

LIPID PEROXIDATION IN FOCAL CEREBRAL ISCHAEMIA AND REPERFUSION

(PART I)

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SUMMARY :

In this study, a rat model of reversible focal cerebral ischaemia is standardized and the involvement of oxygen radicals in focal cerebral ischaemia and reperfusion injury is investigated.

Ischaemia at the frontoparietal cortex and lateral segment of the caudate putamen was achieved in all animals of the occlusion group by inserting a nylon thread through the extracranial carotid artery, ascending it up to anterior cerebral artery and occluding the origin of the middle cerebral artery. Reperfusion was accomplished by pulling the embolic thread out of the wound till resistance was felt. Neurological deficits were graded on the Bederson Scale. The level of lipid peroxidation was estimated by assay of major thiobarbituric acid reactive malondialdehyde. Infarct size was determined by the lack of staining for mitochondrial dehydrogenase activity with triphenyltetrazolium chloride.

In normal animals, the mean MDA level of the frontoparietal cortex and lateral segment of the caudate putamen was 4.82(+0.68) nmol/gr tissue. The ischemic or reperfused area MDA levels were higher than the corresponding area of the contralateral hemisphere ($p < 0.05$) or the normal and sham operated animals ($p < 0.03$). MDA levels of the ischemic areas varied over time; values at the first hour were 20.2 % higher than at the second hour ($p < 0.02$).

MDA level, percent of infarction and neurological grade showed correlation in the occlusion group.

KEY WORDS:

Cerebral ischaemia, free radicals, lipid peroxidation, rat.

INTRODUCTION:

Cerebral ischaemia following thrombosis, emboli, subarachnoid haemorrhage, trauma or iatrogenic interventions during therapeutic procedures, has a high incidence of morbidity or mortality and has been the centre of interest for neurological sciences. The damage depends on the severity and duration of the ischaemia.

Recirculation affects cerebral ischaemia and modifies postischemic events in various ways. Recirculation occurs frequently after spontaneous thrombolysis and break-up of cerebral emboli in a common clinical event.

In recent years, it established that free radical induced lipid peroxidation plays a major role in the tissue damage seen in ischaemia.

reperfusion and traumatic injury (4, 15, 16, 18, 21, 27, 29, 31). Due to the transient nature and the technical difficulties inherent in accurately predicting brain levels of oxygen radicals, peroxidation is usually estimated by assay of thiobarbituric acid (TBA) (6, 13, 18). The major reactive product of TBA is malondialdehyde (MDA) (13). Peroxidation of polyunsaturated fatty acids, amino acids, carbohydrates or nucleic acids result in the formation of MDA (13).

In order to standardize a small animal model of reversible focal cerebral ischaemia and to investigate the involvement of oxygen radicals in focal cerebral ischaemia and reperfusion injury in a rat model of endovascular middle cerebral artery occlusion (MCAO) was studied.

MATERIALS and METHOD:

Animal Groups Studied: Sixty-seven male Wistar rats weighing 200-+20 gr were used. Thirteen animals were used for standardization of the method. The remaining 54 animals were divided into four groups: sham operated, MCAO, reperfusion and normal. The sham operated and MCAO groups consisted of 18 rats each: ten animals for lipid peroxide assay and eight for quantification of the infarct size and neuropathology. The reperfusion group consisted of 13 animals: five for biochemical assays and 8 for quantification of the infarct size and neuropathology. In the 5 normal animals, only biochemical assays were performed.

Surgical Procedure: Rats were allowed free access to food and water before and after all procedures. They were anaesthetized with xylazine 10 mg/kg (Rompun-Bayer) and ketamine hydrochloride 50 mg/kg (Ketalar-Eczacıbaşı) intraperitoneally. Body temperature was maintained within normal limits with a heating pad. Under the operating microscope the right carotid artery was exposed with careful conservation of the vagus nerve through a midline incision of the neck. In the sham operated group, the external (CEA) and the common carotid arteries (CCA) were ligated with 6/0 silk sutures. The tip of a 4/0 monofilament nylon surgical thread (Prolene-Ethicon) was rounded by heating near a flame creating a globular stop-

per for embolization. In 31 of the remaining animals, immediately after ligation of the ECA and CCA, the middle cerebral artery was occluded with the thread introduced through an incision on the ECA. The thread was gently advanced to the internal carotid artery (ICA) and approximately 16-18 mm of it was sent cephalad till resistance was felt and slight curving of the thread was seen. The embolus extended from the bifurcation of the ICA to the proximal portion of the anterior cerebral artery, and the origin of the MCA and posterior communicating artery was occluded by the thread (Figure 1 a).

