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Preclinical evidence of mitochondrial nicotinamide adenine dinucleotide as an effective alarm parameter under hypoxia

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Abstract. Early detection of tissue hypoxia in the intensive care unit is essential for effective treatment. Reduced nicotinamide adenine dinucleotide (NADH) has been suggested to be the most sensitive indicator of tissue oxygenation at the mitochondrial level. However, no experimental evidence comparing the kinetics of changes in NADH and other physiological parameters has been provided. The aim of this study is to obtain the missing data in a systematic and reliable manner. We constructed four acute hypoxia models, including hypoxic hypoxia, hypemic hypoxia, circulatory hypoxia, and histogenous hypoxia, and measured NADH fluorescence, tissue reflectance, cerebral blood flow, respiration, and electrocardiography simultaneously from the induction of hypoxia until death. We found that NADH was not always the first onset parameter responding to hypoxia. The order of responses was mainly affected by the cause of hypoxia. However, NADH reached its alarm level earlier than the other monitored parameters, ranging from several seconds to >10 min. As such, we suggest that the NADH can be used as a hypoxia indicator, although the exact level that should be used must be further investigated. When the NADH alarm is detected, the body still has a chance to recover if appropriate and timely treatment is provided. [DOI: 10.1117/1.JBO.19.1.017005]

Keywords: nicotinamide adenine dinucleotide fluorescence; intensive care; monitoring; hypoxia; alarm; oxygen indicator.

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1 Introduction

Most deaths in the intensive care unit (ICU) arise from multiple organ dysfunction syndrome, which is widely recognized to be a result of tissue hypoxia[1] and is associated with many life-threatening conditions. The brain is the most vital organ that needs to be protected and precisely monitored in patients in the ICU and operating room (OR). Early detection and alarm signals are essential for the implementation of effective emergency measures and for saving lives. Current intensive care monitoring includes the use of electrocardiography (ECG), blood pressure monitoring, central venous catheters, pulmonary artery catheterization, cardiac output, pulse oximetry, airway CO_2 monitoring, transcutaneous blood gases, respiratory mechanics, respired gas analysis, etc.[2] Specifically, respired gas analysis determines the oxygen uptake from inspiration and pulse oximetry provides an estimate of oxygen saturation in arteries. Central venous catheters and pulmonary artery catheterization provide information regarding the oxygen saturation in veins, and transcutaneous blood gases reflect the partial pressure of oxygen at the tissue level.[2] Oxygen is transported in the body as a result of differences in partial pressure at different locations. The partial pressure of oxygen varies from 104 mm Hg in alveoli to 40 mm Hg in veins, 23 mm Hg in tissue cells, and is <1 mm Hg in mitochondria.[3,4] Therefore, fluctuations in oxygen partial pressure in the mitochondria are thought to provide a more sensitive and earlier response (onset) signal than that in other tissues and organelles.

Nicotinamide adenine dinucleotide (NAD^+) in its reduced form, NADH, is an autofluorescent coenzyme involved in the mitochondrial respiration chain in all living cells. Under excitation at 320 to 380 nm, NADH emits fluorescence at 420 to 480 nm, while NAD^+ is unexcitable. Although NADH is an indirect index that reflects oxygen consumption, its particular location at the very beginning of the respiration chain results in it being the most sensitive oxygen indicator in mitochondria and tissues.[5] Since, Chance et al. pioneered *in vivo* NADH measurement in 1962,[6] >1000 related papers on NADH fluorescence combined with other parameters *in vitro* and *in vivo* studies have been published, and descriptions of typical NADH changes in many pathological conditions have been provided.[7] However, to the best of our knowledge, no one has determined the response speed of the NADH signal to hypoxia compared to other parameters.

Most of the parameters that are monitored in the ICU provide information on the systemic respiratory and cardiovascular compartments. Multiparametric monitoring of microcirculatory and intracellular parameters is not available in ICUs or ORs on a daily basis. According to the recently published papers in critical care medicine,[8] measuring "tissue level" parameters are necessary for improving the quality of patient monitoring. Monitoring of the mitochondrial NADH redox state, in addition to other parameters, would help to detect changes in the oxygen balance earlier than would monitoring of other systemic parameters.

