FLOW-MEDIATED SKIN FLUORESCENCE METHOD FOR NON-INVASIVE MEASUREMENT OF THE NADH AT 460 NM – A POSSIBILITY TO ASSESS THE MITOCHONDRIAL FUNCTION

ZALEŻNA OD PRZEPŁYWU KRWI FLUORESCENCJA SKÓRY PRZY DŁUGOŚCI ŚWIATŁA 460 NM DO POMIARU ZAWARTOŚCI NADH – MOŻLIWOŚĆ OCENY CZYNNOŚCI MITOCHONDRIÓW

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Summary: Nicotinamide adenine dinucleotide (NADH) is produced both in the cytoplasm and in mitochondria. However, NADH undergoes oxygenation to NAD^+ only in mitochondria. The NADH molecules, after activation by ultraviolet light, start emitting fluorescence at a wavelength of 460 nm. This phenomenon is used to non-invasively measure mitochondrial function in the forearm epidermis at rest, during transient ischaemia, and during the following reperfusion by the flow-mediated skin fluorescence (FMSF) method. Skin fluorescence derived from NADH comes from the outermost epidermis at a depth of no more than 0.1 mm. This review summarises the fundamental principles of the FMSF method, some of the proposed parameters describing dynamic changes in skin fluorescence, and a comparison with the laser Doppler flow method measuring the function of skin microcirculation. Finally, we present some limitations of this novel and promising method.

Keywords: fluorescence, ischaemia, mitochondria, nicotinamide adenine dinucleotide, reperfusion

Streszczenie: Podczas, gdy dinukleotyd nikotynoamidoadeninowy (NADH) powstaje zarówno w cytoplazmie, jak i w mitochondriach, utlenianie NADH do NAD^+ zachodzi tylko w mitochondriach. Po wzbudzeniu światłem ultrafioletowym cząsteczki NADH emitują fluorescencję o długości fali 460 nm. Zjawisko to zostało wykorzystane do nieinwazyjnej oceny czynności mitochondriów w naskórku przedramienia w spoczynku, w czasie niedokrwienia i reperfuzji przez metodę mierzącą fluorescencję

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skóry zależną od przepływu krwi (ang. *Flow Mediated Skin Fluorescence*, FMSF). Fluorescencja skóry pochodzi z najbardziej zewnętrznej, do 0,1 mm, warstwy naskórka. Przedstawiona praca poglądowa podsumowuje podstawowe zasady metody FMSF, wyjaśnia proponowane parametry charakteryzujące spoczynkową i dynamicznie zmieniającą się fluorescencje oraz porównuje tę metodę z oceną mikrokrążenia skórnego przy pomocy laserowego przepływomierza Dopplerowskiego. W pracy przedstawiono również ograniczenie nowatorskiej i obiecującej metody FMSF.

Słowa kluczowe: dinukleotyd nikotynoamidoadeninowy, fluorescencja, mitochondri, niedokrwienie, reperfuzja

INTRODUCTION

All living cells require energy for their functioning and survival. In the human body, energy is produced from cellular nutrients, i.e. carbohydrates, fats, and proteins, which are gradually oxidised in a series of reactions. The retrieved energy is transferred to special energy carrier molecules such as adenosine 5'-triphosphate (ATP) or nicotinamide adenine dinucleotide (NADH). Whereas some of ATP and NADH are produced in the cytoplasm, for example during glycolysis, the majority of the energy pathways take place in mitochondria. For this reason, mitochondria are called cellular power plants and play a crucial role in cellular homeostasis and well-being [18, 19].

The mitochondria are double-membraned organelles that possess their DNA encoding 37 genes. Mitochondria produce ATP and NADH; they are also involved in cell growth, differentiation processes and finally cellular death [12]. Impairment of mitochondrial function can lead to many acute or chronic diseases (tab. 1).

TABLE 1. Examples of diseases and clinical conditions in which mitochondria are directly involved

TYPE OF DISEASES	EXAMPLES
Metabolic	Diabetes Obesity Body metabolic imbalances
Neurological	Amyotrophic Lateral Sclerosis Huntington's Disease Alzheimer's Disease Parkinson's Disease
Infectious	Hepatitis C Sepsis and septic shock
Cardiovascular	Ischaemic heart disease Myocardial infarction Heart failure Cardiogenic shock
Other	Cancer Tubulopathies

MITOCHONDRIA

STRUCTURE OF MITOCHONDRIA

A double membrane surrounds mitochondria, and the intermembrane space separates the outer and inner layers [22]. (fig. 1)

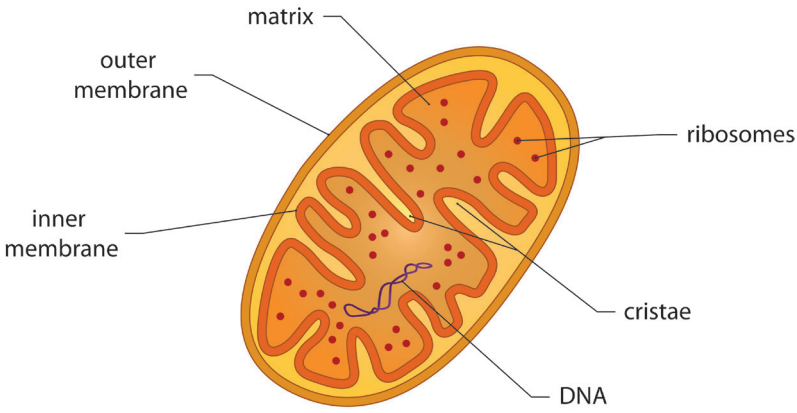

FIGURE 1. The structure of the mitochondrion

Inside the inner membrane, lies the matrix and the mitochondrial genome [12, 25]. The outer layer is permeable to the majority of metabolites, and contains acetyl-CoA synthetase and phosphoglycerol acetyltransferase. The intermembrane space has adenine kinase and creatine kinase, and it is essential for storing protons involved in ATP production (see below). The inner membrane is impermeable to most metabolites (fig. 2).

