Flow Mediated Skin Fluorescence

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Flow Mediated Skin Fluorescence (FMSF) is a new non-invasive method for assessing vascular circulation and/or metabolic regulation. It enables assessment of both vasoconstriction and vasodilation. The method measures stimulation of the circulation in response to post-occlusive reactive hyperemia (PORH). It analyzes the dynamical changes in the emission of nicotinamide adenine dinucleotide (NADH) fluorescence from skin tissue, providing the information on mitochondrial metabolic status and intracellular oxygen delivery through the circulatory system.

flow mediated skin fluorescence FMSF technique NADH fluorescence reactive hyperemia response hypoxia sensitivity normoxia oscillatory index

1. Fundamentals of the FMSF Methodology

Lifestyle diseases, including cardiovascular diseases, are the most common cause of death in industrialized countries. Their treatment incurs tremendous cost to the healthcare system. Since current approaches focus mainly on managing symptoms, there is a need for methods and tools for early diagnosis and monitoring of treatment, especially minimally invasive techniques.

There is convincing evidence of the involvement of endothelial dysfunction in the pathogenesis of vascular diseases. Vascular endothelium is an important metabolically active organ that regulates numerous functions and maintains vascular homeostasis, as well as vascular tone and structure—i.e., the dimensions, elasticity, permeability, and reactivity of arteries and veins, including their ability to constrict and dilate. Healthy endothelium is linked to an intact balance of the release of endothelium-derived vasodilating factors (nitric oxide (NO), prostacyclin (PGI2)), endothelium-derived hyperpolarizing factors (EDHFs), and endothelium-derived vasoconstricting factors (endothelin 1, angiotensin II, and thromboxane A2). Under pathological conditions, such as hypercholesterolemia, hypertension, and coronary disease, the production of vasodilating factors is impaired and the production of constricting factors is activated, which also promotes vascular inflammation, remodeling, and thrombosis. A healthy endothelium is essential for undisturbed functioning of the cardiovascular system, while endothelial-dependent dysfunction has been identified as a common thread linking all cardiovascular risk factors. As both an early event and major risk factor, endothelial dysfunction is an important indicator for medical diagnosis. Endothelial dysfunction may be regarded as a barometer of cardiovascular risk [1][2][3][4].

Most studies focus on assessment of endothelial function in conduit arteries. However, there is compelling evidence that functional changes in circulation in other organs and the pathogenesis of cardiovascular diseases are correlated with microcirculation dysfunction, which may precede endothelial impairment in large blood vessels [5]. Microcirculation is that part of the cardiovascular system located between the arterial and venous systems, where intensive gas and metabolic exchanges take place. The response of the microvascular vessels to ischemia is crucial to limit the degree of tissue damage. Numerous studies have shown that the skin microcirculation, which is a readily accessible vascular bed, is representative for the assessment of the systemic microcirculation, its dysfunction, and pathologies [6]. It has been shown that the reactivity of the skin microcirculation is disturbed in persons with an increased risk of ischemic heart disease [7]. The cutaneous microcirculation is also considered to be an independent predictor of atherosclerotic damage [8]. The epidermal layer of the skin is not vascularized, so oxygen and nutrients are transported from the dermis by diffusion. This makes epidermal cells a sensitive marker of early disorders of vascular circulation.

Since direct observation of microcirculatory vessels is difficult, microvascular function in the human skin is mainly studied based on skin blood flow, with the use of such techniques as Laser Doppler Flowmetry (LDF) and Laser Speckle Contrast Imaging (LSCI). However, it is possible to also test microvascular blood circulation via changes in skin biochemistry, especially the mitochondrial NADH redox state of the dermal and/or epidermal cells, which depends on blood circulation and is sensitive to its changes.

The Flow Mediated Skin Fluorescence (FMSF) technique is based on measurement of nicotinamide adenine dinucleotide (NADH) fluorescence from skin tissue cells. NADH and its oxidized form (NAD^+) play a crucial role in biological systems, as redox coenzymes. The interconversion of the NADH/NAD^+ couple has been studied extensively, including from the mechanistic point of view [9].