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2 Materials and Methods

To provide comprehensive experimental evidence, we established four acute hypoxia rat models with varying causes of hypoxia. These causes cover all the steps of oxygen transport in body, and include hypoxic hypoxia, induced by lowering oxygen tension in the lung; hypemic hypoxia (also called anemic hypoxia), which is caused by a decreased ability of hemoglobin to carry oxygen; circulatory hypoxia (also called stagnant or ischemic hypoxia), which occurs in response to decreased blood flow; and histogenous hypoxia (often confined to histotoxic hypoxia), which reflects disabled metabolic activities in tissues. Due to the variation in the causes of hypoxia, the response order, also referred to as onset order, of systemic pulmonary ventilation, cardiac circulation, and regional microcirculation, should vary significantly. To evaluate the process of death from hypoxia, death was the endpoint for all models. Also, the measuring sustained after all the signs of death happened, such as cerebral blood flow (CBF) reduced to minimum and cardiac and respiratory arrest occurred.

Multiple parameters, including cerebral NADH fluorescence, 366-nm reflectance, microcirculatory CBF, ECG, and respiration, were measured and analyzed from the induction of hypoxia until death.

2.1 *Animal Preparation*

Male adult Wistar rats, weighing 230 ± 20 g, were anesthetized intraperitoneally with a mixture of 10% urethane and 2% chloral hydrate (0.9 ml/100 g body weight). Anesthesia was maintained by adding one-sixth of the initial dose if the animal appeared to be regaining consciousness. The operation and the connection of the fiber optic probe to the cerebral cortex were preformed according to the standard procedures.[9]

2.2 *Monitoring Platform*

The monitoring platform consists of two main parts, which are shown in Fig. 1. One part integrates a direct current (DC) fluorometer/reflectometer[7] and a laser Doppler perfusion monitor and measures regional parameters such as fluorescence, reflectance, corrected NADH signals, and CBF.[10] The other part is a multichannel physiological data acquisition and analysis system (RM6240, Chengdu Instrument Factory, Chengdu, China) and provides systemic and general information, including ECG and respiration.

The anesthetized animal was placed in the supine position, and the fiber optic probe of the monitor was fixed on the cerebral cortex within a 3-mm diameter region of interest that contained fewer blood vessels. Thus, the fluorescence, reflectance, corrected NADH signals, and CBF were collected from approximately the same area in the cortex. The ECG electrodes were placed subcutaneously on the limbs in Lead II mode. The animal's respiration was monitored and recorded with a thorax skin linking convertor. The combined systems were controlled simultaneously through a script run in LabView (National Instruments, Austin, Texas).

2.3 *Experimental Protocols*

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Huazhong University of Science and Technology. The rats were randomly divided into five groups, with eight rats per group. After the operation and stabilization, a preliminary test of brain normality was performed by administering 99.9% nitrogen to the rats for approximately 30 s until they stopped breathing. The rats whose NADH levels were significantly increased passed the test,[9] and

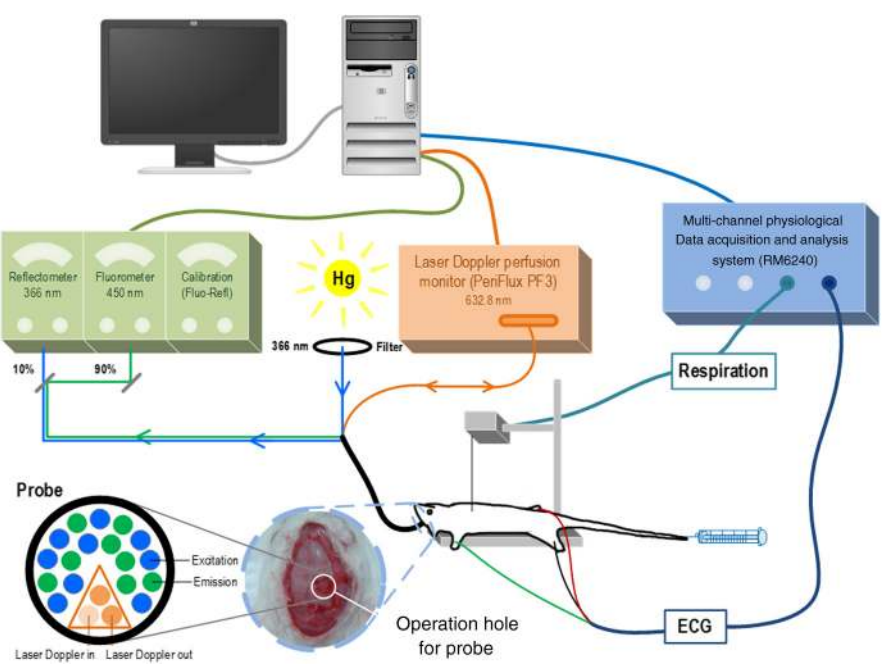

Fig. 1 The multiparametric monitoring platform consisting of two parts. One part integrates a DC fluorometer/reflectometer, which measures fluorescence, reflectance, and calculated nicotinamide adenine dinucleotide (NADH) signals, and a laser Doppler perfusion monitor for cerebral blood flow (CBF). The other part is a multichannel physiological data acquisition and analysis system that monitors ECG and respiration.

