Mitochondrial Dysfunction: Bench- to- Bedside Optical Monitoring of Tissue Vitality

Avraham Mayevsky [1, 2,] Nava Dekel[2], Levi Oren[2], Assaf Deutsch[2] and Eliyahu Pewzner[2]

[1]The Mina & Everared Goodman Faculty of Life Sciences and the Multidisciplinary Brain Research Center, Bar-Ilan University, Ramat-Gan, Israel.

[2]CritiSense Ltd. Giv'at Shmu'el, Israel.

ABSTRACT

In normal cell the mitochondria are the major source of energy for cellular functions. They serve as biosensors for oxidative stress and involved also in termination of cell function by apoptosis. The involvement of mitochondria in pathological states such as neurodegenerative diseases, sepsis, stroke and cancer are well documented. The involvement of mitochondrial respiration and function in cancer development, proliferation and possible therapy were initiated 75 years ago by Otto Warburg.

Monitoring of NADH fluorescence in vivo as an intracellular oxygen indicator was established in the 1950-1970 by Britton Chance and collaborators. In the last 20 years we developed and used a multiparametric monitoring system enabling real time assessment of mitochondria NADH, microcirculatory blood flow and volume as well as HbO_2 oxygenation. In order to use this technology in clinical practice the commercial developed device – the "CritiView" was tested in animal models as well as in patients hospitalized in the critical care departments.

In patients we tested the viability of the urethral wall (a less – vital tissue) by a 3 way Foley urinary catheter that contains the optical probe. The catheter was introduced to patients underwent open heart by-pass surgery or abdominal aorta aneurysm (AAA) operations. The monitoring started immediately after the insertion of the catheter to the patient and was stopped when the patient was discharged from the operation room. The results show that monitoring of the vitality of the Urethral wall provides information in correlation to the surgical procedure performed. In the AAA patients the occlusion of the aorta led to severe ischemia developed in the urethral wall and recovery of signals were recorded after the reopening of the aorta. In patients under went heart bypass surgery the urethra vitality was decreased dramatically during the operation and recovery was noted in most patients after the discharge of the patient from the operation room.

Keywords: mitochondrial dysfunction, patient monitoring, microcirculatory blood flow, NADH redox state, CritiView, hemoglobin oxygenation

1. INTRODUCTION

The intracellular cytoplasmic organelles, the mitochondria (previously named bioblasts) were described by cytologists since the mid 19th century [9]. According to Tzaggolof, (1982) and Scheffler (1999), the name, mitochondrion was given by Benda in 1898. Only in the mid 20th century the role of the mitochondria in oxidative energy metabolism was established [8]. The invaluable pioneering work of Chance and collaborators since the early 1950th opened up a new era of studying mitochondrial function using spectroscopic techniques [2; 3; 6]. Monitoring of NADH redox state in isolated mitochondria [4] and later on under in vivo conditions [5] established the foundations for the understanding of cellular bioenergetics developed under various pathophysiological conditions in experimental animals as well as in patients [11; 14]. In parallel to the role of mitochondria in cellular bioenergetics, the role of mitochondrial dysfunction in various human diseases became very clear and obvious. For example, the involvement of the mitochondria in tumor cells was described initially by Warburg 75 years ago [19] followed by many studies later on [20; 21]. Since then, a large volume of papers showed the involvement of the mitochondria in many human diseases [1; 7; 16-18] as shown in Figure 1. Nevertheless, the translation of the accumulated information into a practical clinical tool or medical device did not happened. The existing devices that are used during operations or in the intensive care units provide real time information on the activity of the respiratory and cardiovascular system. The information on the microenvironment activities, especially mitochondrial

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function, at the tissue level is missing. In our previous papers we described the structure and function of a multiparameter monitoring system that provide information on the viability of the tested tissue [12; 14; 15].