It contains, however, a specific phospholipid – called cardiolipin, enzymes of the respiratory chain, ATP synthase and transmembrane shuttles for transporting selected molecules in and out of the matrix. The inner membrane, to increase its active surface, is folded and forms several smaller folds called cristae [10].

FUNCTION OF MITOCHONDRIA

As mentioned, mitochondria are responsible for generating most of the cell's energy derived from several oxidation processes. In general, these processes require constant oxygen supply during energy retrieval from amino and fatty acids, carbohydrates or ketone bodies. Nutrients are metabolised to substrates which finally enter the citric acid cycle that produces electrons travelling along the protein

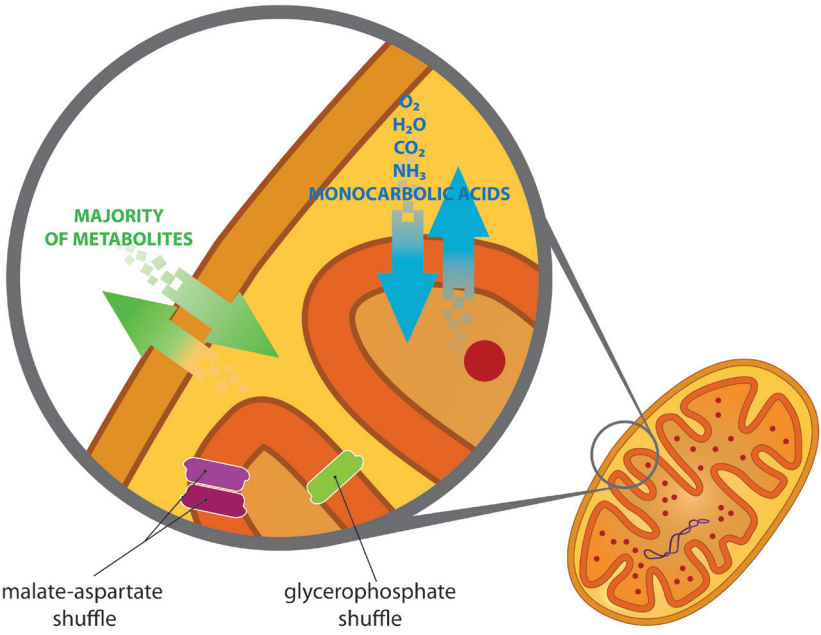

FIGURE 2. The permeability of the mitochondrial membranes and transporting shuttle

complexes of the electron transport chain. The mitochondrial intramembrane space, the inner membrane and the matrix are involved in oxidation which generates the energy gradient across the inner membrane. This gradient is finally transformed into the energy stored in ATP and NADH [1, 8].

NADH is a reduced form of nicotinamide adenine dinucleotide (NAD+) produced by specific dehydrogenases catalysing reactions of glycolysis, the citric acid cycle, and the mitochondrial respiratory chain both in the cytoplasm and in mitochondria. During ATP production through the respiratory chain, electrons are transferred to oxygen mostly with the participation of NADH. Noteworthy, although some of the NADH molecules are also formed in the cytoplasm (via glycerol-3-phosphate dehydrogenase in the oxygen-independent glycolysis), the oxygenation of NADH takes place only in mitochondria. To avoid NADH accumulation in the cytoplasm, two unique cross-membrane shuttles, i.e. the malate-aspartate and glycerophosphate shuttles, transport NADH from cytosol to mitochondria (fig. 2). This transport, however, is active only when oxygen is available (aerobic conditions). During hypoxia or anoxia, when less or no oxygen is available, the NAD+ molecules are regenerated through the process of transforming pyruvate into lactate – as a result, the amount of lactate and protons (H^+) rises [23]. A classic example of hypoxia and/or anoxia is cellular ischaeia. During ischaemia, a restriction in blood supply to organs and tissues leads to a reduction or stopping of oxygen and nutrients needed for cellular metabolism – in such conditions the respiratory chain is blocked [4, 7, 10, 16]. Table 2 summarises cellular processes involving NADH and NAD+.

TABLE 2. List of processes in which NADH and NAD+ are involved in cellular metabolism and function [1]

CYTOPLASM	NUCLEUS	MITOCHONDRION
• Glycolysis (glycerol-3-phosphate dehydrogenase) • Part of second messenger system (precursor of cyclic ADP-ribose) • Pirogronian reduction to lactate	• ADP-ribose transfer reactions • Modification of RNA • Regulation of transcription processes	• Citric acid cycle • Respiratory chain (complexes I, III, IV) • Beta oxidation (matrix)

METHODS TO STUDY MITOCHONDRIA

There are many methods which are applied for studying mitochondria (tab. 3). Some of them can be used for the examination of mitochondrial number, structure and density in cells. These methods use modern light microscopes based on phase contrast or interference contrast optics, optical, fluorescence optical microscopes, high-resolution transmission or scanning electron microscopes and electron tomography. Three significant limitations of these methods are the need to use cell or tissue samples collected either invasively or post-mortem, usually elaborate preparations, and high costs of their use. Another class of methods is used for the examination of different mitochondrial functions, e.g. their enzymatic activity related to the respiratory chain function, handling of specific ions or molecules such as Ca^{2+}, ATP, NADH or even oxygen consumption. Many years ago, it was possible to employ most of these methods only in vitro (e.g. spectrophotometric assays). Nowadays, however, newer methods allow studying mitochondria in vivo either with the use of some cell samples (e.g. biopsy samples, isolated mononuclear cells from the circulating blood) or in living animals and even humans. Magnetic resonance spectroscopy is an example of a sophisticated tool to study mitochondrial function in vivo in various tissues and organs, for instance, liver, kidney or heart. This method measures magnetic resonance signals of specific isotopes of ^{13}C, ^{1}H, ^{31}P and ^{23}Na which can be localised in different mitochondrial structures, e.g. nuclei. Since these methods are very distinct, each of them have specific limitations; some require invasive specimen collection, other expensive reagents or tools or are time-consuming. A different approach to mitochondrial examination is through molecular studies focusing directly on the analysis of mitochondrial genome (mtDNA), e.g. by studying mtDNA point mutations or large-scale mtDNA deletions, applying Southern and Northern blotting, and even next-generation sequencing. Some other molecular methods focus on the examination of genes in the nuclear genome encoding proteins modifying mitochondrial function or proteins (Western blotting) and proteomics. Such methods are very often costly and usually only available to research centres. It is, however, possible to examine