Table 1 Summary of the treatments of the five groups.

Group No.	1	2	3	4	5
Hypoxia type	Hypoxic	Hypemic	Circulatory	Histogenous	Normal
Treatment	Breathe in N_2	i.v. 100 mg/kg NaNO2	Cut the right femoral artery	i.v. 125 mg/kg CPZ	i.v. saline
Number of animals	8	7	6	7	8

they were allowed 15 min of rest for recovery, after which they were subjected to one of the different types of hypoxia (Table 1).

Group 1 was given 99.9% nitrogen to induce hypoxic hypoxia until the rat died. Group 2 was injected with 100 mg/kg sodium nitrite ($NaNO_2$, AccuStandard Inc., New Haven, Connecticut), which can oxidize hemoglobin to methemoglobin and decrease the binding of oxygen via the tail vein at a rate of 0.5 ml/ min. Group 3 was treated by cutting the right femoral artery, causing the rats to die due to blood loss. Group 4 was intravenously injected with 125 mg/kg chlorpromazine hydrochloride (CPZ, injection product from Shanghai Harvest Pharmaceutical Co. Ltd., Shanghai, China), an inhibitor on the respiratory chain between NADH and cytochrome *c*,[11] at the same rate as in Group 2. A high dose of $NaNO_2$ and CPZ was chosen here to ensure 100% death based on our preliminary experiments. Group 5 was intravenously injected with saline at the same volume per body weight as used in the control. The measuring was sustained after all the parameters reached their signs of death, except that Group 5 was monitored for 30 min smoothly.

2.4 *Data Process and Statistics*

For each group, the raw data on fluorescence, reflectance, CBF, ECG, and respiration were monitored simultaneously in LabVIEW and processed to figures by MATLAB (Mathworks, Natick, Massachusetts). The recorded fluorescence, reflectance, and calculated NADH voltages were converted to the percentage of 0.5 V.[7] The baseline was defined as the mean value at 60 s prior to the time inducing hypoxia and set as 100%. CBF was calculated as the change in percentage from the baseline value. In an attempt to capture the change in heart rate and respiration rate more promptly, a period of 5 s was selected for the estimation of a beats-per-minute value at the middle time point.

The time constant of the integrated DC fluorometer/reflectometer and laser Doppler perfusion monitor is 3 s, and the time constant of ECG recorder is 0.2 s. As such, to align the time courses of all monitored parameters, 3 s was subtracted from the times recorded for measuring reflectance, fluorescence, calculated NADH, and CBF, and 0.2 s was subtracted from the time recorded for monitoring ECG waves.

After discarding the samples that reflected obvious dysfunctions during the experimental procedure, the data were based on eight rats in Group 1, seven in Group 2, six in Group 3, and seven in Group 4 and were analyzed by IBM SPSS Statistics for Windows (IBM Corp, Armonk, New York). Statistical significance between hypoxia onset time and the time at which the ultimate value was reached was calculated by the paired *t* tests. The data are presented as the mean ± SEM.

3 Results

The integrated system of the DC fluorometer/reflectometer and the laser Doppler perfusion monitor was developed by Mayevsky and Chance and has been utilized for over 20 years in studies on the combination of NADH and CBF, as well as other parameters, such as electrocorticography, DC potential, extracellular ions, and oxyhemoglobin in the cerebral cortex under hypoxic hypoxia, hypemic hypoxia, and circulatory hypoxia. Typical signal responses have been summarized for various pathological protocols.[7]

In our study, five parameters were simultaneously measured to compare systemic and regional changes from the induction of severe hypoxia until death. The changes in the patterns of NADH and CBF exposed to hypoxic hypoxia were similar to those that have been previously reported.[12] The control group (saline) performed stable in every parameter during the whole monitoring process, while the other four hypoxia groups presented significant fluctuations. If we defined the time of inducing hypoxia is time point 0, as seen in Fig. 2, the response time of each parameter was defined as the point when the curve rose higher than the maximum recorded before time point 0 or when it dropped lower than the minimum recorded before time point 0.

