Real Time Evaluation of Tissue Vitality by Monitoring of Microcirculatory Blood Flow, HbO_2 and Mitochondrial NADH Redox State.

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ABSTRACT

Monitoring of tissue vitality (oxygen supply/demand) in real time is very rare in clinical practice although its use as an early warning alarming system for clinical care medicine, is very practical. In our previous communication (SPIE 2003) we described the Tissue Spectroscope – TiSpec02, by which tissue microcirculatory blood flow (TBF) and mitochondrial NADH fluorescence were measured using a single light source (390nm). In order to improve the measurement capabilities as well as to decrease dramatically the size and cost of this clinical device, we have changed the TiSpec02 into a multi-wavelength illumination system in the new TiSpec03. In order to measure microcirculatory blood flow by laser Doppler flowmetry we used a 785nm laser diode. For mitochondrial NADH fluorescence measurement we adopted a 370nm LED. For the determination of the oxygenation level of hemoglobin (HbO_2) we used the 2-wavelength reflectance technique. This new monitored parameter that was added to the TiSpec03 increases the accuracy of the diagnosis of tissue vitality. The bundle of optical fibers used to connect the tissue to the TiSpec03, was integrated into a special anchoring methodology depending on the monitored tissue or organ. In order to test the performance of the improved TiSpec we have used it in experimental animals brain models exposed to various pathophysiological conditions. Rats and gerbils were anesthetized and the fiber optic probe was located epidurally used dental acrylic cement. During anoxia and ischemia, the lack of O_2 led to a clear decrease in TBF and HbO_2 while NADH shows a large elevation. When brain activation was induced by cortical spreading depression (SD), the elevated O_2 consumption was recorded as a large oxidation (decrease) of mitochondrial NADH while TBF increase dramatically. Blood HbO_2 was not affected significantly by the SD wave.

Key Words: Tissue Vitality, Tissue Spectroscope, Mitochndrial NADH, Laser Doppler Flowmetry, Hemoglobin saturation, Critical Care Medicine.

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1. INTRODUCTION

The involvement of mitochondrial function in various pathophysiological conditions, developed in experimental and clinical situation, is widely documented. Nevertheless, real time monitoring of mitochondrial function In-vivo is very rare due to the lack of suitable instruments that could be operated by minimally trained researchers and clinical staff. The relationship between mitochondrial and neuronal survival was reviewed recently (1) but the authors did not cite papers describing the In-vivo monitoring of mitochondrial function at real time mode. The question is, how the mitochondria of intact tissues behave under In-vivo conditions as compared to the isolated mitochondria. In our previous publication, we described the second generation of the Tissue Spectroscope (TiSpec02) based on the illumination of the tissue with a stabilized 390nm laser diode which enabled the monitoring of three physiological parameters In-vivo (2). The TiSpec02 enabled us to monitor microcirculatory blood flow, tissue reflectance and mitochondrial NADH redox state using the same light source. Although the TiSpec02 was safer and relatively smaller as compared to the first generation TiSpec01, which was based on the He-Cd laser (325nm) as an excitation light (3), its price was quite high. The recent development of UV (i.e. 370nm) LED enabled us to develop a very small Tissue Spectroscope relative to the TiSpec02. Also, we have added another important physiological parameter, the level of blood oxygenation, to the three parameters measured by the TiSpec02.

The aim of the present study is to describe the new TiSpec03 which enables the monitoring of microcirculatory blood hemoglobin oxygenation in addition to the three parameters measured before, namely tissue microcirculatory blood flow, reflectance and mitochondrial NADH redox state. Tissue vitality is correlated to oxygen or energy balance defined as the ratio between O_2 supply and demand. Each tissue in the body is using the generated ATP for specific activities taking place in that tissue as shown in the right side of Figure 1. Therefore in order to monitor the demand for energy of the tissue, one have to use a different technology for each tissue. For example, the brain consumes more than 50% of the total energy consumption for keeping normal ionic homeostasis (4), so one can measure level of extracellular Potassium ions (K^+) as an indicator for brain energy demand (5). The heart is using a major part of its energy consumption for muscle contraction. We have shown that it is possible to monitor the demand for energy by measuring the extracellular level of K^+ in the brain using ion specific electrode (6,7). In contrary to the energy demand, energy or oxygen supply mechanisms are identical in all tissues and therefore could be monitored by the same techniques. As seen in the left side of Figure 1, at least three parameters could be monitored from any tissue in order to identify the supply of O_2 to the tissue. Monitoring of the mitochondrial NADH redox state provides information regarding supply of energy as well as the tissue energy balance (8). In order to understand the functional state of a tissue, exposed to various pathophysiological conditions, the more parameters to be monitored the better the diagnosis of the tissue will be.