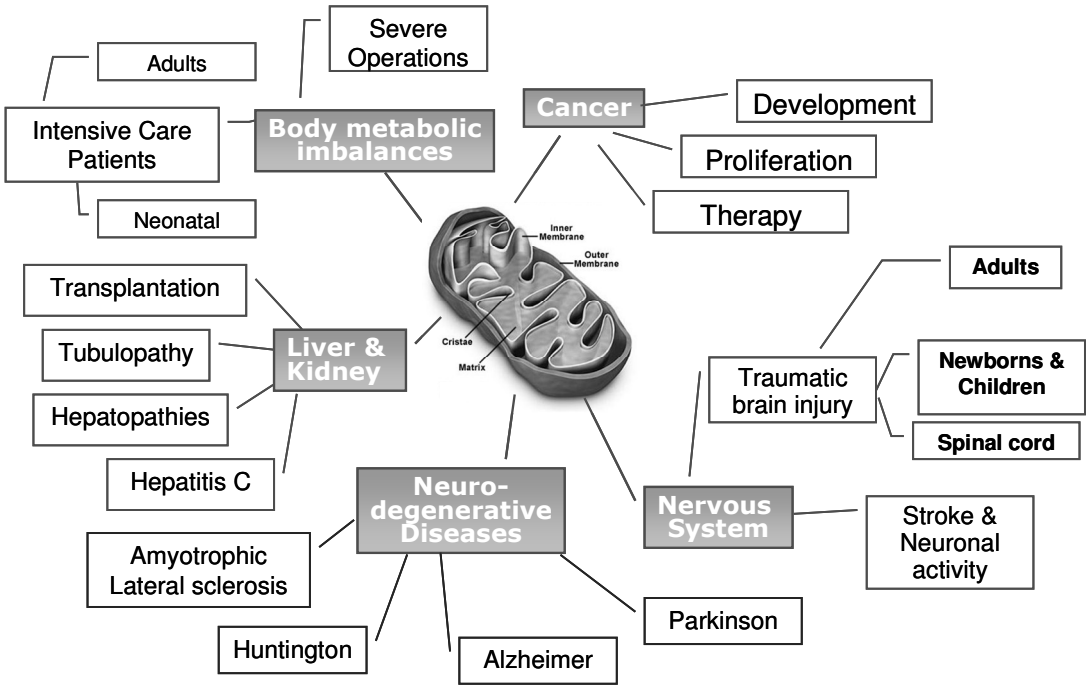

Figure 1: The involvement of mitochondria in various pathological and pathophysiological conditions.

The aims of this paper are as follows:

1. To present the upgraded technology of the CRV3 as compare to the previous model of the CritiView named CRV1 [10].
2. To compare the performance of the CRV3 in vitro and in vivo to the CRV1 or other predicate device cleared by the FDA.
3. To show preliminary results of a clinical study using the Foley catheter in patients.

2. METHODOLOGY

In the present study a new updated version of the CritiView, named CRV3, will be described. This unit, cleared by the FDA in 2007, was tested in small animals (gerbils), large animals (pigs) and in more than 30 patients. The CRV3 device carries out certain in-vivo, spectroscopic measurements as a multi-parametric monitoring device intended for the measurement of tissue metabolic state. The CRV3 consists of the combination of a NADH Fluorometer, a laser Doppler Flowmeter as well as a reflectometer for the monitoring of microcirculatory blood oxygenation. The CRV3 transmits radiation through tissue at known wavelengths and measures these parameters by detecting the intensities of light in the following wavelengths:

1. Mitochondrial NADH fluorescence emitted at 420nm to 480nm.
2. Total backscattered light (375nm) reflected from the tissue. This parameter allows for correction of the NADH fluorescence measurement due to changes in tissue blood volume.
3. Doppler shifted laser light (785nm) reflected from moving blood cells.
4. Two wavelength reflectometry (470 and 530 nm) enabling the monitoring of HbO_2.

The CRV3 does not carry out this assessment. It simply provides the measured information to the user. These parameters contain information pertaining to the redox state of mitochondrial NAD/NADH of the tissue, microvascular blood flow and oxygenation in the tissue, and total blood volume of the tissue. Changes in these values reflect changes in the balance between oxygen supply and oxygen demand. Since the CRV3 is intended as an adjunctive measurement device when used in conjunction with conventional patient monitors, this further supports the low safety risk of the CRV3.The basic scientific principles and technology of the CRV3 are identical to the predicate device (CRV1) described 2 years ago [10].

2.1 Description of the Device –CRV3

The light source unit (see Fig 2.) of the CRV3 comprises a 785nm CW laser diode which serves for laser Doppler measurement, a UV LED (375nm) for NADH fluorescence excitation and for total back scatter (or reflection) measurement, a Blue LED (470nm) and a Green LED (530nm) for HbO_2. In order to enable a very high measurement dynamic range of fluorescence and reflection parameters the light source unit is designed to enable a very wide range of the excitation intensities. To enable this wide excitation range and linearity the system is designed according to a three floors concept. Each floor comprises three LEDs one UV LED with emission peak at 375nm, one Blue LED with emission peak at 470nm and one Green LED with emission peak at 530nm. The different wavelengths from all LEDs of the same floor are assembled together and coupled into a single fiber by a set of dichroic mirrors and appropriate collimation and focusing lenses. The current of each one of the discrete LEDs is set by the appropriate electronics drivers directly controlled by a D/A of the DSP processor. The difference between the floors is the output intensity. There is a High, Medium and Low intensity floors. The different excitation intensities are achieved by utilizing various pinholes while maintaining all other electro optical properties as the same for all three floors. The light from all three floors and a laser diode is combined into single mixer fiber therefore enabling precise setting of the excitation intensity within a very wide excitation range. The near IR laser diode at 785nm, for laser Doppler measurements, operates in Continuous Wave (CW) operation mode. The UV LEDs, Blue LEDs and Green LEDs operate in chopping mode. This enables usage of synchronous detection techniques in order to detect the NADH fluorescence and total backscatter light. Additionally the chopping operation mode enables one to perform NADH measurements with very low excitation intensities well below the limits specified by the laser safety standards.