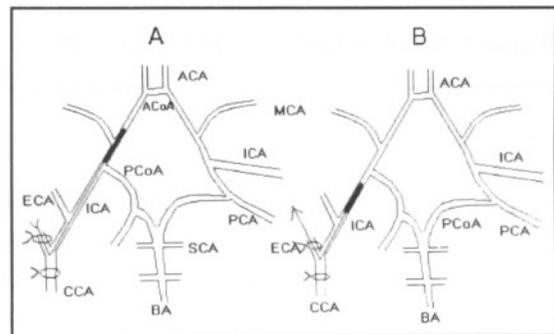


Fig. 1 : Schematic representation of position of nylon thread during MCAO (A), and reperfusion (B).

Recirculation was performed in the reperfusion group after an hour of MCAO by pulling the proximal end of the thread out of the wound till resistance was felt. The ischemic area could be reperfused via the circle of Willis through the contralateral carotid artery and basilar artery (Figure 1 b). Surgery was completed within 15 minutes.

Neurological examination: Animals recovered from anaesthesia 45 minutes after the injection of xylazine and ketamine. Neurological examination was performed at the second and 24th hours prior to sacrificing. A neurological grading system described by Bederson (2) was used (Grade 0= normal, Grade 1= forelimb flexion, Grade 2= decreased resistance to lateral push, Grade 3= circling).

Tissue preparation and lipid peroxide assay: Five normal animals and after an hour of survival, 5 rats from the each of sham operated and MCAO groups; after two hours of survival, 5 other rats from each of the three

groups were sacrificed by decapitation. The heads were immediately immersed in liquid nitrogen to stop metabolic processes and kept there till evaluation. Prior to assay, the brains were removed from the skulls in a cold room (+4°C) and a 2mm thick coronal slice was made 5mm from the frontal pole. Samples from the ischemic (or normal in the normal group) right frontoparietal cortex and the lateral segment of the caudate putamen and the corresponding area of the left hemisphere, were prepared (Figure 2 a). Tissues were transported in falcon tubes in ice.

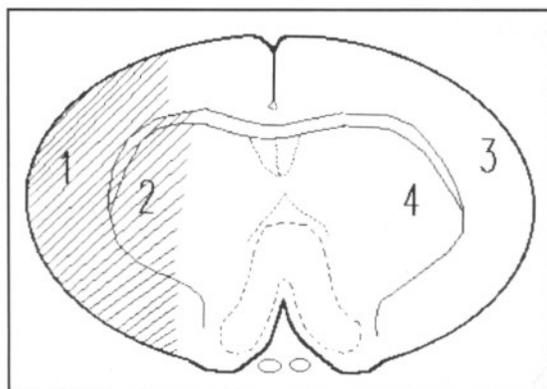


Fig. 2a : Anatomical regions of coronal section at 5mm from the frontal pole: A: Schematic drawing. 1 and 3 frontoparietal cortex. 2 and 4 lateral segment of caudate putamen. Shaded area represents ischaemic area.

Lipid peroxidation was measured by a TBA test (6). Homogenates of the samples (10 % in trichloroacetic acid) were centrifuged (3000/sec) at +4°C for 15 minutes. The supernatant was added to an equal volume of TBA (0.67 %) and heated in boiling water for 15 minutes. After cooling, the absorbancy was determined at 532 nm on a spectrophotometer. The absorbancy reading was multiplied by $1.56 \times 10^5 \text{ M}^{-5} \text{ cm}^{-1}$ and the MDA value was expressed as nmol/gr tissue.

Quantification of Ischemic Brain Damage:

Rats were sacrificed at the 24th hour of ischaemia or ischaemia and reperfusion. After decapitation, the brains were immediately and carefully removed from the skull using a microrongeur. The position of the intraluminal thread was confirmed each time. Within 2 minutes of sacrifice a 2mm thick coronal slice was made 5mm from the frontal pole. The slices were immersed in a 2 % solution of 2,3,5 triphenyltetrazoliumhydrochloride (Sigma T-8877) in saline at 37°C for 30 minutes (3). Later the sections were fixed in 10 % formalin and within 5 days the specimens were photographed (Figure 2 b). The

photographs were analyzed under a scale and the percentage of the infarction site to the brain slice was calculated.