The aim of the current presentation is to describe a new integrated medical device – TiSpec03 which enabled, for the first time, to monitor four parameters from the tissue using a single optical probe (consists of two fiber-optic bundles) attached to the tissue.

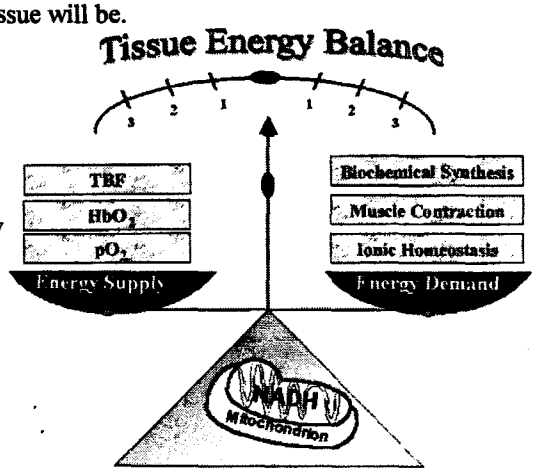


Fig. 1. Schematic presentation of the relationship between energy demand and supply in a typical tissue in the body. The demand processes are specific to each tissue while the supply mechanisms are identical in all tissues.

NADH – Nicotine amid adenine dinucleotide
TBF – Tissue Blood Flow
HbO_2 – Hemoglobin Oxygenation
pO_2 – Partial Pressure of Oxygen

2. METHODOLOGY

In our last communication (2), we described the TiSpec02 which employed a single UV laser source to optically measure three physiological parameters: Tissue blood flow using laser Doppler flowmetry, NADH fluorescence excited by the UV light, and the reflectance of the tissue by measuring the intensity of backscattered light.

Laser Doppler flowmetry requires an extremely stable laser source, usually within the red or near Infrared region. However, the excitation spectrum of the NADH lies in the near UV region. In the TiSpec02, we synthesized these apparently opposing spectral constrains by developing a unique, ultra-low noise UV light source.

The light source of the TiSpec02 was based on an external cavity stabilized UV laser diode, chopped by means of an Acousto-Optic modulator (AOM). Beam chopping is necessary to neutralizing the detected signals from external room illumination. Moreover, the chopping reduces the UV irradiation upon the tissue. We preferred keeping the laser running on CW mode and modulate the AOM rather than modulating the driving current, in order to maintain highest stability in the laser cavity.

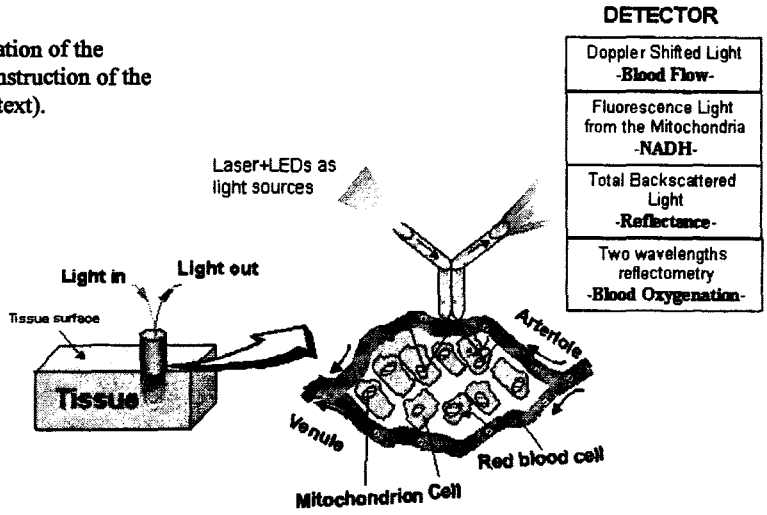
It became clear that the complexity of system must be dramatically scaled down in order to produce an affordable and smaller device. We relied on the recent developments of UV LEDs, to design a completely new light source unit (LSU) comprising of a NIR laser diode for Doppler measurement and a UV LED for reflectance measurement and NADH excitation. Additionally, The new TiSpec03 harbors a completely new module for the determination of the oxygenation level of hemoglobin (HbO_2), based on LEDs. The TiSpec03 hence utilizes four solid-state light sources to simultaneously monitor four types of optical signals, essential for the assessment of tissue vitality (Figure 2).