NADH fluorescence has been widely used to determine mitochondrial function in vivo [10][11]. The metabolism of keratinocytes in human skin has also been studied in terms of NADH fluorescence and mitochondrial dynamics [12][13].

As circulation at rest rarely provides significant information on its proper functioning or dysfunction, the FMSF technique, like LDF, uses mechanical stimulation to assess vascular status. It measures changes in the intensity of NADH fluorescence from the skin in response to post-occlusive reactive hyperemia (PORH), which is accomplished by blocking and releasing blood flow in the forearm using a typical occlusion cuff (as used for blood pressure measurement devices). This enables assessment of both vasoconstriction and vasodilation.

PORH is the most popular test used to assess vascular reactivity in both macro and microcirculation, based on the use of mechanical provocation. Studies on the mechanism of action of PORH prove that several important mediators are involved, with nitric oxide (NO) acting as a potent vasodilator of muscle-type arteries [14].

One of the most extensively investigated non-invasive methods using PORH is Flow Mediated Dilation (FMD), which is based on monitoring the diameter of brachial arteries after reactive hyperemia with a two-dimensional

ultrasound and Doppler ultrasound [15]. This method has been applied widely in clinics and in large-scale epidemiological research, proving that endothelial dysfunction is associated with cardiovascular diseases. It has been shown in many clinical trials that brachial artery FMD independently predicts the risk of future cardiovascular events and enables monitoring of the effectiveness of treatment [16][17][18][19][20][21][22][23]. Low-Flow Mediated Constriction (L-FMC) can be considered as a complementary method and in combination with FMD gives an integrated score of stimulated vascular function [24][25][26].

The PORH test accompanied by observation of blood flow changes in the skin microcirculation (by Doppler methods or tonometry, such as Peripheral Arterial Tonometry (PAT)) is also a popular method of assessing endothelial functioning. The blockage of blood flow in main arteries and its subsequent resumption cause quantitative and dynamic changes related to the bioavailability of NO. Laser Doppler Flowmetry assesses blood flow with a high sampling frequency and very good time resolution, enabling analysis of changes in the skin perfusion after stimulation of the microcirculation [6][27]. The time evolution of the post-occlusive signal resembles that observed by FMD. A major disadvantage of LDF is its poor reproducibility, which is caused mainly by the regional heterogeneity of skin perfusion due to skin anatomy and the small size of the monitored region [28].

NADH fluorescence from human skin at rest provides limited information on the level of the NADH coenzyme in skin cells and the redox equilibrium of the NADH/NAD^+ pair, as indicators of mitochondrial function. However, the reduced form of the coenzyme (NADH) accumulates under ischemic conditions and during hypoxia, and undergoes oxidation during hyperemia. The degree of change in NADH/NAD^+ imbalance as a result of the PORH test can be used to assess the vascular response to ischemic conditions. In the last decade, substantial evidence has linked vascular diseases with a dysfunctional response to hypoxia.

Some similarity in laser Doppler and FMSF post-occlusive signals can be observed, although dynamical changes in NADH fluorescence are slower than the dynamic changes of skin blood flow observed by simultaneous FMSF and LDF measurements. In fact, the measured changes in NADH fluorescence reflect the intrinsic changes of NADH levels in skin tissue, providing important information on the mitochondrial metabolic state in terms of oxygen delivery. Both LDF and PAT measurements remain blind to the ischemic period. In contrast, in the FMSF technique the temporal evolution of the ischemic and hyperemic periods resemble those observed by L-FMC and FMD, respectively. Monitoring the changes in NADH fluorescence from the skin reflecting chances of oxygen and nutrient delivery to epidermal cells by the vascular circulation seems an attractive alternative of the circulatory system monitoring without requiring direct observation of the blood flow itself [29][30].

