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REAL-TIME MONITORING OF MITOCHONDRIAL FUNCTION AND CEREBRAL BLOOD FLOW FOLLOWING FOCAL ISCHEMIA IN RATS

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Focal ischemia due to reduction of cerebral blood flow (CBF), creates 2 zones of damage: the core area, which suffers severe damage, and penumbra area, which surrounds the core and suffers intermediate levels of injury. *Objectives*: A novel method is introduced, which evaluates mitochondrial function in the core and in the penumbra, during focal cerebral ischemia. *Methods*: Wistar rats underwent focal cerebral ischemia by middle cerebral artery occlusion (MCAO) for 60 minutes, followed by 60 minutes of reperfusion. Mitochondrial function was assessed by a unique Multi-Site — Multi-Parametric (MSMP) monitoring system, which measures mitochondrial NADH using fluorometric technique, and CBF using Laser Doppler Flowmetry (LDF). *Results*: At the onset of occlusion, CBF dropped and NADH increased significantly only in the right hemisphere. CBF levels were significantly lower and NADH significantly higher in the core than in the penumbra. After reperfusion, CBF and NADH recovered correspondingly to the intensity of ischemia. *Conclusion*: Application of the MSMP system can add significant information for the understanding of the cerebral metabolic state under ischemic conditions, with an emphasis on mitochondrial function.

Keywords: Mitochondrial function; cerebral blood flow; focal ischemia.

1. Introduction

Brain focal ischemia involves a shortage of cerebral blood flow (CBF).[1,2] Since the brain function is completely dependent on a continuous blood supply, CBF deterioration leads to a disruption of cerebral oxygen balance (supply versus demand) resulting in diverse changes in the tissue, creating a new microenvironment in the ischemic area.[3–5] These changes include the disruption of extra-cellular ion balance, increased Lactate levels, decrease in extra-cellular pH, Glutamate and free radicals accumulation and suppression of spontaneous electrical activity.[1,2,5–7]

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Oxygen shortage in the brain affects the intracellular respiratory chain activity, resulting in a decrease in adenosine triphosphate (ATP) synthesis, and an increase in mitochondrial NADH levels.[3,8] Due to its high sensitivity to intracellular oxygenation, mitochondrial NADH level can serve as an indicator of tissue pathological state.[3,9]

Brain focal ischemia entails gradual injury, spreading from severe injury in the lesion center (core) toward moderate damage in periphery regions (penumbra), due to collateral blood supply,[1] affecting the energy balance and leading to mitochondrial injury.[5,8] In reperfusion, mitochondrial recuperation is more clearly observed in the core than in the penumbra.[8,10]

The present study introduces a novel method, which combines the evaluation of mitochondrial function and cerebral blood flow in the core and in the penumbra, during and following focal cerebral ischemia of various severity.

2. Methods

The monitoring of the rat brain was performed by a unique Multi-Site–Multi-Parametric (MSMP) monitoring system, designed for real-time and continuous measurements.[11,12] The MSMP system includes 2 channels, each comprising a probe, with a bundle of optical fibers. Each probe combines NADH monitoring using the fluorometric technique, and cerebral blood flow (CBF) monitoring using Laser Doppler Flowmetry (LDF), as specified in previous articles.[11–13]

The experiments were performed on male Wistar rats (200–300 g), according to the NIH guidelines for the care and use of laboratory animals, and approved by the Institutional Animal Care committee of Bar-Ilan University. Animals were anesthetized by an IP injection (0.3 ml/100 g) of Equithesin,[11] and additional injections were given every 30 minutes during monitoring (0.1 ml/100 g). A heating pad was placed under the rat to maintain body temperature at 37°C. Preliminary experiments, as well as results from other studies, show no significant changes in blood pressure, heart rate, blood pH, pO_2 and pCO_2 following the MCAO procedure.[14] Therefore, and in order to simplify the current model, no physiological parameters were monitored.