For the monitoring of blood oxygenation we utilized a 2-wavelength reflectance technique to analyze the absorption of light in the blood, exploiting the difference in absorption spectra of oxyhemoglobin (hemoglobin saturated with oxygen) and deoxyhemoglobin. The principles of the technique were demonstrated years ago using a time-sharing system (9). Generally, Absorption coefficient of oxyhemoglobin differs from that of deoxyhemoglobin, along most of the spectrum (10). However in some certain isosbestic wavelengths, the absorption curves cross each other and represent the same value. The change in reflectance in an isosbestic wavelength is affected only by blood volume, while a change in reflectance in a non-isosbestic wavelength is affected by the oxy-deoxy ratio as well. By subtracting the back-reflectance in these two wavelengths, one can consider the difference between the measurements as qualitative representation of blood oxygenation level.

As seen on Figure 3, the former time-sharing system used two Hg-lamp emission bands to determine the oxy-deoxy ratio in-vivo by measuring the reflectance in 585nm (R585) and 577nm (R577). Calculating –(R577-R585) yields the relative change in oxyhemoglobin state. We used the same principle with contemporary 525nm (nearly isosbestic) and 470nm (non-isosbestic) super bright LEDs. The relative change in oxyhemoglobin state is therefore derived by –(R470-R525).

It is important that the two wavelengths are close and roughly within the same range of absorption coefficient. Thus, the absorption of the tissue may be considered constant and get neutralized.

Fig. 2. Schematic presentation of the principles used for the construction of the TiSpec03 (for details see text).



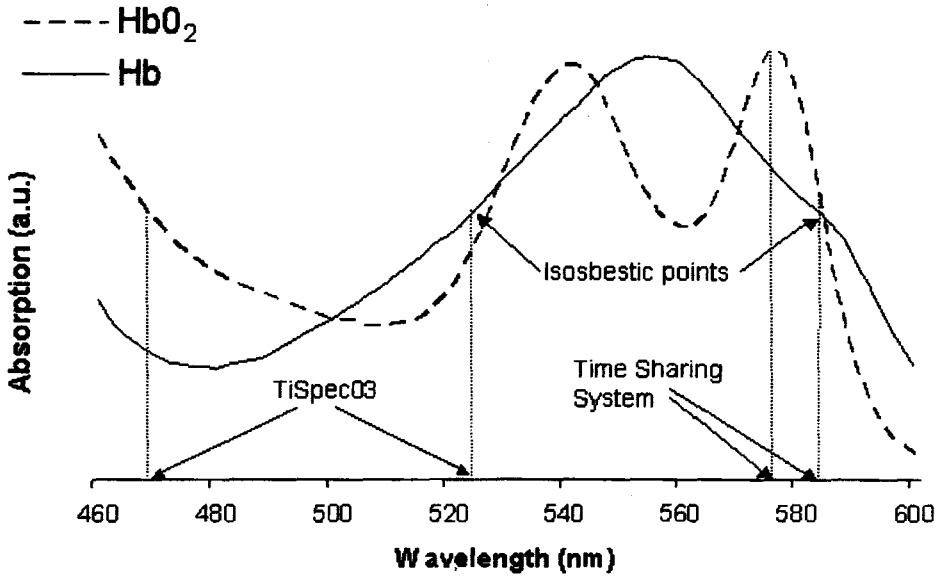


Fig. 3. Absorption spectra of oxy- and deoxyhemoglobin. Wavelengths involved in monitoring tissue blood oxygenation by the time-sharing system and by the TiSpec03 are marked.

TiSpec03 uses the same A/D converter as TiSpec02, now connected to an external laptop PC. Software was rebuilt on LabVIEW 6.0 development environment (National Instruments, Austin, TX). The software employs two virtual digital oscilloscopes for signals synchronization and identification. Data is acquired, calibrated and smoothed (using a median filter algorithm). The processed data is then displayed on the screen and stored on a local hard drive. Operator may add remarks or notes as required. Data storage format is compatible with Microsoft Excel electronic spreadsheet, and is readily available for off-line review and analysis.

A schematic blocks diagram of the new TiSpec03 is shown in Figure 4.