As expected, a significant difference in the order of the response times of these parameters was observed among

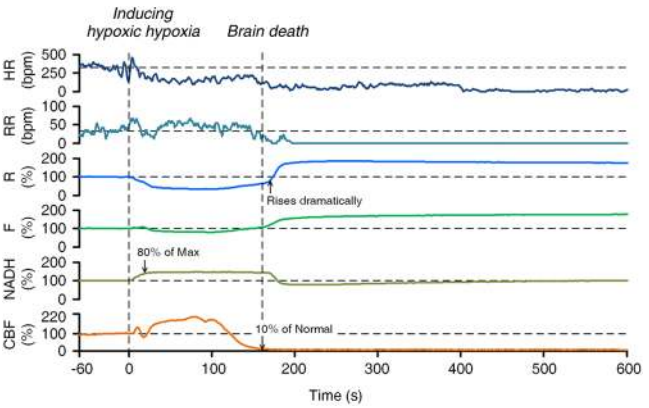

Fig. 2 Representative multiparametric monitoring during the procedure of hypoxic hypoxia till death. The horizontal axis represents time (s). Monitored parameters are listed from top to bottom as heart rate (HR), respiration rate (RR), reflectance change (R), fluorescence change (F), corrected NADH change (NADH), and cerebral blood flow (CBF). The rat breathed in nitrogen at the time point 0, and the baselines, shown as horizontal dashes, were calculated as the mean values of each parameter during the 60 s before breathing in nitrogen. The vertical dashes tell the time when starting breathing in nitrogen and brain death. Brain death is defined as CBF decreased to below 10%. The time, when NADH, reflectance, and CBF reached their ultimate values were pointed out, were defined as 80% of the maximum, rising dramatically, and 10% of normal value (baseline), respectively. However, for respiratory and ECG, the ultimate value was defined when the raw waves recorded by RM6240 became flat, not based on the calculated rate curves as shown in this figure.

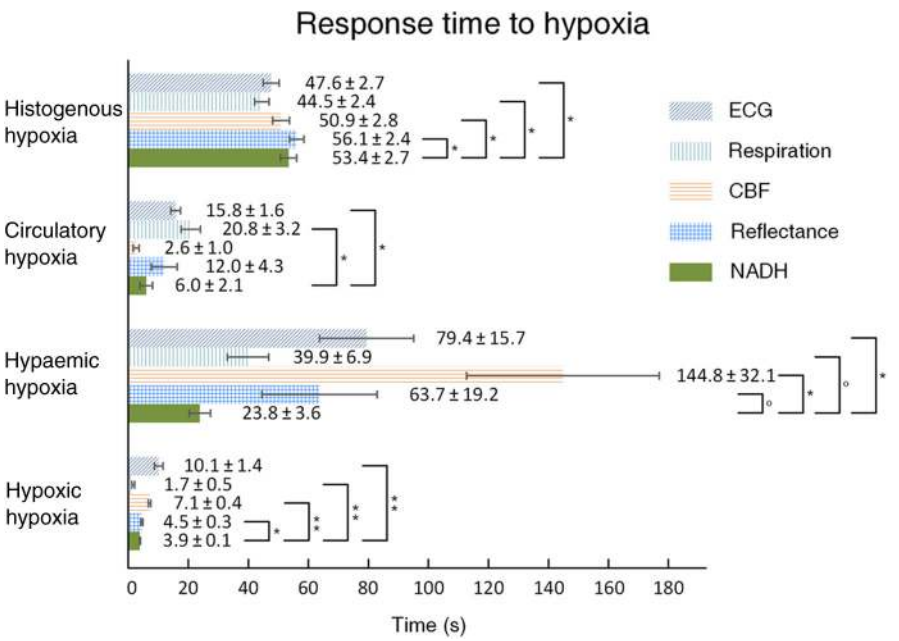

Fig. 3 Comparison of the response time of the monitored parameters to hypoxia. The horizontal axis represents time (s), and the vertical axis shows the four types of hypoxia, each with the five monitored parameters: NADH, reflectance, CBF, respiration, and ECG. The bar length represents the mean response time to hypoxia for each parameter, and the error bar is the standard error of the mean (SEM). A single asterisk (*) indicates that the significance of the response time between NADH and any other parameter is $p < 0.05$, a double asterisk (**) indicates $p < 0.01$, and a circle indicates $p < 0.1$.

the four hypoxia models. As can be summarized in Fig. 3, the response time of each parameter was quite close in the hypoxic hypoxia group, and the respiration signal responded the most promptly to the disturbance of breathing. In this group, NADH was the second signal to respond. In the hypemic hypoxia group, NADH responded first. Blood flow was the most directly affected parameter in the circulatory hypoxia group, and accordingly, NADH, reflectance, and CBF were not statistically different from each other.