Each animal was placed in a ventral position, and the skull was exposed by a midline incision. Holes were drilled in the parietal bones for cannulas, in which the probes were placed. The cannulas were placed epidurally and fixated to the skull using dental acrylic cement, with additional screws for better fixation. Two models of cerebral measurement during and after MCAO were developed (Fig. 1). In the bilateral model ($n = 8$), 2 probes were placed in parallel locations on both hemispheres (each on a different hemisphere), 1 mm posterior and 5 mm lateral to the Bregma. In the unilateral model ($n = 9$), both probes were placed on the right hemisphere in medial and lateral positions, using a double cannula that was placed 1 mm posterior and 1 mm lateral to the Bregma. At this point, the rat was turned over on its back, and the MCAO surgery was performed on the right

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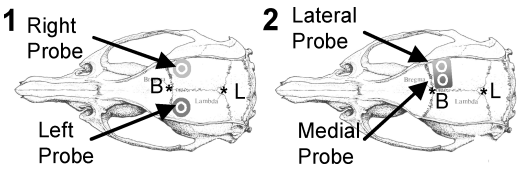

Fig. 1. Schematic representation of the location of the MSMP system fibers on the monitored sites in the bilateral model (1) and unilateral model (2). B-Bregma, L-Lambda.

artery, according to the protocol of Longa *et al.*,[15] with several adaptations. A 4/0 nylon filament, with a heated bulb head covered with Poly-L-Lysine, was inserted through a puncture to the external carotid artery, onto the common carotid artery. The MSMP probes were then placed in their cannulas, and the monitoring began. After a short anoxia, the nylon filament was removed from the common carotid artery and inserted 21 mm into the internal carotid artery, occluding the basis of the MCA. The animals were monitored before, during and after focal ischemia for 60 minutes. At the end of each experiment, the rats were sacrificed by N_2 inhalation.

Mean ± S.E. values were calculated for the following parameters: reflectance (Ref), fluorescence (Flu), NADH and CBF. T-test was used to determine significant differences between each parameter and its baseline, and between 2 areas in the brain.

3. Results

During the bilateral monitoring of the brain (Fig. 2), CBF dropped to a level of $22 \pm 5\%$ at the onset of the occlusion only in the right hemisphere. This decrease was significant during the whole ischemic period, as compared to basal levels ($p < 0.001$) and to the left hemisphere ($p < 0.001$). Fluorescence levels in the right hemisphere showed an increase to a level of $170 \pm 15\%$ 5 minutes after the occlusion, with significant differences from the baseline ($p < 0.05$) and from the contra-lateral hemisphere ($p < 0.01$) during 30 minutes of the ischemic period. Reflectance levels showed an increase of $23 \pm 9\%$, which was significant as compared to the contra-lateral hemisphere for one minute ($p < 0.05$), then gradually decreased until stabilizing. Due to these changes, NADH levels showed a $50 \pm 5\%$ increase in the right hemisphere, which was significantly different from the contra-lateral hemisphere during 30 minutes after the occlusion ($p < 0.001$), and then returned to the baseline. In reperfusion, all parameters returned to the baseline, except for a significant decrease in NADH levels to $75 \pm 7\%$ ($p < 0.05$). No significant changes were observed in the left hemisphere, during the entire experiment.

During the unilateral brain monitoring (Fig. 3), CBF levels decreased to $20 \pm 7\%$ in the lateral site ($p < 0.001$, compared to the baseline) and to $40 \pm 10\%$ in the medial site ($p < 0.001$), exhibiting significant differences between the sites ($p < 0.05$). Reflectance increased to a level of $170 \pm 19\%$ in the lateral site 3 minutes after the occlusion onset; these values were significantly higher than the