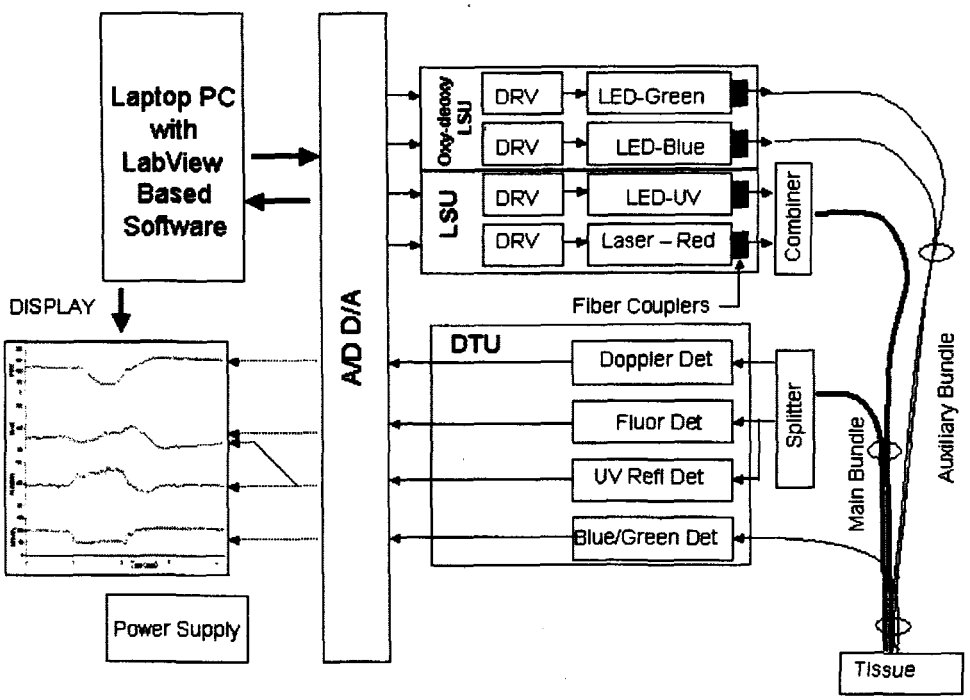


Fig. 4. Blocks Diagram of the TiSpec03 (for details see text).

Light Source Unit (LSU)

The light source unit of the TiSpec03 comprises a temperature stabilized and collimated 785nm CW laser diode module (Power Technology Inc., Alexander, AR). An adjustable lens couples the laser to an optical fiber. Attenuation of the laser intensity and elimination of back-reflection are achieved by an APC fiber connector and non-paraxial coupling.

A 370nm LED by Nichia Chemical Industries (Anan, Japan) serves as a UV light source. The UV LED is mounted on a miniature XYZ stage, coupled to a 0.22NA fused silica fiber by means of an AR-coated aspheric lens from Thorlabs Inc. (Newton, NJ). Since the UV LED does not serve as a light source for the delicate Doppler measurement, a direct modulation of the LED is enabled. Consequently, actual current to the LED in each pulse may be about 3 times higher than the nominal recommended CW current, yields a similar amplification of the intensity during the pulse. Yet, no degradation or major life-time shortening were noticed so far.

Oxy-Deoxy LSU

Considering the above-mentioned requirements for measuring hemoglobin saturation and LEDs availability, we chose super-bright LEDs as light sources. A 525nm, 1.6mW Super-Green LED as an isosbestic wavelength and a 470nm, 3.3mW Super- blue LED for the non-isosbestic wavelength, both from Ledtronic Inc. (Torrance, CA). Having a narrow viewing angle of 15 degrees, these LEDs are ideal for coupling into 0.22NA fibers. LEDs are mounted in a two miniature XYZ stages, to optimize fiber coupling. The LEDs were sequentially modulated on and off using a 40Hz pulse train.

Fiberoptic bundles

Two separate external fiber bundles serve the TiSpec03: the main bundle is dedicated to the measurement of blood flow, reflectance and NADH while an auxiliary bundle is dedicated to the oxy-deoxy hemoglobin measurement. The bundles has "excitation" channels that transmits light from the LSUs towards the tissue on the distal end of the bundle, and "collection" channels, transmitting the light signals from the tissue into the detection units (DTUs) . The proximal ends (device side) of the fibers are terminated using SMA connectors, providing interchangeability of bundles using the connection ports located on the front panel of the TiSpec03. The distal ends (tissue side) of fibers in each bundle are compactly packed in a thin hypodermic stainless steel tube tips. The tips should be placed adjacently on the monitored tissue, enabling monitoring of roughly the same tissue area.

We put much effort in reducing the number of fibers running in the main bundles from the device toward the tissue. Among the reasons for fibers reduction are: lowering price and labor of the long bundle, cutting down the number of optical connectors, and minimizing fuss when connecting new bundles to the TiSpec.

In order to achieve this goal, we multiplexed the fibers from the UV and NIR sources into a larger core fiber. Using a similar method inversely, we demultiplexed light signals from the detection channel into two detection channels. All multiplexing/demultiplexing stages where permanently and internally embedded in the TiSpec03.