Observations of blood flow changes in the skin microcirculation or NADH fluorescence from epidermal cells by blocking/restoring the blood flow in the brachial artery raises the question of whether macro- and micro-circulation responses to PORH can be distinguished in the overall response to hypoxia.

Blood flow in microvessels is not a homogeneous process, constant over time between successive heart contractions. To be effective, it must be accompanied by vascular oscillations. Oscillations in the microcirculation, known as flowmotion, are a well-recognized feature of cutaneous blood flow [31][32]. The mechanistic aspects of

flowmotion have been the subject of extensive study [33]. The main assessment techniques used are Doppler blood flow tests, using for example a Laser Doppler Flowmeter (LDF), which allow for semi-quantitative characterization of changes in cutaneous blood flow [34][35]. Analysis of the signal recorded with an LDF in the frequency domain makes it possible to extract the components of microcirculation oscillations, which are classified as follows: endogenous, independent of NO (<0.0095 Hz); endogenous NO-dependent (0.0095–0.021 Hz); neurogenic (0.021–0.052 Hz); myogenic (0.052–0.15 Hz); respiratory (0.15–0.62 Hz) and cardiac (0.62–2.00 Hz) [36][37].

Vascular changes are determined by changes in the intracellular calcium level and by the membrane potential of vascular smooth muscle cells. The most important processes related to Ca^{2+} activity include the regulation of muscle contraction and, indirectly, the activation of ion pumps, numerous enzymes, and other target proteins including NO synthase, which determines the release of nitric oxide. Nitric oxide synthase, as a result of increased demand for oxygen, also activates the vascular endothelial growth factor (VEGF) receptor. Hypoxia also activates the hypoxia inducible factor (HIF), which is the stimulus that accelerates the transcription of the VEGF gene. Stabilization of HIF-1α is responsible for the regulation of many physiological processes, including wound healing and adaptation to high-altitudes or physical exercise [38].

Myogenic microcirculatory oscillations, associated with changes in the diameter of microvessels (vasomotion), are a very sensitive measure of the microcirculatory response to hypoxia and can be monitored with high precision using the FMSF technique. Thus, the FMSF technique in conjunction with the use of PORH test allows diagnostics of vascular circulation including not only on dysfunctional blood flow in major arteries, but also microcirculatory response to hypoxia.

2. Technical Aspects of the FMSF Measurement

The FMSF method measures NADH fluorescence from the skin of the forearm in response to blocking and releasing blood flow. Blood flow is blocked in the forearm using a typical occlusion cuff, commonly used to measure blood pressure. Measurements are performed using the AngioExpert, a device constructed by Angionica Ltd. (Lodz, Poland).

The AngioExpert measures the NADH fluorescence (the emitted wavelength of NADH fluorescence is around 460 nm) at a sampling frequency of 25 Hz, excited by ultraviolet radiation with a wavelength of 340 nm [39]. The penetration of exciting light (340 nm) in skin tissue is low (about 0.3–0.5 mm). A substantial fraction of the exciting light is therefore absorbed by the epidermis and papillary dermis [40]. In these skin regions, the density of blood microvessels is low and the changes in NADH fluorescence depend mainly on the supply of oxygen diffused from deeper layers. The inefficient absorption of the excited light by blood components is limited.

For fluorescence measurements, AngioExpert uses a light-emitting diode (LED-UV) with an emission maximum of approx. 340 nm as the NADH excitation light source and a second photodiode as the NADH fluorescence detector at 460 nm. In the optical head, a set of interference filters is used to ensure proper selection of excitation and emitted light. This is an important aspect for the selective observation of the changes in NADH fluorescence,

although alone it would not be sufficient to limit the excitation of other endogenous fluorophores present in the skin, such as collagen, tryptophan, elastin, flavin adenine dinucleotide or some glycated products present. However, to a large extent, such limitation is achieved as a result of NADH excitation primarily in the keratinocytes of the epidermal layer.