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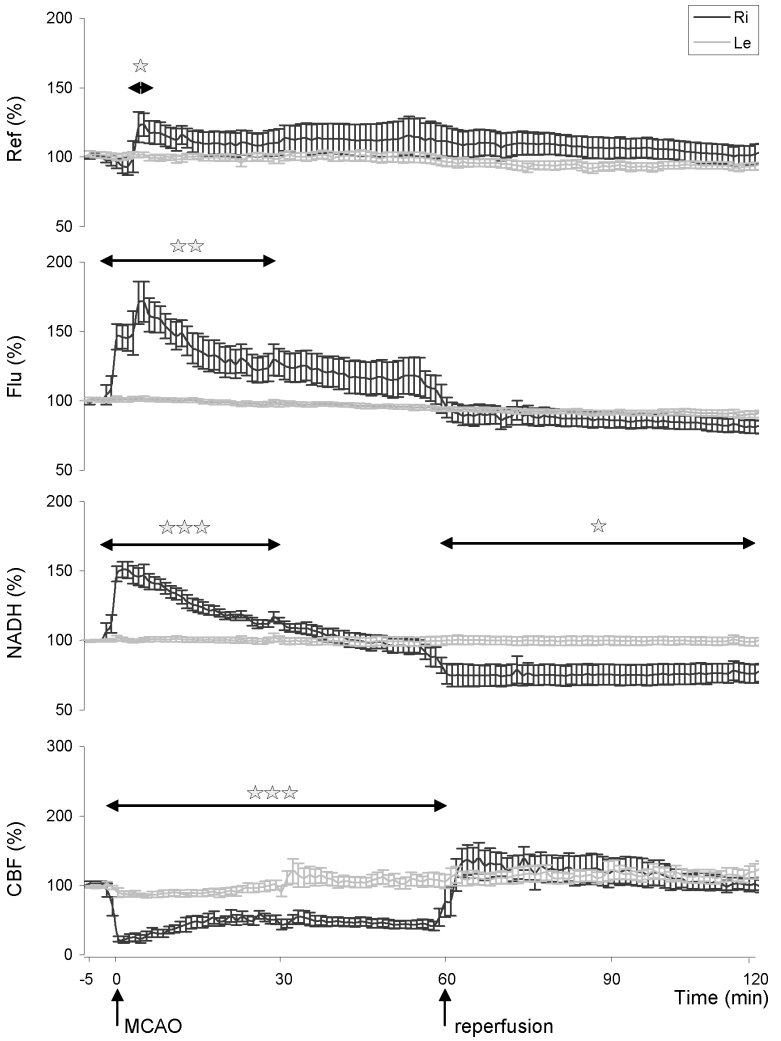

Fig. 2. Hemodynamic and metabolic changes in the bilateral monitoring model, following right MCAO for 60 minutes and reperfusion. Right hemisphere — Ri, Left hemisphere — Le. The results are presented as mean ± S.E. ($n = 8$). Significant differences between the two hemispheres (in each minute) are marked by asterisks: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

baseline ($p < 0.001$) and when compared to the medial site ($122 \pm 9\%$, $p < 0.05$). Fluorescence reached peak levels of $213 \pm 22\%$ in the lateral site ($p < 0.001$, compared to the baseline) and $167 \pm 19\%$ in the medial site ($p < 0.01$), and then stabilized and remained higher than the baseline for the rest of the ischemic period, exhibiting a significant difference between the 2 sites ($p < 0.05$). NADH increased to peak levels of $145 \pm 10\%$ in both sites ($p < 0.01$, compared to the baseline), followed by a decrease to the baseline in the lateral site and to $120 \pm 13\%$ in the medial site.

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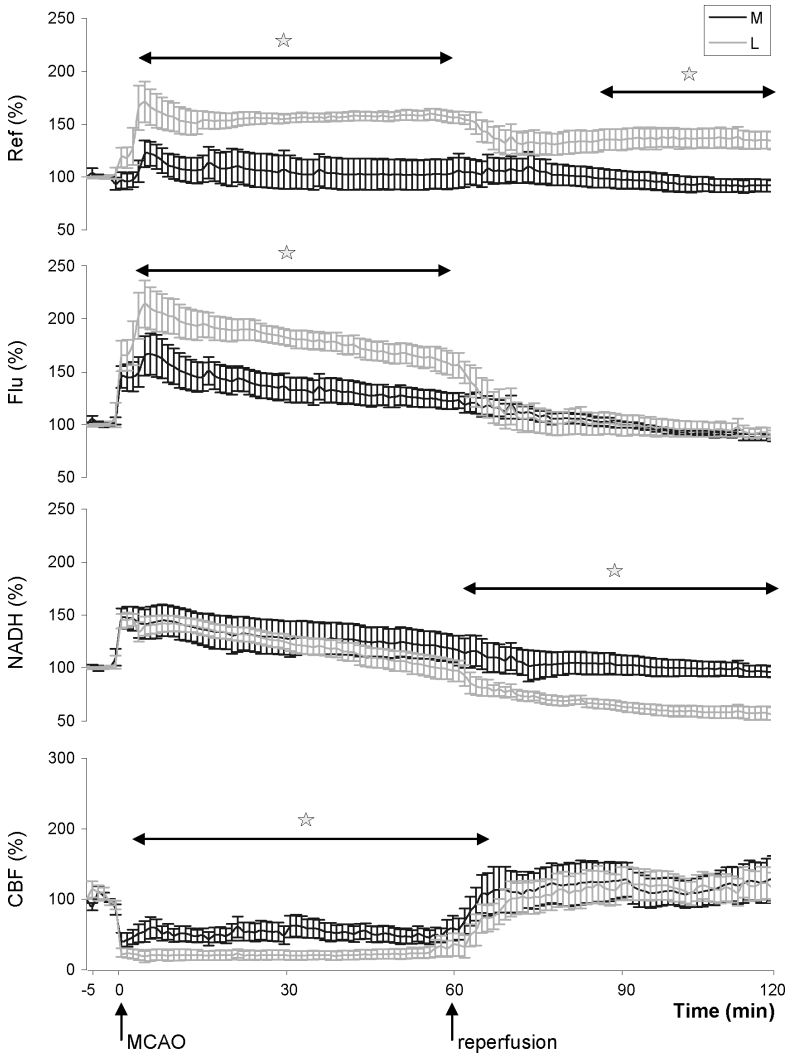