mitochondrial function in vivo, entirely in a non-invasive way, and at low cost by studying skin fluorescence characteristic for NADH. The remaining part of this review will focus on such a method.

TABLE 3. Examples of different methods for the examination of mitochondrial structure, function, genes or proteins

CLASS OF METHOD	METHOD NAME	PRINCIPLE OF OPERATION	COMMENTS
Mitochondrial structure	High-resolution electron microscopy and electron tomography [16]	3D presentation of the mitochondrion, *cryo-ET combined with 3D view provides close to atomic resolution	shows many details, requires careful sample preparation, time-consuming, accessible only in scientific centres, expensive, invasive, in vitro
Enzyme activity	Spectrophotometric enzymes assays [16]	Measurement of mitochondrial enzymes activity	requires small tissue sample, expensive, invasive, in vitro
	P-MRS of mitochondrial citric acid cycle [16]	based on magnetic resonance spectroscopy (MRS), bounded with respiratory chain activity	provides an insight into mitochondrial function, time-consuming acquisition, poor reproducibility, expensive, non-invasive, in vivo
Respiratory chain	Bioluminescent measurement of ATP production [16]	quantifying the rates of ATP production	it is possible to measure the smaller sample of isolated mitochondria than in traditional respirometry, cheap, invasive
	Polarographic measurement of oxygen consumption [16]	the level of oxygen consumption in the presence of specific substrates	time-consuming, small biopsy samples, cheap, invasive
	P-MRS of mitochondrial O2 consumption or ATP production [16]	based on magnetic resonance spectroscopy (MRS)	limited clinical application, poor reproducibility, expensive non-invasive, in vivo
	Positron emission tomography (PET) [3]	Measures ATP in intact organs	broad clinical application, especially in oncology, ATP measurement is not used in clinical practice, expensive, non-invasive, in vivo

CLASS OF METHOD	METHOD NAME	PRINCIPLE OF OPERATION	COMMENTS
Mitochondrial genome	genetic testing [13]	searching for primary disorders affecting, for example, respiratory chain due to inherited disorders	tested from a blood sample, only some centres, for rare mutations, expensive, limited invasiveness, in vitro
Nuclear genome	genetic testing [14]	searching for nuclear DNA defects causing mitochondrial disorders, e.g. related to oxidative phosphorylation and respiratory chain	tested from a blood sample, only some centres, for rare mutations, expensive, limited invasiveness, in vitro
Proteins	Electrophoresis and Western-blotting [16]	analysis of mitochondrial proteins, e.g. from the respiratory chain	highly specific and sensitive method, determine protein composition, different kind of samples (cells, tissues) time-consuming, frequent artefacts, expensive, invasive, in vitro
	Molecular probes for immunofluorescence [3]	possibility to visualise mitochondrial membranes, calcium flux, oxidative phosphorylation	a tissue sample is needed, expensive, invasive, in vitro
	FRAP (fluorescence recovery after photobleaching) [21]	different markers allows choosing which protein/membrane is examined	a tissue sample is needed, determining the kinetics of diffusion in living cells, expensive, invasive, in vitro
NADH fluorescence spectroscopy	CritiView (CRV) [19]	spectroscopy of mitochondrial NADH, tissue blood flow, blood oxygenation, tissue reflectance	measures parameters related to tissue viability, used in clinical scenarios, non-invasive, in real-time, cheap, in vivo
	Flow Mediated Skin Fluorescence FMSF AngioExpert [11, 24, 26]	NADH fluorescence	limited to the epidermis, easy-accessible, easy to transport, non-invasive, real-time dynamic changes in NADH due to ischaemia and reperfusion, cheap, in vivo

NADH AS A MARKER OF MITOCHONDRIAL FUNCTION

In the 40's of the 20th century, Warburg discovered that NADH activated by ultraviolet light starts emitting fluorescence at a wavelength of 340 nm [18]. More detailed studies found out that NADH absorbs ultraviolet light at a wavelength range of 320-380 nm, and emits autofluorescence at a length of 420-480 nm. It appears that emission of fluorescent light is specific only for NADH but not NAD+ [4, 27]. This phenomenon has been used to quantify NADH amount in different solutions as well as in cell and tissue samples. Mayevsky et al. [18] also found that NADH is the most substantial component responsible for fluorescence at 460 nm in the human skin, and it estimates the skin redox state.

In vitro studies on cellular redox state back to 1955 [6], whereas the first in vivo animal experiments date to 1962 [5, 15]. Balu et al. [2] used a clinical multiphoton microscope to monitor NADH fluorescence in vivo and noninvasively. During arterial occlusion, they observed a reduction in NADH fluorescence in a human keratinocyte laying close to stratum basale, and the change in NADH fluorescence was connected with a decrease in oxyhemoglobin.

Not so long ago, Mayevsky et al. [18, 19] developed a new device, named CritiView, for monitoring patients in intensive care units and/or undergoing some surgery. Very recently, Angionica, a Polish company, has developed a new device called the AngioExpert for Flow Mediated Skin Fluorescence (FMSF). The FMSF measures forearm skin NADH fluorescence change in time during arterial occlusion and post-ischaemic reperfusion [24], i.e. after restoring blood flow to the forearm.