However, in the histogenous hypoxia group, NADH only responded earlier than the reflectance ($p < 0.05$). A reduction in the respiration rate occurred first, and this was followed by a reduction in the heart rate, indicating that the drug, CPZ, damaged the pulmonary, and cardiac circulation when it entered the veins and subsequently spread to the lungs and heart, before arriving at the brain. Therefore, the mitochondria of the lung and the heart were damaged successively before the brain was.

By systematically comparing the response times of the five parameters under the four types of hypoxia, we concluded that the order in which the monitored parameters respond is affected by the type (cause) of hypoxia. Notably, among the five parameters studied, NADH is not always the first parameter to respond. As such, multiparametric monitoring may be required to increase the sensitivity of hypoxia detection. Any parameter that responded earlier than the NADH merits attention. As indicated in Fig. 3, the lag time between the NADH response and the directly affected parameters, such as respiration in hypoxic hypoxia, CBF in circulatory hypoxia, and respiration, ECG, and CBF in histogenous hypoxia, is only between 2.2 and 8.9 s. In contrast, the lead time between the NADH response and the indirectly affected systemic parameters, such as ECG in hypoxic hypoxia, respiration, ECG in hypemic hypoxia, and respiration and ECG in circulatory hypoxia, is between 6.2 and 55.6 s. The results suggest that among the five parameters studied, NADH is the parameter that is most sensitive to hypoxia. This implication excludes the directly affected parameters caused by different types of hypoxia.

We also compared the time required for each parameter to reach its ultimate value before the animal died. According to the literature, when an animal dies, NADH reaches its maximum level.[9] Additionally, reflectance increases, which is also called the secondary reflectance increase.[7] In contrast, CBF drops to its minimal level and the respiration and ECG waves become flat. Therefore, we defined the time point when each parameter reached its ultimate value as follows. NADH reached 80% of the maximum, which in most cases represented the beginning of a plateau. Reflectance rose dramatically. CBF decreased to below 10%, and it is known that when CBF decreases to 10% of its normal value, brain death occurs. The respiration and ECG waves disappeared. As shown in Fig. 4, we found that with the exception of the histogenous hypoxia group, the monitored parameters reached their ultimate values in a similar time sequence: NADH, reflectance, respiration, CBF, and ECG. Notably, CBF and respiration reached their ultimate values at nearly the same time, confirming the signs that when the brain death occurs, both CBF and spontaneous breath disappear.[13]

However, we found that in all four hypoxia models, NADH was the parameter that reached its ultimate value first. As shown in hypoxic hypoxia in Fig. 4, NADH reached its ultimate value 156.3 s earlier than did CBF ($p < 0.01$), which represents brain death. In hypemic hypoxia, NADH reached its ultimate value 343.0 s earlier than did CBF ($p < 0.1$). In circulatory hypoxia, NADH reached its ultimate value 596.0 s earlier than did reflectance ($p < 0.01$), and the second one reached the ultimate value 926.2 s earlier than did CBF ($p < 0.01$). In histogenous hypoxia, NADH, CBF, respiration, and ECG reached their ultimate values without significant difference ($p > 0.05$). In other words, when