Fig. 3. Hemodynamic and metabolic changes in the unilateral monitoring model following right MCAO for 60 minutes and reperfusion. Lateral monitoring location — L, Medial monitoring location — M. The results are presented as mean ± S.E. ($n = 9$). Significant differences between the two hemispheres are marked with asterisks (*) $p < 0.05$.

In reperfusion, CBF levels increased and returned to the baseline one hour following reperfusion. The recovery of CBF was more rapid in the medial area, as compared to the lateral area. Reflectance levels declined to the baseline in the medial site and to $135 \pm 9\%$ in the lateral site ($p < 0.01$), demonstrating significant differences between the 2 sites ($p < 0.05$). Fluorescence decreased gradually until reaching baseline levels 30 minutes after reperfusion. NADH levels decreased significantly in the lateral site to $80 \pm 7\%$ as compared to the baseline ($p < 0.001$),

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while a gradual decrease toward the baseline was observed in the medial site, resulting in significant differences between the 2 sites ($p < 0.05$).

4. Discussion

The monitoring of the brain during an ischemic stroke induced by MCAO, may contribute to the understanding of the events occurring in ischemia and reperfusion. The effect of right MCA occlusion on both brain hemispheres was tested to validate the model integrity, and indeed, our results showed that ischemic injury was restricted to the right hemisphere, as demonstrated by the decrease in blood supply and the increase of reduced NADH, while the left hemisphere showed no significant changes, as documented in the literature.[1,7,8] These findings verify the restricted and local nature of the damage in the cerebral tissue following MCAO, as well as the reliability of the MSMP monitoring system in detecting focal brain ischemia.

In the current study, the lateral site of the unilateral model represents the core and the medial site represents the penumbra of the focal ischemia, as can be seen from the differences in CBF between the two sites and as was reported in earlier works.[1,7] The higher levels of CBF in the penumbra are due to a partial blood supply by collateral blood vessels, resulting in a minor degree of damage compared to the core.[1,7] In addition, the recovery phase of CBF was delayed in the core, as compared to the penumbra, indicating that tissues in different degrees of ischemia have different oxygen demands.[1,4]

The ischemic core showed a secondary reflectance increase (SRI) 3 minutes after the occlusion onset, which was absent in the penumbra. This phenomenon, known as ischemic depolarization (ID),[5] is caused by an increase in extra-cellular potassium levels, due to oxygen shortage in the brain, and leads to the absence of cerebral electrical activity.[16] The high levels of reflectance for the entire ischemic period, lead to an artifact in NADH calculation, as previously observed in cases of ischemia and at death.[9,16] As a result, low NADH levels were observed during ischemia and at reperfusion. In such cases, the non-corrected fluorescence might serve as a better indicator of the mitochondrial state,[9,16] and indeed, fluorescence levels were higher in the core than in the penumbra, suggesting a more severe mitochondrial injury in the former area,[3,13] as seen in other studies.[1,8]

In conclusion, the application of the MSMP monitoring system can contribute additional novel information regarding the mitochondrial function and cerebral tissue state following focal ischemia, and may be potentially used in clinical practice.

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