The AngioExpert device activates skin with ultraviolet light at a wavelength of 340 nm and measures the fluorescence at a length of 460 nm. Figure 3 shows an example of skin NADH fluorescence recorded at rest for 3 minutes, then during 100-second ischaemia evoked by arterial occlusion caused by inflation of the brachial cuff, followed by instant cuff deflation leading to reperfusion. Proposed parameters describing dynamic changes in NADH fluorescence during ischaemia and reperfusion are presented in figures 3, 4 and 5, and table 4.

Figure 3 shows an example of the skin fluorescence signal acquired at rest, during 100-second ischaemia followed by reperfusion from a healthy 47-year-old man. The fluorescence increases gradually during ischaemia and then rapidly decreases within the first few seconds of reperfusion and recovers to baseline level less steeply. There are apparent oscillations of this signal both at rest and during reperfusion. Interestingly, these oscillations completely fade away during ischaemia. Whereas panel a shows the original recording in the fluorescence units (u), panel b shows the same recording with each sample of the corresponding signal but normalised to the mean value of baseline. Consequently, the original shape of the curve and the rate of change are preserved but all values oscillate around 1.

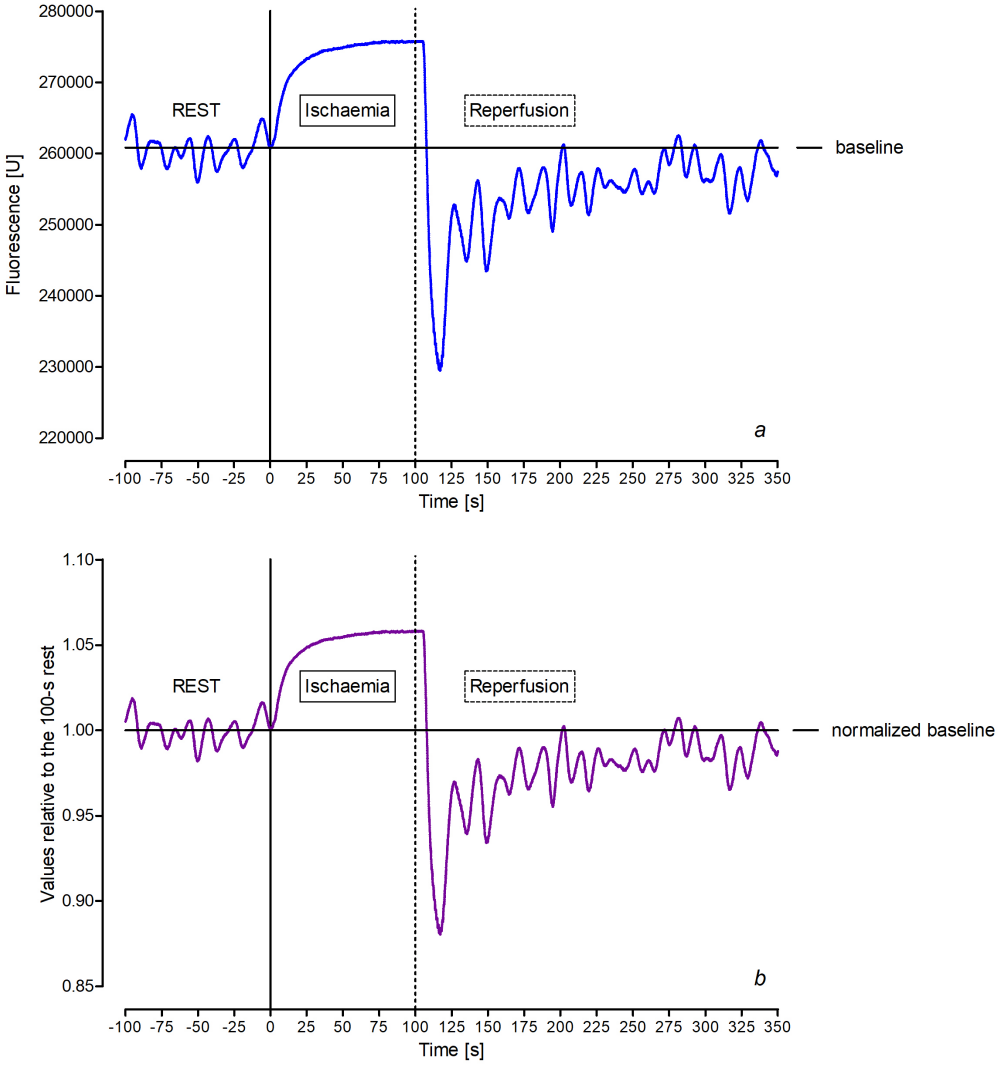

FIGURE 3. An example of typical epidermal fluorescence at a wavelength of 460 nm recorded in the forearm of a 47-year-old healthy man during rest in a seated position, then during 100-second ischaemia and then following reperfusion. Panel a shows fluorescence in its absolute units (u) whereas panel b the same signal normalised to the mean value of baseline measured over a 100-second period before ischaemia. After the resting recording, there is a gradual increase of the fluorescence during ischaemia with a subsequent drop at the time of reperfusion. Of note, apparent oscillations are present both at rest and during reperfusion