The Detection Unit (DTU) All six collection fibers of the fiber optic probe are assembled into a single male SC optical connector. The light from the probe passes though the panel connector into a single thick optical fiber that delivers the light to the DTU. At the DTU entrance the collimation lens collimates the fiber output light. The collimated light is split according to the different wavelengths into the respective photo detectors by means of dichroic beam splitters. The first dichroic beam splitter reflects the total backscatter signal at 375nm towards the photodiode detector. The higher wavelengths pass through the first beam splitter towards the second dichroic beam splitter. The second dichroic beam splitter reflects the NADH fluorescence signal at 450nm and total backscatter signals at 470nm and 530nm towards the photomultiplier detector. Due to the chopping operation of the LED's the photomultiplier detector detects each one of the above mentioned signals at different time, i.e., time sharing operation detection mode. The second dichroic filter enables the laser Doppler signal at 785nm to pass through it towards the photodiode detector. All acquired signals are digitized into the DSP processor by high resolution 16bit A/D.

The DSP processor is responsible for whole system control, initial data processing and calculation of Doppler parameter. The DSP is build around Tern Inc. 586-Engine-P controller board with AMD SC520 CPU. After initial data processing the calculated values are transmitted to the panel computer for final data processing display through RS-232 serial interface.

The CRV3 device utilizes medical grade main power supply for all electronic circuits including the panel computer.

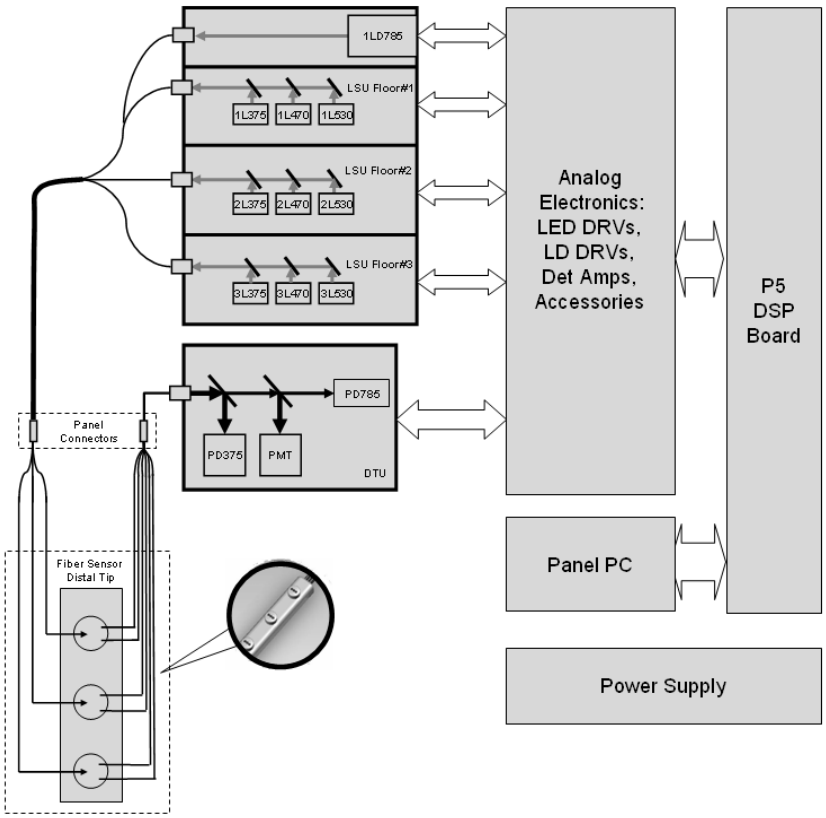

Fig. 2: The CRV3 block diagram and its Foley catheter probe design.

2.2 The CRV3 optical probes

1. The pencil style optical probe has one measurement point to be used on exposed skin/tissue. The excitation and collection fibers are held together in a stainless steel element. It is comprised of one excitation fiber connected to respective excitation connector on the CRV3 panel; and 6 collection fibers connected to the single collection connector on the CRV3 front panel.
2. The Foley catheter optical probe for measuring the urethral wall. It is based upon a standard 3 lumen Foley catheter (The Foley catheter is an inflatable balloon retention type catheter inserted through the urethra, and used to drain the bladder). This probe construction enables the measurement of tissue metabolism at the urethral wall while draining the bladder. The CRV3 Foley catheter optical probe is designed to perform optical measurements from three adjacent points on the tissue 3.5mm apart. Each measurement point is comprised of one excitation fiber and two adjacent collection fibers. The 3 excitation fibers from the LSU are connected to the excitation connector on the CRV3 panel. All 6 collection fibers from the three measurement points are connected to the single collection connector on the CRV3 front panel. Figure 3 shows the finished probe.