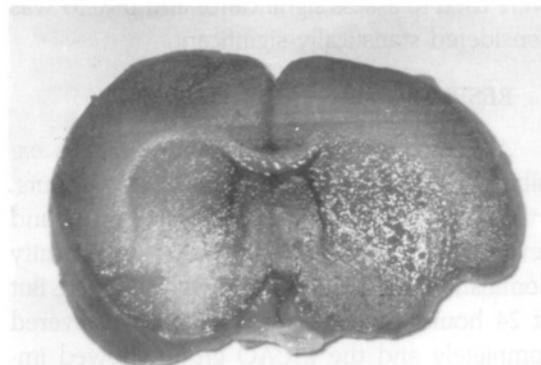


Fig 2 : b. Photograph of TTC immersed 2mm thick section. Pale areas represent ischaemic area.

Neuropathology: Three rats from each group apart from the normal animals were sacrificed at the second hour of occlusion; the brains were immediately removed and stored in 10 % formalin. With the addition of the TTC immersed slices which were used in quantification of the infarction area, all specimens were embedded in paraffin wax and sectioned. 5 microns thick sections were stained with hematoxylin and eosin and examined by light microscopy (Figure 3).

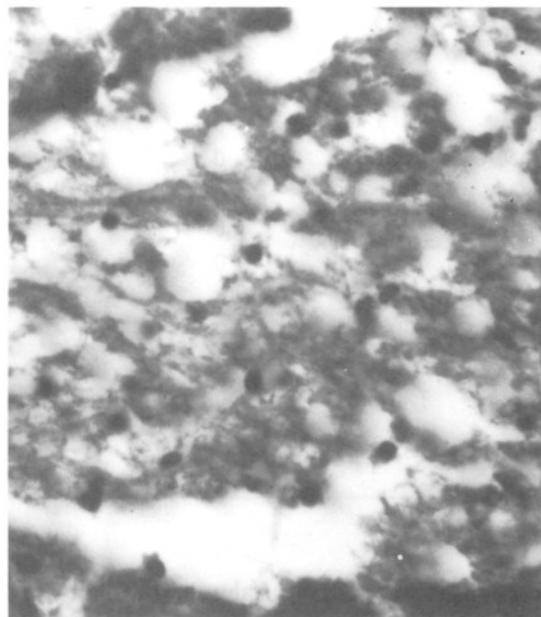


Fig 3 : Photomicrograph of infarct area in the MCAO group (Hematoxylin Eosin x 500). Shrinkage of the cells, heavy staining of nucleus and cytoplasm and vacuolisation is seen.

Statistical Analysis: Data is presented as real or mean [+standard deviation (SD)]. Mann-Whitney U and Wilcoxon-Signed Ranks tests were used to assess significance and $p < 0.05$ was considered statistically significant.

RESULTS:

All rats apart from the normal animals exhibited right Horner's syndrome. At two hours, mild hemiparesis in the reperfusion group, and severe left hemiparesis with upper extremity dominant in the MCAO group was observed. But at 24 hours, the reperfused animals recovered completely and the MCAO group showed improvement. Neurological deficits were graded on the basis of the Bederson Scale (Table 1). Only one animal showed mild hemiparesis in the early phase in the sham operated group, but had recovered completely at 24 hours.

TABLE 1

| GROUP | NO | 2hrs | NEURO. GRADE 24 hrs | ISCHAEMIA PERCENT |
|---------|----|------|---------------------|-------------------|
| MCAO | 1 | 3 | 1 | 38 |
| " | 2 | 3 | 1 | 29 |
| " | 3 | 3 | 2 | 34 |
| " | 4 | 2 | 1 | 26 |
| " | 5 | 2 | 0 | 24 |
| SHAM | 1 | 0 | 0 | 0 |
| " | 2 | 0 | 0 | 0 |
| " | 3 | 1 | 0 | 0 |
| " | 4 | 0 | 0 | 0 |
| " | 5 | 0 | 0 | 0 |
| REPERF. | 1 | 1 | 0 | 0 |
| " | 2 | 1 | 0 | 0 |
| " | 3 | 1 | 0 | 0 |
| " | 4 | 1 | 0 | 0 |
| " | 5 | 2 | 0 | 0 |

The position of the intraluminal thread was confirmed in each case of the MCAO group, and the tip was found to be in the anterior cerebral artery in all. No subarachnoidal haemorrhage was observed in any case.