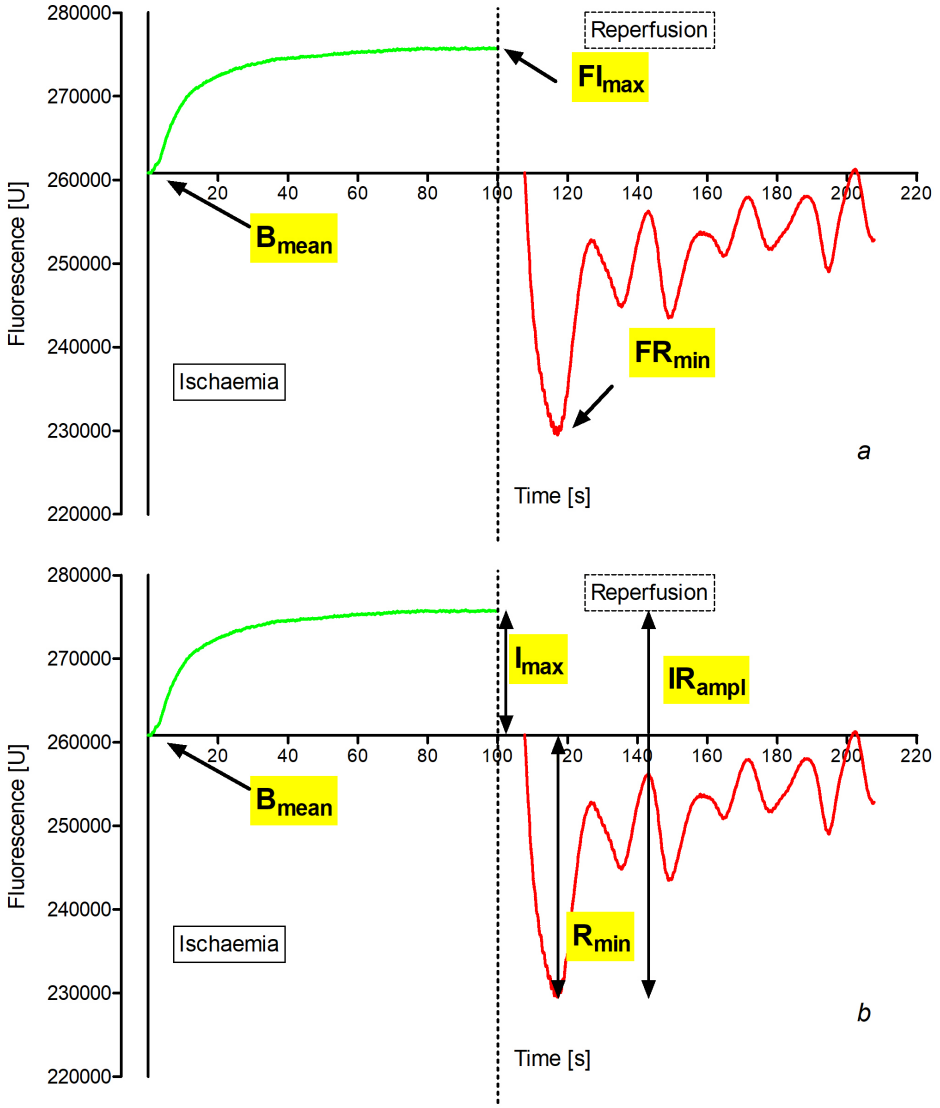

FIGURE 4. The same signal as in Figure 3 with a focus on the ischaemia and reperfusion phases. Panel a shows the three basic points in the signal, i.e. the B_{mean}, FI_{max} and FR_{min} which are starting points for the computation of other parameters shown in panel b and Figure 5, and explained in table 4. Panel b shows three derivative parameters, i.e. I_{max}, R_{min} and IR_{ampl}