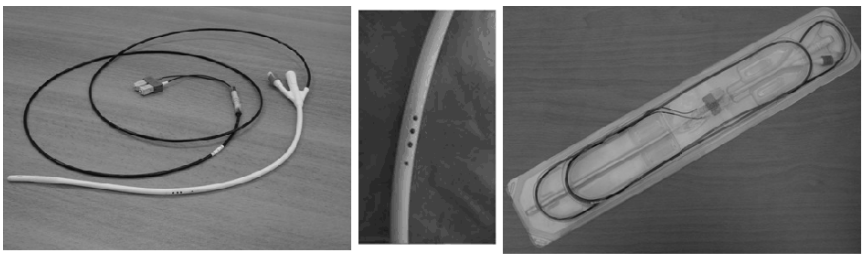

Figure 3: Foley catheter probe assembly. (A) The fiberoptic tip element embedded inside a standard 3 way Foley catheter. (B) A close-up on the catheter measurements points. (C) The Foley catheter probe packed in its blister package.

2.3 Testing the CRV3 in Vitro

The aims of the study were to demonstrate the efficiency of the CRV3 device for the measurement of NADH concentration, in two steps:

1. Demonstrate the correlation between the NADH concentration in solution and the fluorescence intensity measured by the CRV3.
2. Compare the correlation between NADH fluorescence in solution measured in this study by the CRV3 to that measured by the CritiView-CRV1.

Figure 3: Foley catheter probe assembly. (A) The fiberoptic tip element embedded inside a standard 3 way Foley catheter. (B) A close-up on the catheter measurements points. (C) The Foley catheter probe packed in its blister package.

The experimental methodology is straightforward. Various solutions of NADH were measured with the CRV3 as well as with the CritiView-CRV1.Insofar as the measurement with the CRV3 or the CritiView-CRV1 involves placing its probe tip into the solution one might ask if this measurement is the same as placing the tip of the probe against a tissue surface. The equivalence of these two measurements can be understood if one considers that although immersion in aqueous solution and placement adjacent to tissue are, clearly, two different physical situations, nevertheless, the process and principle of measurement that takes place is the same for both. Aside from their contiguity, there is no interaction between the probe and the solution or any adjacent tissue. The probe merely collects emitted light from its immediate environment, i.e., a hemispherical volume adjacent to the probe tip, and transfers the collected light to detectors for measurement Therefore, for the purposes of comparison of CRV3 spectroscopic characteristics to those of control NADH solution and to a clinical fluorometer, the CRV3 probe can be positioned on the surface of a tissue or a liquid, or it can be inserted into the tissue or liquid. Three sets of solutions with known concentrations of NADH + Double Distilled Water (DDW) were prepared in the following way: Stock solution of 1mM NADH was prepared by dissolving 14.188mg NADH in 20ml DDW. The samples of NADH solutions (5ml) were prepared in glass vials by the appropriate dilution of the stock solution with DDW which also used as a blank that was subtracted from the actual reading made by the CRV3. The "pencil type" probe of the CRV3 was placed in the vials for 10 seconds and a reading was taken. The probe was taken out of the solution and a second reading was recorded. This was repeated again so that three reading were averaged. A second and a third set of diluted solution were prepared by the same procedure and were measured in the same way. The same procedure was used with the predicate CritiView-CRV1 with on set of NADH solutions.

2.4 Testing the CRV3 in Vivo

The aim of this study and report is to describe in detail the in vivo control study of the CRV3 device in order to demonstrate substantial equivalence to its predicate CritiView-CRV1. The performance of the CRV3 device in measurement of changes in TBF (Tissue Blood Flow), NADH fluorescence, tissue reflectance and HbO_2 resulting from standard metabolic perturbations in an animal model (gerbils) was done. All signals that are monitored by the CRV3 as well as the CRV1 are expressed as relative values. This brings all the calculations to be presented as “percent change of the signals”. The TBF outputs of the CRV3 and the CRV1 are curves of signal measurements as functions of time. In order to assess the performance of CRV3 in measurement of TBF we performed a series of experiments in which the TBF of animals was measured simultaneously by the 2 instruments. The same basis was applied to the other three parameters, namely NADH, Reflectance and HbO_2 in the same statistical analysis principles. The purpose of the statistical analysis is to demonstrate that the 2 curves of each experiment are equivalent. Since the scales of the instruments are not identical, it is meaningless to compute the difference between 2 curves. In fact, equivalence in this case means “having the same direction of change”. That is, when one curve goes up, the other goes up; and when one goes down, the other goes down. The appropriate statistical measure for such behavior is the correlation coefficient. It is desirable to have a correlation coefficient as close to +1 as possible between every pair of corresponding curves. Thus, by computing correlation coefficients between two curves, it is possible to measure the statistical equivalence between them.