The intention of reducing the total number of external optical connectors is currently fulfilled only in the main bundle. The main bundle does have only one input and one output connectors (although it supports 2 light sources and 3 detectors of different wavelengths), whereas the auxiliary bundle has 3 connectors – each one faces a single LED or detector. However, we will shortly use the same multiplexing technique to reduce the number of connectors in the auxiliary bundle as well.

Detection Unit (DTU)

The modifications in light sources essentially resulted in appropriate modifications in the detection system. The detection system and primary signal processing of the basic 3 wavelengths (370, 460 and 785nm) transmitted by the main bundle uses the same two-channels opto-mechanical design of the former TiSpec02. However, some essential adaptations were performed as follows. On one channel, the 785nm light is collimated after emitting the fibers, then discriminated using high-pass mirror. The collimated beam is focused into the active area of a fast photodiode with a joined pre-amplifying circuit.

On the other channel, the light is collimated and directed through a dichroic mirror. The Back-reflected 370nm light is reflected by the mirror into focusing lenses towards the active area of a second pre-amplified photodiode. NADH fluorescence, around 460nm is merely affected by the mirror and directed towards a photomultiplier tube (PMT) via a band-pass filter. The band pass filter attenuates residual 370 and 785nm signals.

PMT and photodiodes are the same as in TiSpec02 (2).

The detection system of the auxiliary bundle is much simpler. It consists of a single pre-amplified photodiode that measures the back-reflected intensity of both green and blue-pulsed light. Due to the

different on/off timing of the green and blue LEDs, the system can easily separate the two signals from the alternating output voltage of the single photodiode.

The efforts for improving the hardware and the software resulted in the portable briefcase-size device, shown in Figure 5.

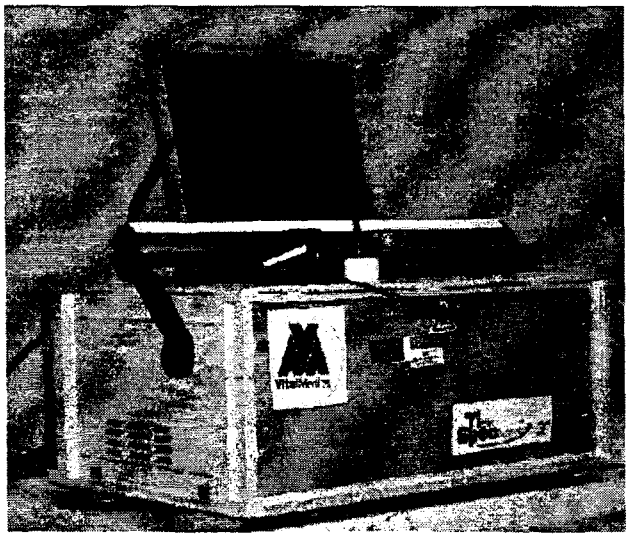


Fig. 5. The TiSpec03

3. RESULTS

The TiSpec03 used in the present study is different from the previous device, Tispec02, described in our previous publication (2). We have changed the light source for the excitation of the NADH and also added the monitoring of hemoglobin oxygenation (HbO_2). Therefore we checked the performance of the TiSpec03 by using 4 rats and 4 gerbils exposed to various perturbations.

The typical results to be presented were taken from one of the gerbils used. Figures 6 and 7 show the responses to two metabolic perturbations, namely, Anoxia and Ischemia. In the two events a wave of cortical spreading depression (SD) was developed (5, 11) and the responses were recorded as well.

As seen in Figure 6, exposing the gerbil to anoxia ($100\%N_2$) led to a clear decrease in HbO_2 correlated very well to the increase in NADH (calculated by subtracting the reflectance (R) from the fluorescence (F) signal). The cerebral blood flow (CBF) did not changed dramatically during the anoxia but a large hyperemic response was recorded during the recovery phase in parallel to the recovery of the NADH and HbO_2 to its baseline levels. About one minute after the recovery, a wave of SD developed led to massive activation of the energy metabolism as expected (12). Here we can see a clear correlation between the oxidation of the NADH and the increase in CBF. The HbO_2 response was biphasic starting with a small decrease followed by a relatively larger increase. Within 2-3 minutes all signals returned to the baseline values. During the ischemic episode induced by the occlusion of the 2 carotid arteries (Fig. 7) very similar responses were recorded with the exception of the CBF response. As soon as the arteries were occluded the CBF decreased to its very low values and a recovery, including a short hyperemia, was recorded immediately after the opening of the carotid arteries. The other difference was the significant increase in the reflectance signal during the ischemia. The

responses to the SD developed after the recovery from the ischemia were similar to that recorded after the anoxia.

