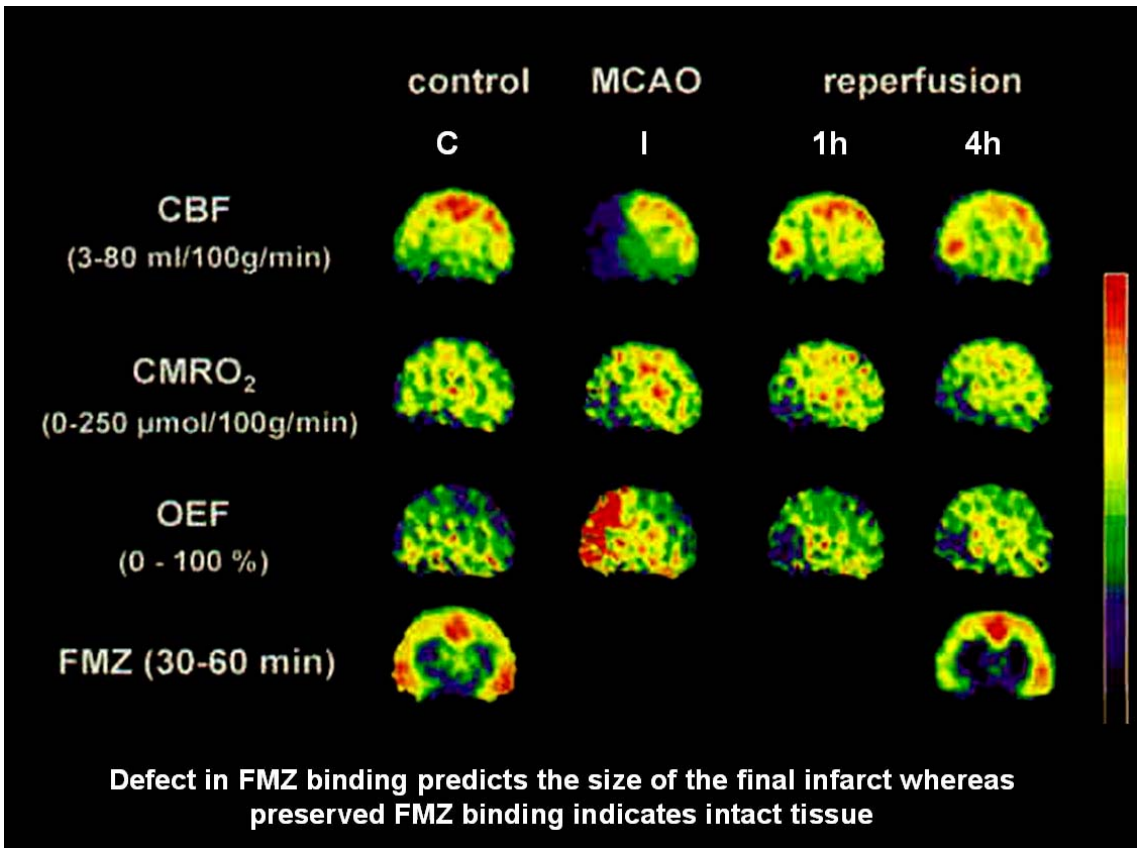

Fig. 7 : reduction of ^{11}C-flumazenil binding in a cat model of MCAO; from Heiss et al.[14]

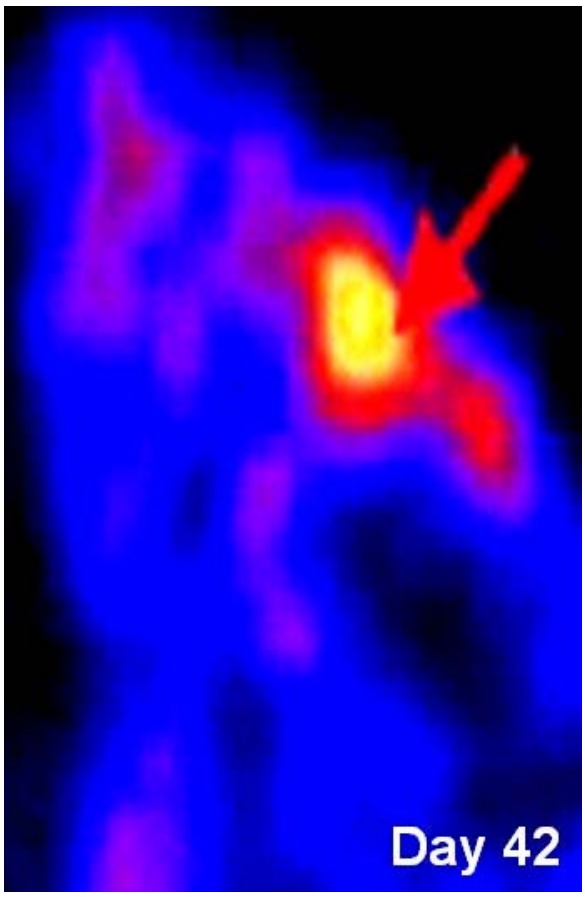

Fig. 8 : microPET angiogenesis imaging with ^{18}FFB-RGD injected in mouse after inoculation of 105 U87MG human glioblastoma cells to the forebrain; from Chen et al.[26]