Animal preparation: In order to test the depth of anesthesia in each gerbil monitored, we used a surrogate systemic parameter that represents the function and the intactness of the cardiovascular and respiratory systems. The parameter is the level of the hemoglobin oxygenation in the systemic blood -SpO_2 measured by a standard vetinerinary pulse oximeter suitable for small animal experiments. In Figure 4, a record of the SpO_2 during the entire experimental protocol of the gerbil is presented. As one can see, the SpO_2 was very high and stable during the entire period of monitoring. During the 4 perturbations imposed on the gerbil a clear decrease of the SpO_2 was recorded. This decrease in SpO_2 during anoxia,

hypoxia and terminal anoxia is according to the availability of oxygen to the body. The decrease in SpO_2 during cerebral ischemia is probably due to stimulation of the sympathetic and parasympathetic nerves that are affected by the puling of the carotid arteries during the ischemic episode.

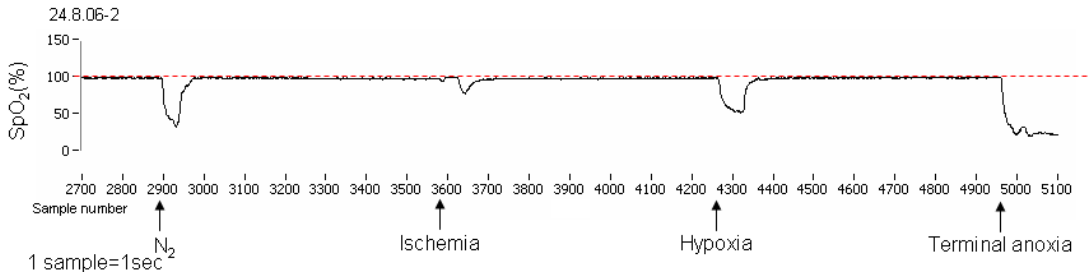

Figure 4: Continuous record of the SpO_2 in one gerbil that was involved in the comparative study. N_2=Anoxia

Indeed in each of the experiments included in the comparative study we monitored the SpO_2 in addition to the parameters monitored from the brain.

Operation procedure: Male Mongolian Gerbils (50-75gr) were used. We had operated 25 gerbils but only 19 are reported in the results section. Six gerbils were excluded according to the predetermined criteria. The two common carotid arteries were isolated just before brain surgery and ligatures of 4-0 silk thread were placed around them. The gerbil was placed in a head holder in the supine position. After a midline incision of the skin, an appropriate elliptic hole (about 3x8mm) was drilled in the parietal bone of the right hemisphere. The dura mater remained intact and a light guide holder –cannula was placed in the drilled hole and extra pressure on the tissue was avoided. Two stainless steel screws in the left parietal bone were used to fix the cannula, with dental acrylic cement Body temperature was measured by a rectal probe (YSI) and was regulated to be at the range of 35-37°C using a heating blanket. The animals were kept anesthetized during the operation as well as during the entire monitoring period, by IP injections of Eth 0.03-0.05ml every 30 minutes according to the existence of a pain response.

Metabolic Perturbations

Anoxia: Exposure of the animal to oxygen deficient atmosphere by spontaneous breathing of 100% N_2, for a period of time (approx. 25 sec.) until changes are observed on the monitor, and the animal has stopped breathing. The animal is then allowed to breathe normal room air by spontaneous breathing or by short artificial respiration.

Ischemia: Reversible occlusion (30 sec.) of the 2 common carotid arteries by constricting them with threads.

Hypoxia: Exposure of the animal to spontaneous breathing of low oxygen concentration (6% oxygen in air) for a short period of time (60sec.). The animal is then allowed to breathe normal room air by spontaneous breathing.

Terminal Anoxia: Exposure of the animal to complete irreversible anoxia by exposing the animal to 100% N_2 until the death of the gerbil.

The application of the various gases was done by placing a small tube, connected to the gas cylinder, around the nose of the gerbil. The flow of the gas was very slow (below 1 liter per minute) in order to avoid respiratory disturbances.