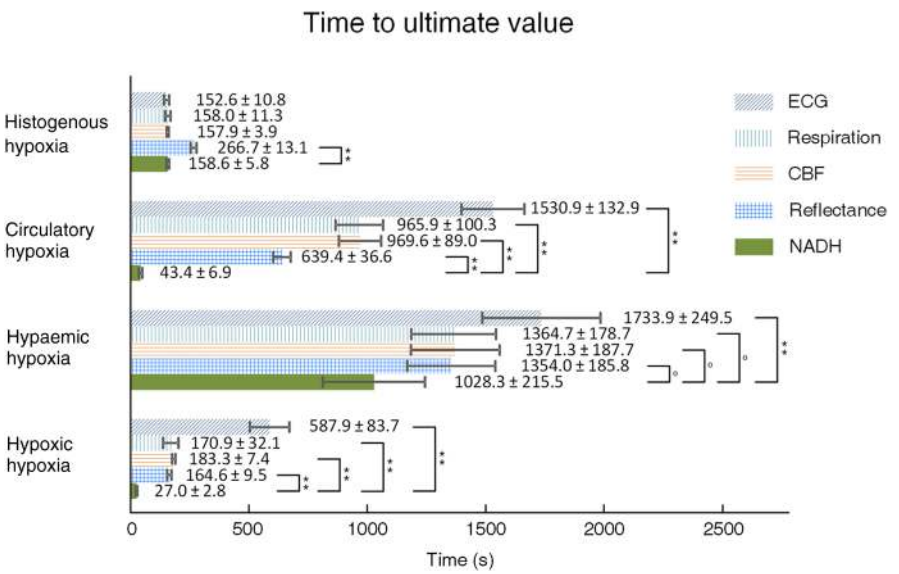

Fig. 4 Comparison of the time required for the monitored parameters to reach their ultimate values. The horizontal axis represents time (s), and the vertical axis shows the four types of hypoxia. The ultimate values are defined when the NADH reaches a plateau, secondary reflectance increases, CBF decreases below to 10% of the normal level, and the respiration and ECG signals stop. The bar length represents the mean time and the error bars are the standard error of mean (SEM). A single asterisk (*) indicates that the significance of the response time between NADH and any other parameter is $p < 0.05$, a double asterisk (**) represents $p < 0.01$, and a circle represents $p < 0.1$.

NADH reaches its ultimate value, brain death follows in from several seconds to >15 min.

4 Discussion and Conclusion

It is accepted that NADH is the most sensitive oxygen indicator at the mitochondrial level.[7,9] However, previous studies have not paid attention to the time comparison between NADH and other simultaneously monitored parameters. In this study, we induced hypoxia in four different ways and investigated the responses of NADH and other parameters during the severe pathological processes. We found that NADH plays a leading role as an early and sensitive indicator of hypoxic, hypemic, and circulatory hypoxia. It is known that any type of hypoxia can induce adaptions and changes in pulmonary ventilation, hemoglobin saturation, and cardiac output systems. The turbulent response of ECG, respiration, and CBF not only shows their state of dysfunction but also reflects autoregulation by the body to maintain homeostasis.

Because NADH was shown to have an exciting advantage in terms of response time to hypoxia, a "gold standard" should be suggested for clinical application. We found that the maximum of NADH increase was $(149.3 \pm 2.1)\%$ in the hypoxic hypoxia group, $(143.0 \pm 6.1)\%$ in the circulatory hypoxia group, and $(144.5 \pm 3.9)\%$ in the histogenous hypoxia group. These differences were not statistically significant. In contrast, in hypemic hypoxia, NADH only rose $(126.3 \pm 2.4)\%$. The explanation for this finding might be that NADH can reduce the methemoglobin induced by $NaNO_2$ back to oxyhemoglobin in red blood cells.[14] Therefore, under normal conditions, when a rat dies, NADH levels may rise 136.9% to 151.4%.

In our study, the time required for NADH to rise to a plateau of approximately 80% of the maximum value was defined as the time at which the ultimate value was reached. Therefore, we suggest that 80% of the lower limit (increase of 36.9%) is a possible indicator of NADH increase, which peaks at approximately 130%. However, in our preliminary tests involving 30 s breathing N_2, NADH levels were observed to rise by >50% during hypoxia, even though the animal could totally recover after respiration was restored. As such, if the appropriate treatment is provided in a timely manner, recovery is possible, even if NADH reaches its alarm limit (130%).

In addition to an elevation of 130%, the reduced rising slope of the NADH monitoring curve is also noteworthy, as this result indicates that NADH levels are reaching a plateau.

In summary, based on the theory that was established and developed by Chance et al. in the last century, we have systematically studied four different types of hypoxia, specifically, quantitatively and simultaneously monitoring NADH, reflectance, CBF, respiration, and ECG from the induction of hypoxia until death. We reported the onset order in which the parameters responded to hypoxia, as well as the order in which the parameters reached their ultimate values. Our results support NADH as a sensitive oxygen indicator comparing to regional microcirculatory and systemic signals. Moreover, even if the NADH curve reaches its alarm limit, appropriate and timely treatment can still prevent mortality.

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Biographies of the authors are not available.