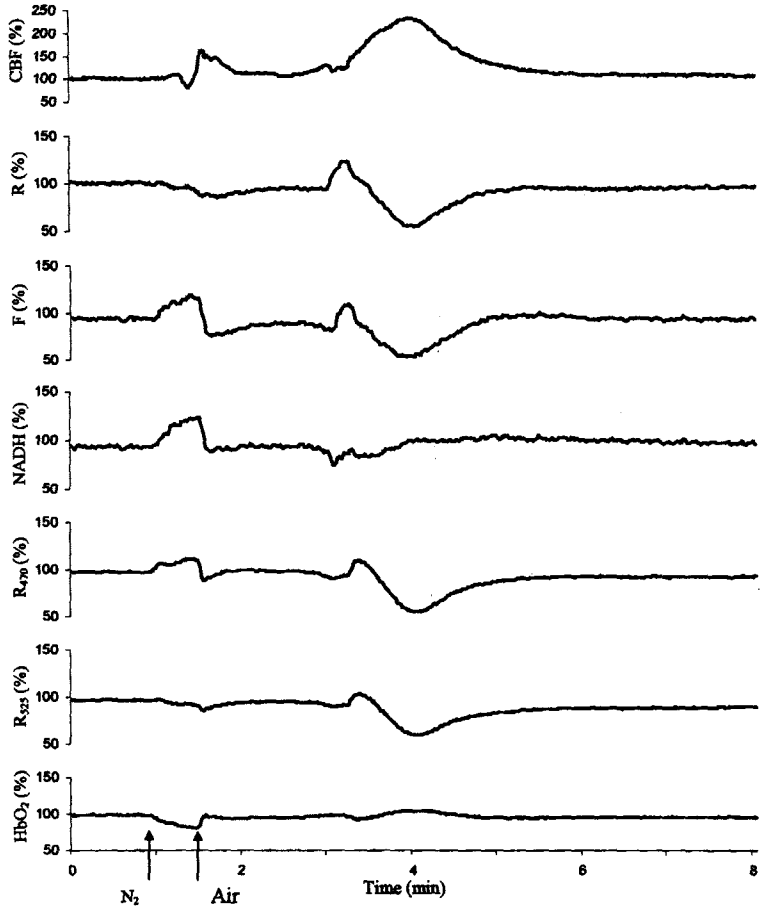


Fig. 6. Typical responses to anoxia (N_2) recorded from the cerebral cortex of a gerbil. CBF – Cerebral blood Flow, R – Reflectance, F – Fluorescence, R_{470}, R_{525} –Two wavelength reflectance signals used to calculate the oxygenation of hemoglobin-HbO_2.

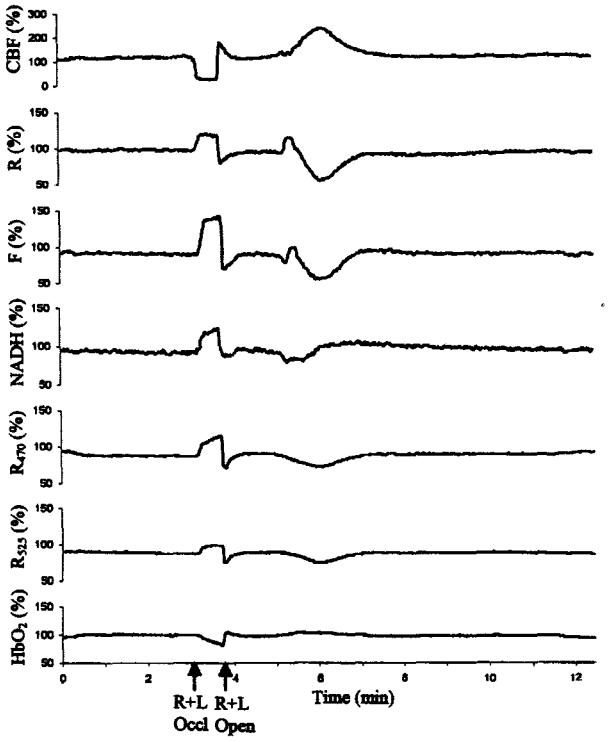


Fig. 7. Typical responses to a short complete ischemia induced by bilateral occlusion of the two carotid arteries.
Abbreviations are as in Fig. 6