Experimental Protocol: Monitoring the various parameters was started immediately after the end of operation. This was done by using two needle type optical probes connected to the CRV3 and the Critiview-CRV1 and placed inside the cannula cemented to the skull. In order to check the intactness and the physiological status of the brain, a 20 seconds anoxia was induced. Gerbils that present abnormal response were discarded before running the experimental protocol. When body temperature reached the range of 35-37°C the exposure of the gerbils to the various perturbations started. The gerbils were exposed to the various conditions having interval of about10 minutes between each test to allow recovery. The pulse oximeter value was used in order to assure the complete recovery. The data was collected at a rate of 1 sample per second, and stored in different channels of a computerized data acquisition program.

3. RESULTS

3.1 In vitro testing

The data presented in figure 5 was collected from the 3 sets of NADH aqueous solutions. Final concentration of the 24 samples numbered as 1-24 prepared by diluting the stock solution with. The results of the 3 dilution sets are presented. Each sample was measured three times and the mean is shown. The fluorescence arbitrary unit's values of the CRV3 were plotted against concentration values as seen in Fig. 5. A linear plot is expected for the concentration range used and the 3 regression lines related to the three sets of measurements were calculated and plotted. From Figure 5 one can see the linear correlation between NADH fluorescence to control NADH concentration in aqueous solution. There is no significant difference between to three sets of measurements.

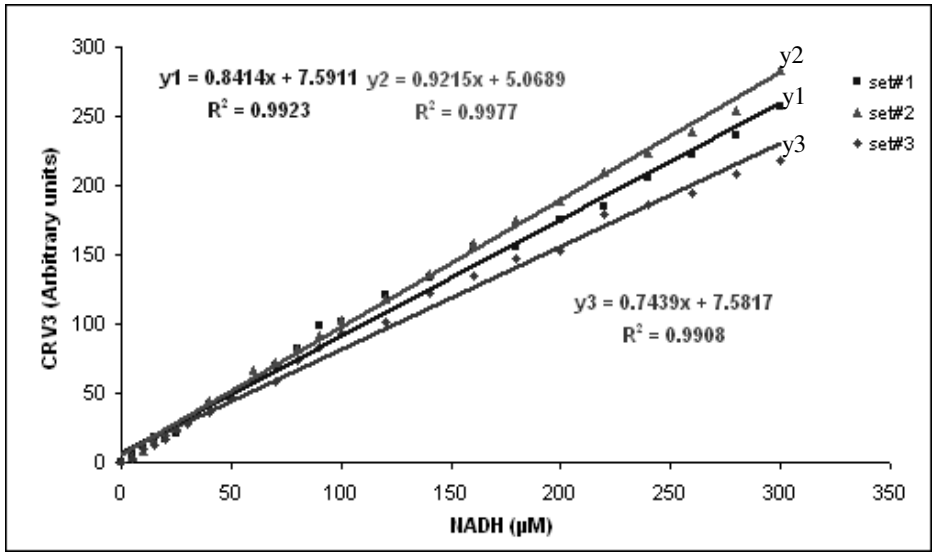

Fig.5: Fluorescence readings (three sets) of the CRV3 plotted against NADH concentration after subtraction the reading of the blank solution. A linear regression was calculated and the equation and R^2 are presented for the three sets of control solutions.

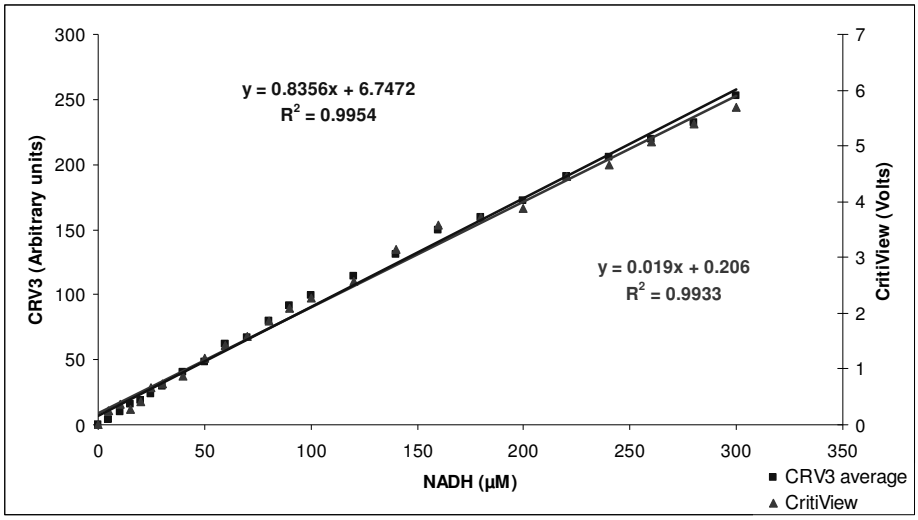

Figure 6: Readings of the predicate CritiView-CRV1 (on the right Y axis), and readings of the CRV3 (on the left Y axis) plotted against NADH concentrations after subtraction of the reading of the blank solution.