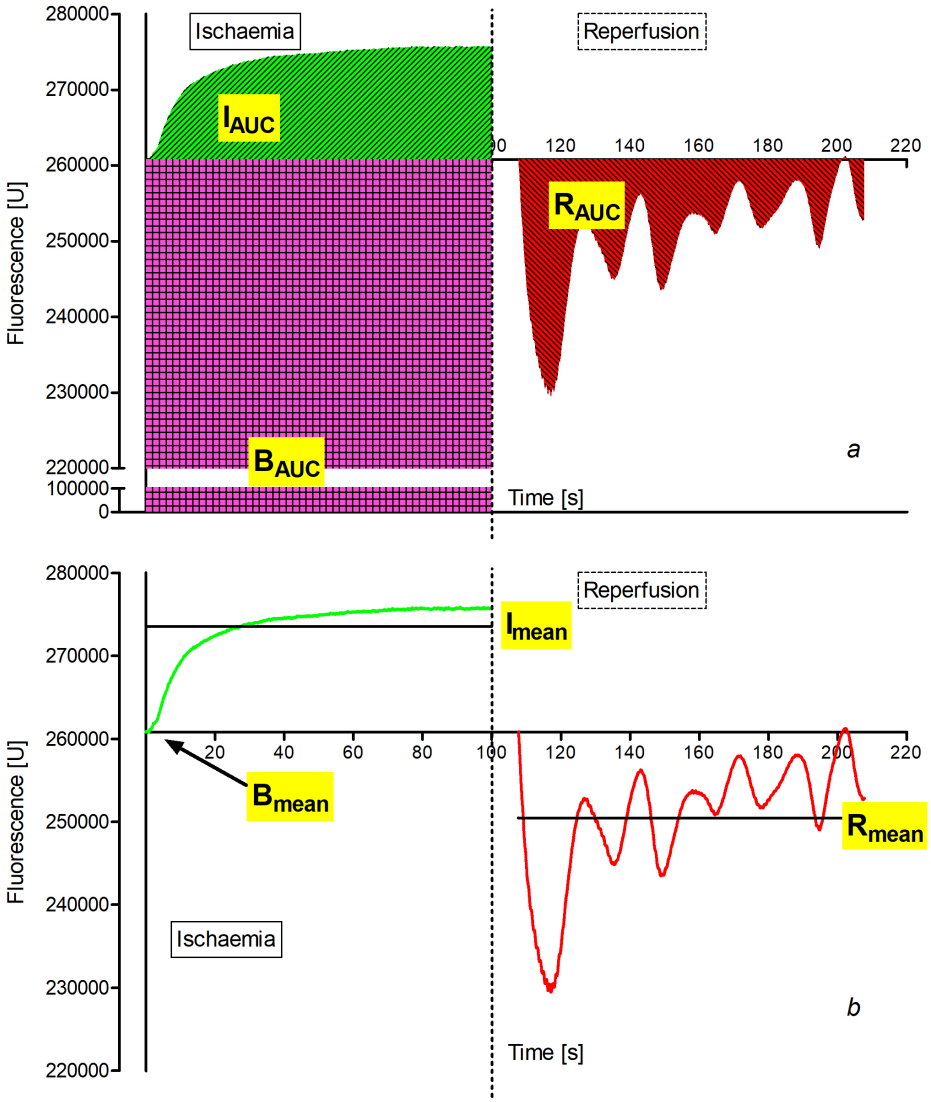

FIGURE 5. The same signal as in Figure 3 with a focus on the ischaemia and reperfusion phases. Panel a shows the three areas defined for the baseline (B_{AUC}), ischaemia (I_{AUC}), reperfusion (R_{AUC}). Panel b presents the positioning of the mean fluorescence during ischaemia (I_{mean}) and reperfusion (R_{mean}) and their relation to B_{mean}

TABLE 4. Parameters used to quantify and describe various features of the forearm flow mediated skin fluorescence signal during rest, ischaemia and reperfusion

PARAMETER	DEFINITION	PROPOSED INTERPRETATION	FORMULA
t_I [s]	Duration of ischaemia.	Time of arterial occlusion caused by inflation of the brachial cuff with the pressure occluding brachial artery.	Varies between protocols.
t_R [s]	The length of the reperfusion period included in the analysis.	This time, for methodological reasons, should be identical with t_I	Varies between protocols.
t_B [s]	The length of the resting period included in the analysis.	This time, for methodological reasons, should be identical with t_I	Varies between protocols.
B_{mean} [u]	Mean fluorescence at 460 nm recorded during rest as the baseline value.	The reference skin fluorescence, related mostly to the amount of NADH being in the balance with NAD^+.	The mean fluorescence over specific time, e.g. 100-second, recorded at rest before ischaemia.
FI_{max} [u]	The maximal fluorescence that increased above the baseline during controlled forearm ischaemia	A measure of the maximal fluorescence during ischaemia of a specific length.	The highest fluorescence value during ischaemia above B_{mean}.
I_{max} [u]	The difference between FI_{max} and B_{mean}	An index of maximal increase of NADH content at the end of ischaemia.	$FI_{max} - B_{mean}$
FR_{min} [u]	The minimal fluorescence that decreases below the baseline during reperfusion.	A measure of the minimal fluorescence during reperfusion.	The lowest fluorescence value during reperfusion below B_{mean}.
R_{min} [u]	The difference between B_{mean} and FR_{min}	An index of the rapid reduction in the NADH content due to its oxidation to NAD+ right after the blood flow and oxygen supply restoration to the forearm during reperfusion.	$B_{mean} - FR_{min}$
IR_{ampl} [u]	The maximal range of the fluorescence change during ischaemia and reperfusion.	An index of the potentially maximal amount of NADH that is generated during ischaemia and then subsequently oxidised to NAD+ during reperfusion.	$I_{max} + R_{min}$
CI_{max} []	The contribution of I_{max} to IR_{ampl}.	An index estimating the relative contribution of the NADH generation during ischaemia to the total amount of NADH turned over during ischaemia and reperfusion. CI_{max} helps to compare how proportional are the effects of different interventions on ischaemia and reperfusion.	I_{max} / IR_{ampl}

PARAMETER	DEFINITION	PROPOSED INTERPRETATION	FORMULA
B_{AUC} [us]	The area under theoretical horizontal line constructed over values of B_{mean} for a specific, protocol-dependent duration, e.g. 100-second.	An index that estimates the baseline load of NADH which is in the balance with NAD+ at rest.	A product of the sum of each fluorescence sample and the duration of the specific recording (e.g. 100 s) divided by the sampling rate of the fluorescence signal.
I_{AUC} [us]	The area under the curve of the fluorescence signal during ischaemia of a specific length, e.g. 100-second, which is above the baseline level.	An index that estimates the accumulated increase of NADH content above the baseline during ischaemia.	A product of the sum of the differences in the fluorescence between each sample and B_{mean}, and the duration of the specific recording (e.g. 100 s) divided by the sampling rate of the fluorescence signal.
R_{AUC} [us]	The area between the baseline and above the fluorescence signal during reperfusion of the same length as the proceeding ischaemia, e.g. 100-second.	An index that estimates the accumulated reduction in NADH content below baseline values during reperfusion, and then gradually recovers towards the baseline.	A product of the sum of the differences in the fluorescence between B_{mean} and each sample, and the duration of the specific recording (e.g. 100 s) divided by the sampling rate of the fluorescence signal.
$IRtot_{AUC}$ [us]	The total area under the fluorescence curves for the ischaemia and reperfusion.	An index of the total amount of NADH generated during ischaemia and then subsequently oxidised to NAD+ during reperfusion.	$I_{AUC} + R_{AUC}$
CI_{AUC} []	The contribution of the I_{AUC} to the $IRtot_{AUC}$.	An index showing the relative contribution of the NADH accumulated during ischaemia to the total amount of NADH turned over during ischaemia and reperfusion. This index is useful for estimating whether the effects of different interventions on ischaemia and reperfusion are proportional or not, and it is less sensitive to random effects than CI_{max}.	$(I_{AUC} - B_{AUC}) / IRtot_{AUC}$
I_{mean} [u]	The average change of the fluorescence above the baseline during ischaemia.	An index of the mean NADH increase during ischaemia.	I_{AUC} / t_I
R_{mean} [u]	The average reduction in the fluorescence below the baseline during reperfusion.	An index of the mean NADH reduction during reperfusion.	I_{AUC} / t_R