The test is performed with the patient in a comfortable sitting position, after a minimum adaptation period of 5 min, in a quiet room with a controlled air temperature (24 ± 1 °C). The resting NADH fluorescence value emitted by the epidermal layer of the forearm is recorded for the first 3 min (180 s). The brachial artery is then occluded by inflating the cuff of the device to 60 mm Hg above the systolic pressure. The ischemic response is recorded over a period of 3 min (180 s). During this time, ischemic changes in the NADH fluorescence signal are recorded. Upon completion of occlusion, the cuff pressure is released abruptly, restoring flow in the brachial artery and inducing a hyperemic response for a minimum duration of 4 min (240 s).

Although there is no time limit for fluorescence measurements, the time available for measurement and analysis is constrained by the requirement that the patient remain still during the examination, to avoid artifacts and resulting errors. The data are analyzed using analytical software installed on the AngioExpert device or commercially available programs. The initial section of the measurement, during which the patient adjusts to the examination conditions, is discarded from the analysis. Because cuff occlusion is limited to 3 min and the hyperemic/reperfusion period following occlusion also lasts 3 min, analysis of the signal during ischemic and hyperemic periods is limited to that time.

3. Definition of the Measured FMSF Parameters

3.1. Reactive Hyperemia Response (RHR)

During the initial stage of the measurements, the baseline is collected (for 3 min). The FMSF signal is normalized with respect to the mean value of the fluorescence in first 1–2 min of this stage of the measurements. Normalization of the signal makes the result of its analysis independent of measurement conditions related to the individual characteristics of the patient's skin (for example, skin pigmentation, suntan) and/or different technical reasons, as only relative changes are analyzed.

In the second stage of measurements, known as the ischemic response (IR), an increase in NADH fluorescence is observed due to the occlusion of the brachial artery as the cuff is inflated to 60 mm Hg above the systolic blood pressure of the patient. After 3 min, the cuff pressure is released and the NADH fluorescence falls below the baseline, reaching a minimum followed by a return to the baseline. This third measurement stage, called the hyperemic response (HR), consists of a very rapid decrease in NADH fluorescence due to hyperemia (20–30 s) followed by a slow return of NADH fluorescence to baseline due to reperfusion (approximately 3 min).

Based on the combined response from both the ischemic and hyperemic parts of the measured FMSF trace, a Reactive Hyperemia Response (RHR) parameter can be defined [41]. This is a powerful diagnostic tool for

characterization of vascular circulation. The RHR parameter characterizes endothelial function related predominantly to the changes in the production of nitric oxide (NO) in the vasculature, mainly in the macrocirculation, due to ischemia and reactive hyperemia ($RHR = IR_{max} + HR_{max}$).

Some additional parameters can be defined that combine the magnitude of changes observed with the rate of NADH fluorescence growth (IR_{index}) during ischemia and the return of fluorescence to the baseline after hyperemia (HR_{index}). In some cases (some patients with type 1 diabetes), the final level of fluorescence does not reach the level of the baseline. This difference is called the Metabolic Recovery (MR) parameter. A detailed definition of these parameters can be found elsewhere [42].

All parameters mentioned above measure the relative changes in NADH fluorescence, expressed as percentages. Using this approach, perturbations caused by the variability of the skin condition are avoided, and comparisons can be made between unhealthy and healthy populations, or between patients.