In the normal animals, the mean MDA level of the frontoparietal cortex and lateral segment of the caudate putamen was $4.82(+0.68)$ nmol/gr tissue, and there was no significant difference between hemispheres (Table 2). The ischaemic area MDA levels of the MCAO groups had significant

TABLE 2

| GROUP | NO | TIME (hr) | NEURO. GRADE | | MDA RIGHT | LEVEL LEFT |
|---------|----|-----------|--------------|--|-----------|------------|
| | | | 2hrs | | | |
| MCAO | 1 | 1 | | | 15.17 | 12.66 |
| " | 2 | 1 | | | 12.90 | 5.88 |
| " | 3 | 1 | | | 12.13 | 10.82 |
| " | 4 | 1 | | | 14.32 | 12.05 |
| " | 5 | 1 | | | 15.64 | 8.06 |
| " | 1 | 2 | 3 | | 10.68 | 3.58 |
| " | 2 | 2 | 3 | | 11.66 | 8.37 |
| " | 3 | 2 | 3 | | 13.33 | 8.00 |
| " | 4 | 2 | 2 | | 10.09 | 8.67 |
| " | 5 | 2 | 2 | | 12.58 | 8.47 |
| SHAM | 1 | 1 | 0 | | 8.15 | 6.25 |
| " | 2 | 1 | 0 | | 5.97 | 6.19 |
| " | 3 | 1 | 0 | | 6.42 | 6.20 |
| " | 4 | 1 | 0 | | 6.72 | 6.98 |
| " | 5 | 1 | 0 | | 5.70 | 5.05 |
| " | 1 | 2 | 0 | | 7.23 | 6.22 |
| " | 2 | 2 | 0 | | 5.27 | 6.02 |
| " | 3 | 2 | 1 | | 9.80 | 6.61 |
| " | 4 | 2 | 0 | | 5.72 | 6.13 |
| " | 5 | 2 | 0 | | 6.52 | 6.03 |
| REPERF. | 1 | 2 | 1 | | 8.28 | 7.51 |
| " | 2 | 2 | 1 | | 9.53 | 8.62 |
| " | 3 | 2 | 1 | | 9.84 | 5.02 |
| " | 4 | 2 | 1 | | 10.48 | 8.53 |
| " | 5 | 2 | 2 | | 12.35 | 6.91 |
| NORMAL | 1 | 0 | | | 4.50 | 4.59 |
| " | 2 | 0 | | | 4.50 | 4.59 |
| " | 3 | 0 | | | 5.01 | 4.86 |
| " | 4 | 0 | | | 5.99 | 5.69 |
| " | 5 | 0 | | | 3.55 | 4.02 |

differences from the right frontoparietal cortex and lateral segment of the caudate putamen MDA levels of the sham operated and normal groups ($p < 0.03$) (Figure 3). In the MCAO and reperfusion groups, the MDA levels of the ischaemic or reper-

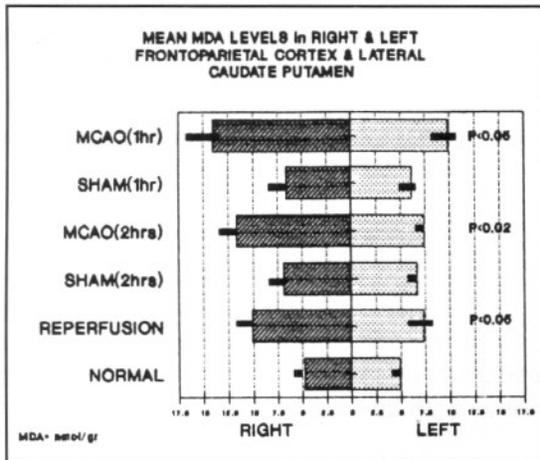


Fig. 4 :

fused areas were significantly higher than the corresponding area in the contralateral hemisphere (Figure 4). Although MDA levels of the sham operated group were higher than in the normal groups, there were no statistically significant difference between hemispheres.

MDA levels of the ischaemic areas varied with time; values at the first hour were 20.2 % higher than the second hour findings ($p < 0.02$). Results of the reperfusion group were lower than the occlusion group at the second hour, but this was not statistically significant ($p > 0.05$) (Figure 5).

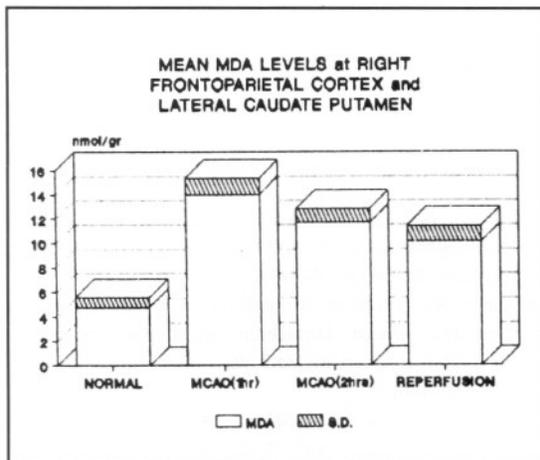


Fig. 5 :

The percentage of infarction in the MCAO group ranged between 24 and 32 (Figure 2 B), but no animals from the sham operated or reperfused group showed infarction at 24. hours (Table

1). MDA levels and neurological grade showed positive correlation in the MCAO group.