PARAMETER	DEFINITION	PROPOSED INTERPRETATION	FORMULA
$I_{t1/2}$ [s]	The halftime of the fluorescence increase from baseline to the maximum.	An index measuring the time required to reach the half of I_{max}.	
nI_{max} []	I_{max} normalised to B_{mean}	An index of the maximal ischemia-related increase of NADH relative to B_{mean}.	I_{max} / B_{mean}
nR_{min} []	R_{min} normalised to B_{mean}	An index of the rapid reperfusion-related reduction in NADH relative to B_{mean}.	R_{min} / B_{mean}
nIR_{ampl} []	IR_{ampl} normalised to B_{mean}	A normalised to the B_{mean} index of the potentially maximal amount of NADH turned over during ischaemia and reperfusion.	IR_{ampl} / B_{mean}
nI_{AUC} []	I_{AUC} normalised to B_{AUC}	A normalised to the B_{AUC} index of the relative accumulation of NADH content during ischaemia.	I_{AUC} / B_{AUC}
nR_{AUC} []	R_{AUC} normalised to B_{AUC}	A normalised to the B_{AUC} index of the relative reduction in NADH content during reperfusion.	R_{AUC} / B_{AUC}
n$IRtot_{AUC}$ []	$IRtot_{AUC}$ normalised to B_{AUC}	A normalised to the B_{AUC} index of the relative change in the total NADH content turned over during ischaemia and reperfusion.	$IRtot_{AUC}$ / B_{AUC}
nI_{mean} []	I_{mean} normalised to B_{mean}	A normalised to the B_{mean} index of the relative mean increase of NADH during ischaemia.	I_{mean} / B_{mean}
nR_{mean} []	R_{mean} normalised to B_{mean}	A normalised to the B_{mean} index of the relative mean reduction in NADH during reperfusion.	R_{mean} / B_{mean}
n$I_{t1/2}$ []	$I_{t1/2}$ normalised to t_I	An index showing what proportion of the ischaemia duration is required to achieve the half of I_{max}.	$I_{t1/2}$ / t_I

PENETRATION OF THE FLUORESCENCE IN FMSF

To better understand what is measured by the skin fluorescence at 460 nm length, some information about the skin is required (fig. 6).

The epidermis is the outermost layer of the skin containing four strata that represent the cycle of an epidermal cell's (keratinocytes) life. The deepest part of

the epidermis is a basal or germinal stratum which is the most active one. Nearly all skin cells localised there are proliferating. During maturation, skin cells become a compound of spinous stratum, where they produce lamellar bodies. The next level is granular, where keratinocytes gradually die and lose their nuclei. The most superficial layer is the cornified stratum – it protects the epidermis against external factors and injury, and consists only of dead cells [25].

The epidermis has no direct blood supply – its blood microcirculation like small arteries, veins and capillaries can lie beneath in the dermis. Nutrients, water, metabolites, and most of the oxygen are delivered to the epidermis by diffusion from dermal microcirculation. It is also speculated that the most external epidermis can use some of the oxygen diffusing directly from the air [2, 15].

Whereas penetration of the excited ultraviolet light applied in the FMSF is only up to 0.5 mm, it is suggested that over 90% of a fluorescence signal comes from the depth below 0.1 mm (fig. 6) [9, 24]. It means that skin fluorescence at 460 nm arises from NADH from the cells building the most superficial epidermis layer, which, as mentioned above, gets nutrients and oxygen through diffusion [9, 26]. Therefore, skin NADH fluorescence represents both the function of microcirculation [11] and diffusion of the oxygen and other substances required for NADH metabolism.

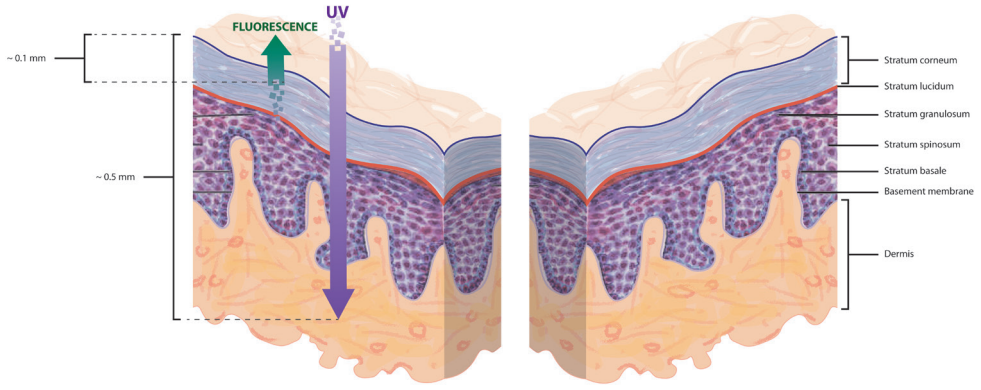

FIGURE 6. The layers of the epidermis with the depth of ultraviolet (UV) light penetration exciting NADH to emit fluorescence light. Of note, whereas the UV penetration reaches 0.5 mm, the fluorescence comes from the outermost layer of the depth of less than 0.1 mm. At this depth there are no capillary vessels, and all cells which are living there are dependent on the diffusion of nutrients and oxygen from the deeper strata