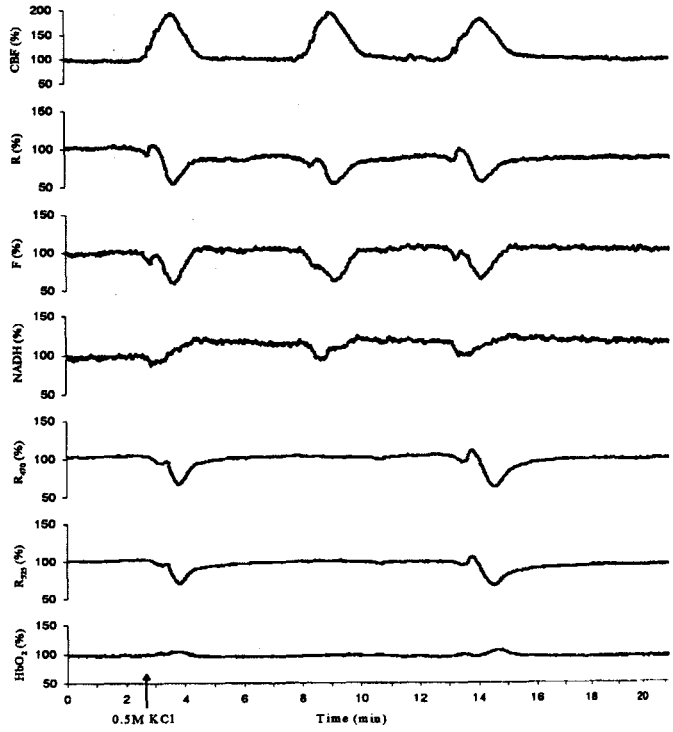


Fig. 8. Effects of cortical Spreading Depression (SD) on Cerebral blood flow, mitochondrial NADH redox state and the level of hemoglobin oxygenation. Abbreviations are as in Fig. 6.

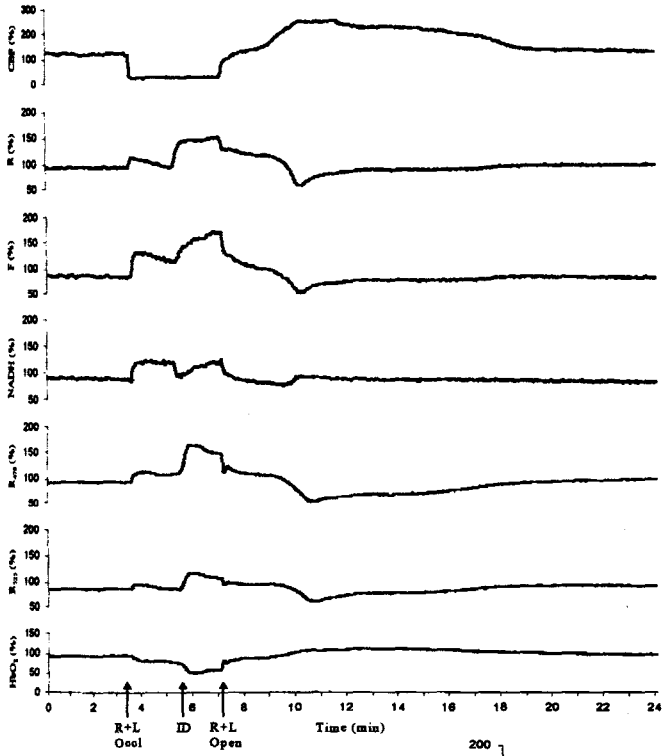


Fig. 9. Effects of 5 minuets complete ischemia, induced by bilateral occlusion of the carotid arteries in a gerbil model.
ID – Ischemic Depolarization. Other abbreviations are as in Fig. 6.