Histological examination showed swelling of the cells of the ischaemic area at the second hour of the occlusion in the MCAO and reperfusion series. The sham operated group was normal. At the 24th hour, shrinkage of the cells, heavy staining of nucleus and cytoplasm and vacuolisation was seen in the MCAO group (Figure 3), while the oedema in the reperfusion group persisted.

DISCUSSION:

Our aim was to standardize a small animal model of reversible focal cerebral ischaemia and determine the involvement of oxygen radicals in focal cerebral ischaemia and reperfusion injury. To achieve this goal, we chose a rat model of intraluminal MCAO without craniectomy. Laboratory rat have been used widely for focal cerebral ischaemia because of their anatomical similarity to human cerebral circulation. They are relatively inexpensive and easily available.

Many models of reversible focal cerebral ischaemia have been defined (24, 26). Transcranial interventions such as clip application or ligation of MCA disrupts autonomic nerve supply to the vascular bed or injures brain tissue and causes neurogenic artifacts complicating physiological and biochemical data analysis (24). Craniotomy also causes changes in the intracranial pressure and affects cerebral blood flow (25). Development of ischaemia after extracranial occlusion of a major cerebral artery is usually prevented by an efficient intracranial collateral circulation. Additional stress such as hypoxia or hypotension have many complicating factors.

The model that we used was a slight modification of Koizumi (20) which was repeated and modified by Nagasawa (25) and Longa (22). We inserted the thread through the ECA as recommended by Longa, but did not ligate the pterygopalatine artery. In our pilot studies, without ligating the PPA, the thread entered this artery if it was introduced through the ICA; but if it was introduced through the ECA, the embolus ascended by following the counter wall of the PPA and did not enter it. This finding shortened the surgical procedure by approximately two

minutes. Our results were similar to Nagasawa's (25) and Longa's (22) findings; the position of the intraluminal thread was found to be in the anterior cerebral artery occluding the origin of the middle cerebral artery and an infarct of the frontoparietal cortex and lateral segment of the caudate putamen was observed each time. The histological findings were also compatible with the literature (5).

Due to the transient nature and the technical difficulties inherent in accurately predicting brain levels of oxygen radicals, peroxidation was estimated by assay of thiobarbituric acid. Ferrendelli (11) determined that intact mice brains immersed in liquid N₂ cooled to 0°C. between 2-19 seconds; and in our study, metabolic processes were stopped in approximately 45 seconds. Gutteridge (13) showed that peroxidation of polyunsaturated fatty acids, amino acids, carbohydrates or nucleic acids all result in the formation of malondialdehyde (MDA), the major thiobarbituric acid reactive product.

Free radicals are molecules that have an odd number of electrons. They are continuously produced in tissues during normal aerobic metabolism, but can lead to tissue damage if they are generated in excess (10, 12, 14, 23). With the onset of focal or global ischaemia, the supply of oxygen and glucose ceases. This triggers free radical processes and causes reduction in cerebral tissue concentrations of the endogenous antioxidants (14, 17). The result of this sequence is the excess production of radicals such as superoxide (O₂⁻) or hydroxyl (OH[•]) radicals (12). The brain is rich in oxidizable substrates, mainly catecholamines and unsaturated lipids (9). Lipid peroxidation associated with a free radical attack on cellular membranes has been implicated as a cause of cellular injury in animal central nervous system trauma and ischaemia models (8, 16, 17, 27, 28, 31, 33). We also found a positive relationship between MDA levels, proportion of the ischemic area to normal brain tissue and neurological grading of the MCAO groups. This study also showed that the lipid peroxide level after cerebral ischaemia was at its highest value at the first hour of MCAO as in spinal ischaemia (1, 19)

Previously, it was found that reperfusion af-

ter global ischaemia caused a burst of superoxide radical production which again resulted in membrane lipid peroxidation (7, 30, 32). Recirculation after MCAO occlusion in the acute stage of ischaemia has been known to cause blood-brain barrier damage and vasogenic oedema in other animal species (26). In this first study of reversible focal cerebral ischaemia in rats, the MDA levels of the reperfused tissues were found to be higher than the corresponding area of the contralateral hemisphere or sham operated and normal animal brain tissues; but not as high as in the MCAO groups.

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