3.2. Hypoxia Sensitivity (HS)

The FMSF signal oscillates both at rest (basal oscillations called flowmotion at rest (FM)) and even more strongly during the reperfusion period (called flowmotion at the reperfusion period (FM(R)). During the ischemic stage, the signal remains quite smooth because of the blockage of the blood flow, especially in the microcirculation. The altered strength and frequency of oscillations after post-occlusive reactive hyperemia (PORH) reflects the reaction of the vascular microcirculation to hypoxia caused by transient ischemia. Using FMSF signal normalization, two methods can be used to assess the strength of microcirculatory oscillations. The first is based on evaluating the oscillations in terms of the mean square error (MSE), which describes the deviations of the experimental signal points (at a sampling frequency of 25 Hz) from the baseline. In most cases, the baseline around which the FMSF signal oscillates (corresponding to the average fluorescence characteristic for a given patient at rest) is straight. However, in some cases it deviates from a straight horizontal line. Moreover, since during hyperemia the baseline is always an ascending line reaching a plateau the baselines can be defined using the second order polynomial regression method. The mean deviation of the fluorescence signal from the baseline can be used as a measure of the mean magnitude of oscillations. This parameter is objective and patient specific. As MSE values are extremely low, the FM parameters are defined as the MSE values multiplied by a factor of 10^6, to keep them in the number range of units to hundreds. As mentioned, the fluorescence changes are normalized so the FM parameters remain unitless values. The oscillations of the fluorescence signal relative to baseline, both for the signal before and after occlusion of the brachial artery.

The second assessment of the strength of oscillations contained in the FMSF signal can be performed using the fast Fourier transform (FFT) algorithm. Power spectral density (PSD) calculated as a mean squared amplitude with rectangular windowing is very well correlated with flowmotion parameters (FM(R)) defined above ($r = 0.996$). Fast Fourier transform analysis provides an estimate of the signal power at a given frequency and its relative contribution to the total power of the signal. The calculated power is grouped into three frequency intervals: ≤0.021

Hz, (0.021–0.052 Hz) and (0.052–0.15 Hz). These correspond to endothelial, neurogenic, and myogenic activities, respectively.

Among low frequency oscillations, the fraction of FM(R) (or PSD × 10^6) values covering the intensity of flowmotion related to myogenic oscillations (0.052–0.15 Hz) is especially interesting. Recorded during reperfusion, this value shows what is called Hypoxia Sensitivity (HS), as it is entirely responsible for the increased activity of the vessels after post-occlusive reactive hyperemia. Thus, the HS parameter, similarly to efficient stabilization of HIF-1α in microvascular smooth muscle cells during transient hypoxia, reflects the microcirculatory response to hypoxia.

Whereas the HS parameter varies within quite a broad range, log(HS) remains normally distributed.

3.3. Normoxia Oscillatory Index (NOI)

Although the microcirculation at rest rarely provides significant information about its normal functioning or dysfunction, which requires the use of provocations such as PORH, some exceptions seem to be of particular interest. One such exception may be the analysis of the flowmotion at rest, especially the relative ratio of endothelial and neurogenic oscillations to myogenic oscillations. Thus, a new parameter representing the contribution of endothelial and neurogenic oscillations relative to all oscillations detected at low frequency intervals (<0.15 Hz) can be introduced [43]:

$$NOI = [PSD(endothelial) + PSD\ (neurogenic)]/[PSD(endothelial) + PSD\ (neurogenic) + PSD(myogenic)] \times 100\%$$

Despite of the decrease of flowmotion (FM) at rest with age, the NOI parameter remains age-independent. Moreover, in patients with some diseases affecting the vascular system and thus also basal flowmotion, such as diabetes mellitus, a similar distribution of NOI is observed as for healthy subjects [44].

As has been shown for hundreds of patients investigated using the FMSF method, less than 15% of individuals have an NOI parameter below 60%.

There is convincing evidence that a chronic decrease in NOI is associated with various types of stress, such as emotional stress, physical exhaustion, or post-infection stress. It is important to mention that such deviation from the normal NOI distribution may be the result of a significant decrease in endothelial and neurogenic oscillations, with a relatively unchanged value of myogenic oscillations, or, conversely, a significant increase in the myogenic component of basal oscillations at rest.

RHR and log(HS) are the key diagnostic parameters derived from FMSF measurements. They can be used for efficient characterization of vascular circulation based on the response to transient ischemia. NOI is an auxiliary parameter to assess the state of microcirculation under stress of various origins. Chronically low NOI values can lead to the development of serious vascular circulatory disorders.

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