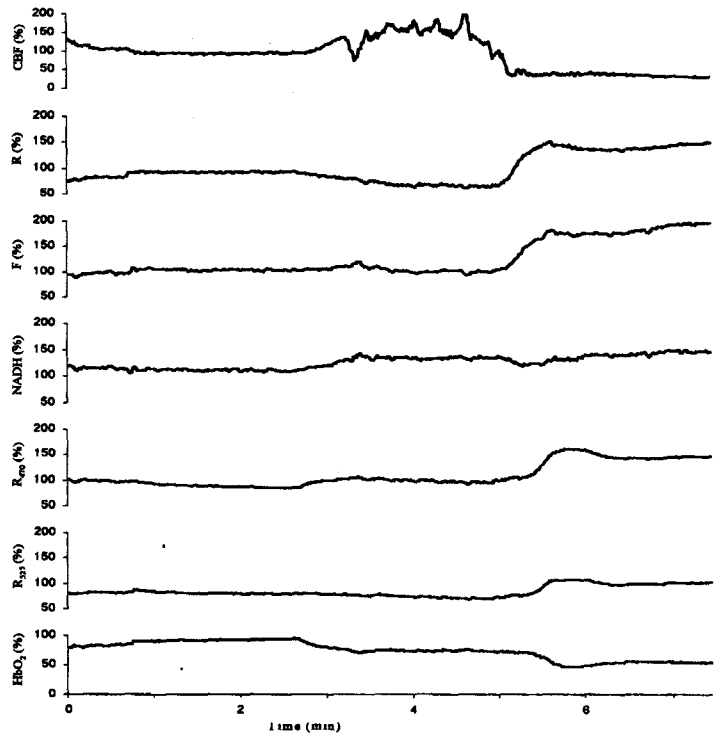


Fig. 10. Effects of terminal anoxia on the responses of the various parameters monitored from the gerbil brain.
Abbreviations are as in Fig. 6.

Figure 8 shows the responses of the brain to repetitive waves of SD induced by epidural application of KCl solution .The three responses were very similar to those recorded after the anoxia and ischemia (Figs. 6 and 7). Here again, activation of the energy metabolism due to the efflux of potassium from the intracellular to the extracellular space led to the oxidation of NADH (decrease in fluorescence) and a large increase in CBF.
The SD waves led to a 100% increase in CBF in all 3 cycles recorded. It can be seen that there is a clear correlation between the oxidation of NADH and the increase in CBF. The relative change in the oxygenation of the hemoglobin was very small due to the normoxic state of the animal. The effect of 5 minutes ischemia is presented in figure 9. In this recording the responses could be divided into two stages. In the initial step, due to the occlusion of the 2 carotid arteries, a dramatic drop in CBF was accompanied by a clear decrease in Oxy-hemoglobin and a large increase in NADH. After about 1.5 minutes, an ischemic depolarization was developed and due to large increase in the reflectance traces in 365 and 525 nm, an artifactual oxidation of NADH and decrease in oxyhemoglobin were recorded. After the opening of the two carotid arteries the various parameters recovered to the pre ischemic levels within 15 minutes. As can be seen the NADH signal recovered to base line much faster as compared to the other 2 parameters. The effects of long- term terminal anoxia are presented in figure 10. As seen, during the initial phase of the anoxia a decrease in oxyhemoglobin was accompanied by a large increase in NADH. Blood flow was elevated initially for about 2 minutes and than CBF fall to the minimal levels indicating the death of the gerbil. Here also, due to the anoxic depolarization, an apparent oxidation of NADH was recorded together with apparent large decrease in the oxyhemoglobin levels.

4. DISCUSSION

Real time optical monitoring of tissue vitality could be applied to many clinical situations in order to diagnose the metabolic state of the patient. In our previous presentation (2) we described the advantages of the TiSpec02 having smaller dimensions, and other advantages, as compared to the first TiSpec described in many details recently (13). The present communication present the recent version of our Tissue Spectroscope-TiSpec03, having two major new features that make this model very attractive to be used in clinical practice. The first issue is the addition of a new parameter to the 3 existing indicators of tissue vitality, namely, the oxygenation of hemoglobin. The second advantage is the technical improvement of the monitoring system and its smaller dimensions. Despite the fact that the presentation of the parameters are not in absolute values, it appears that the multiparametric nature of the system make it very practical for real time evaluation as a function of time in the same tissue. The redox state of the NADH as measured in vivo is the most sensitive parameter of intracellular oxygen levels as compared to the other parameters monitored by the TiSpec. The changes in the NADH levels are also the best indicator of cellular energy balance in the tissue exposed to various pathophysiological conditions (for review see ref. 14). The other 3 parameters monitored by the TiSpec are very important for the understanding of tissue vitality but having them measured without the NADH decreased the level of data interpretation. For example, in figure 6, under anoxia the changes in CBF are not significant at the early stage while the HbO_2 and NADH showed a better correlation to the availability of oxygen. Under Ischemia, all 3 parameters are very well correlated to the oxygen supply to the tissue. When the brain was exposed to spreading depression the more significant responses were in NADH, CBF and Reflectance while the HbO_2 changes were minimal due to the positive energy balance achieved by the large increase in CBF to compensate for the extra oxygen needed for the mitochondria. The decrease in dimensions of the TiSpec03 will enable to use it in the operating room and in the various intensive care units (ICU). In critically ill patients the needs for better monitoring system are obvious and could be beneficial to the better outcome of the patients. The TiSpec could provide real time information on various parameters representing changes in tissue vitality. These measurements may be performed on a so called non-vital organ and will provide information that will serve as an early warning signal for the deterioration of the body (2, 15). This

approach was tested in preliminary clinical studies where tissue vitality was monitored in the urethral wall using a 3-way Foley catheter (Mayevsky et al., unpublished results).

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