Figure 6 presents readings of the predicate CritiView-CRV1 (on the right Y axis), and readings of the CRV3 (on the left Y axis) plotted against NADH concentrations. A linear regression was calculated for each instrument and the equation and R^2 are presented

It can be concluded that linear relationship was established between the NADH concentrations and the fluorescence signals measured by the CRV3 with correlation coefficients that are very close to 1.0.

The correlation coefficients between CritiView-CRV1 or CRV3 and NADH concentration (C), were:

r(CritiView-CRV1) = 0.9933; r(CRV3) = 0.9954

This study proves the efficacy of the CRV3 in measurement of NADH concentration in solutions and also that the CRV3 is substantially equivalent to its predicate CritiView-CRV1 for the NADH measurement efficiency.

3.2 In vivo testing

The comparison between the two devices provided a large quantity of data that can not be presented in the current paper due to page limitation. Therefore only the results of the NADH measured by the 2 instruments are presented.

The responses of mitochondrial NADH to the lack of O_2 is unidirectional namely an increase of signal in all perturbations used. As can be seen in Figure 7 (A-D), under all perturbations the CRV3 and the control instrument present very similar responses and the correlations were statistically significant ($r > 0.87$). Under terminal anoxia, the duration of monitoring was 1 minute before the perturbation and 1 minute after the fast increase in NADH without recovery phase.

3.3 Statistical Analysis

In almost all events induced in the various animals and type of perturbation and recorded, the correlation coefficients were statistically significant. This indicates that mitochondrial responses and other parameters are very similar regardless the instrument used or the perturbation induced.

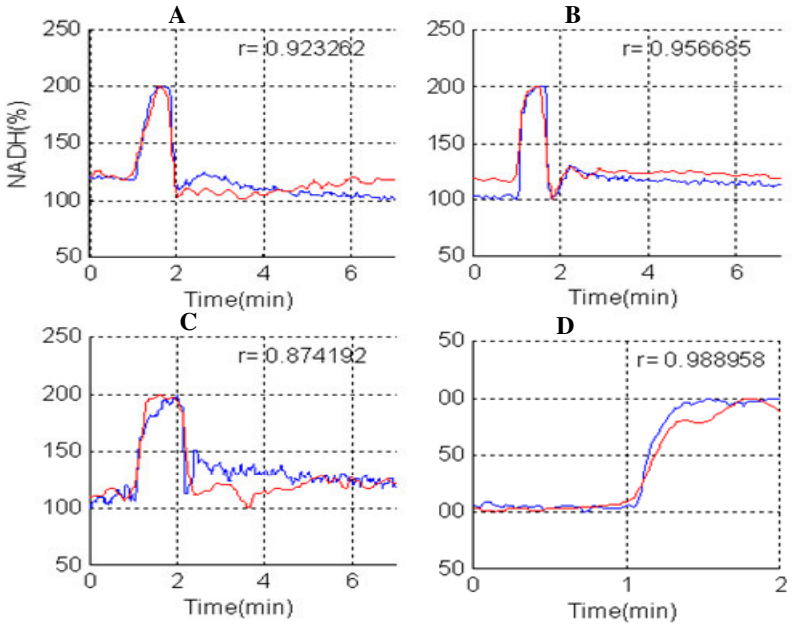

Figure 7: Time plot of typical NADH response to (A) anoxia, (B) ischemia.(C) hypoxia, (D) Terminal anoxia. The two plots in each perturbation were recorded by the CRV1 and CRV3.

4. DISCUSSION

4.1 Responses to Metabolic Perturbations

In Figure 7 (A-D), one sees the response of NADH to all four perturbations used. As seen in all 4 figures, the typical increase in the NADH level is very significant. This is due to the decrease in the available oxygen to the brain's mitochondria that shifts the [NAD]/[NADH] ratio to a more reduced state. This is followed by complete recovery when the animal is allowed to receive oxygen again.

When oxygen is deprived from the tissue either by ischemia, hypoxia or anoxia the expected increase in NADH due to the inhibition of the respiratory chain located in the mitochondria was recorded. Under such conditions the supply of energy is diminished while the demands for energy try to stay the same. Due to the negative oxygen balance, the brain will not be able to perform its various activities. Tissue blood flow is a primary event under ischemia while under anoxia or hypoxia is secondary. The responses of tissue blood flow in the four perturbations were not identical. Under anoxia the TBF response is showing a biphasic nature. The responses recorded by the reflectance trace indicate that this parameter is correlated to blood volume in the tissue. The reflectance was elevated under ischemia indicating a decrease in blood volume.