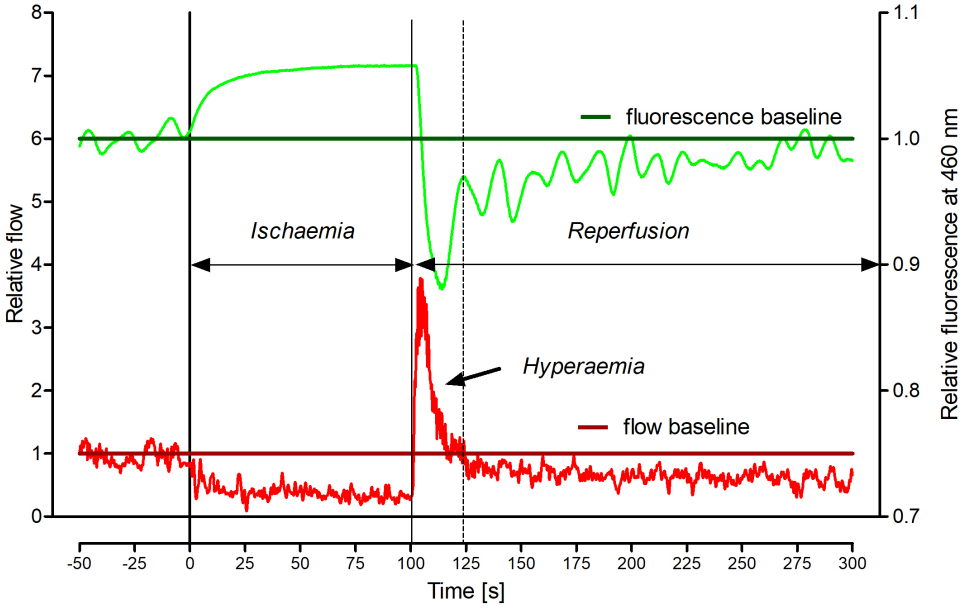

FIGURE 7. Simultaneous recordings of the blood flow in the microcirculation by the Laser Doppler Flow method and cell NADH amount by the fluorescence at 460 nm. The microcirculatory flow was measured by the Laser Doppler Perfusion Monitor (PeriFlux System 5000, Perimed, Sweeden – the device made available, courtesy of Prof. Dorota Zozulinska-Ziolkiewicz from the Department of Internal Medicine and Diabetology of University School of Medical Sciences in Poznan, Poland). The skin fluorescence was measured by the Angioexpert device (Angionica, Poland). Both the flow and fluorescence signals are shown after normalisation of each signal sample by the mean baseline value recorded during the last 100 seconds of the rest in supine position. Both sensors measuring flow and fluorescence were placed at the same time on the same foream at a distance less than 10 cm apart. This figure shows that there is a short-lasting hyperaemia in the early phase of reperfusion, and whereas the flow signal returns to the baseline level, the fluorescence signal needs much more time for recovery to baseline

Figure 7 shows synchronised and simultaneous measurement of FMSF and the microcirculatory flow with the use of the laser Doppler flow technique in the same person. Both sensors were placed on the same forearm approximately 10 cm from one another. It is precisely visible that during forearm ischaemia there is a sudden drop in microcirculatory flow which is accompanied by a gradual increase in fluorescence (and NADH). During reperfusion, after blood supply to the forearm is restored, there is a massive increase in microcirculatory flow with short-lasting hyperaemia accompanied by a dramatic reduction in the fluorescence signal. Whereas, the microcirculatory flow recovers to baseline level within less than 20 seconds of reperfusion, the time needed for restoration of the fluorescence signal to its baseline value is much longer. It shows that the FMSF method visualises

dynamic changes of NADH which are partially explained by blood flow in the microcirculation. However, it is evident that some other factors significantly contribute to this process. It is also worth noticing (Figures 3, 4, 5 and 7) that the FMSF signal shows some slow oscillations at the rate of 3-4 times a minute or frequency between 0.05 and 0.067 Hz. These oscillations are present at rest and are much stronger and regular during reperfusion. In contrast, all oscillations disappear during ischaemia.

METHOD LIMITATIONS

The FMSF pattern is not a constant individual feature, it varies among different people, and it also differs in the same person under different conditions. Although the method has several highly demanded properties, some of which are unique (complete non-invasiveness, in vivo, low cost, safety, allows for examination of the dynamic changes in NADH), it also has some limitations.

Due to the construction of the AngioExpert device, FMSF can be measured only on the forearm skin with subjects sitting on a chair. Due to the properties of fluorescent light at 460 nm, which has very short penetration, it shows the changes in NADH only in cells which are no deeper than 0.1 mm from the skin surface. To correctly perform the study, each participant must be seated in a quiet position for several minutes (usually 10 minutes). Probably, remaining motionless for the entire examination is the most demanding task for studied individuals, particularly older ones. In case they do not optimally comply with the instructions, even tiny arm or forearm movements may produce severe artefacts in the signal. Not rarely, yet if there are no movements, for unknown reasons, an atypical shape of the fluorescence signal during ischaemia is recorded. Instead of an increase, there is either no significant change or a decrease of the fluorescence. Interestingly, such atypical patterns are repeatedly found in the same people who are apparently healthy. It suggests that our understanding of the mechanisms behind FMSF is not complete. Skin fluorescence varies among different people because of differences in skin pigmentation. For this reason, the preferable approach to quantify FMSF signal is the use of readings normalised to individual baseline rather than absolute values of fluorescence. Finally, the proposed parameters for the description of different features of FMSF mainly focus on the static properties of this signal, whereas it appears that there is a lot of interesting information hidden in the dynamic parts of the fluorescence increase during ischaemia and dramatic drop during reperfusion. Another exciting feature requiring further investigation is the presence, quality and intensity of oscillation of fluorescence at rest and during post-ischaemic reperfusion. However, it is important to underline that the FMSF method is a novel method which is still under development.

CONLUCIONS

In summary, mitochondria are essential organelles for all living cells. They play many different functions, with energy production as one of the most crucial. There are many methods to examine their structure and function. The flow-mediated skin fluorescence is a very recent development which has many exciting features that appear to be useful in the research on mitochondria and NADH metabolism in the skin. We are, however, aware that understanding all possibilities and recognising the clinical utility of this method require further and extensive investigations.

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