4.2 Correlations

Since all responses to any of the perturbations are measured simultaneously by the same two devices, i.e., CRV3 and the CRV1 one might expect that all groups would have similar correlation coefficients. Nevertheless, we find differences between the correlations of different perturbations. This is understandable since the physiological responses are slightly different for the various perturbations. As a result of the difference in physiological responses, including autoregulatory effects on local blood flow, it can be expected that the correlation between the measured signals at two adjacent sites in the tissue would be more similar for certain perturbations than for others. Similarly, when autoregulatory mechanisms are active, such as anoxia, variations in blood flow at different tissue sites can be expected. Additionally, the intensity of the results of a perturbation depends both on its duration and character. Duration is significant insofar as autoregulatory mechanisms are more active during a short perturbation and attempt to reject the unwanted perturbation, whereas after a while (a few seconds), their ability to act decreases dramatically.

4.3 Preliminary clinical testing of the CRV3

In order to assess the ability of the CRV3 to measure the vitality of the urethral wall in patients a preliminary clinical study was performed. In patients we tested the viability of the urethral wall (a less – vital tissue) by a 3 way Foley urinary catheter that contains the optical probe. The catheter was introduced to patients underwent open heart by-pass surgery or abdominal aorta aneurysm (AAA) operations. The monitoring started immediately after the insertion of the catheter to the patient and was stopped when the patient was discharged from the operation room. Figure 8 presents the data collected from the urethral wall during the occlusion period as well as after the reperfusion of the urethra in one of the 6 patients monitored. During the preparation for the occlusion of the large arteries, a small transient decrease in microcirculatory blood flow (TBF) together with an increase in NADH levels. When the two arteries were occluded completely, the TBF decreased to near 0 levels while NADH reached its maximal levels. The HbO_2 decreased in parallel to the decrease in TBF. Under the ischemic conditions developed the Reflectance signal increased due to the decrease in blood volume in the monitored area. Immediately after the reopening of the two blood vessels, all signals returned to the area of the baseline values. The results show that monitoring of the vitality of the Urethral wall provides information in correlation to the surgical procedure performed. In the AAA patients the occlusion of the aorta led to severe ischemia developed in the urethral wall and recovery of signals were recorded only after the reperfusion of the urethra. In patients under went heart bypass surgery the urethra vitality was decreased dramatically during the operation and recovery was noted in most patients after the discharge of the patient from the operation room.

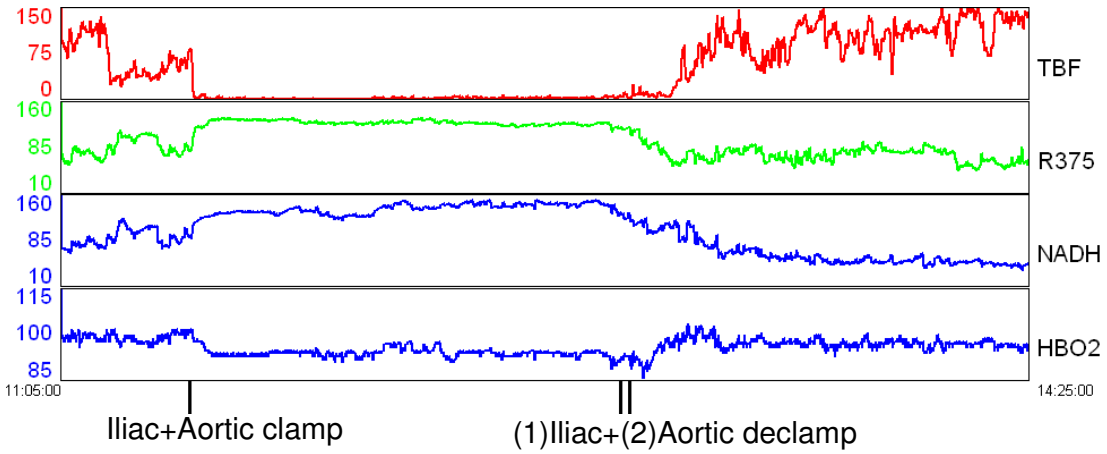

Figure 8: Effects of occlusion of the Iliac artery and the abdominal aorta on urethral wall parameters represent the vitality at the tissue level in a AAA operation. The time scale represents a period of 3 hours and 20 minutes.

5. CONCLUSIONS

The CRV3 and the CRV1 are substantially similar when used in different physiological states. This is consistent with the concept of "identical principles of physiological function between different organisms", that forms the rationale for the ability to use the CRV3 in any living tissue. The results of the comparisons between different sites in the same tissue, different tissues and different species were published recently [13] confirms and emphasizes the ability of the CRV3 to measure all types of living tissues in animals as well as in patients.

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