Akademia Wychowania Fizycznego im. Eugeniusza Piaseckiego w Poznaniu

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Rozprawa doktorska

Zmiany fluorescencji NADH w skórze pod wpływem wysiłku fizycznego u wysokowytrenowanych sportowców badane metodą Flow Mediated Skin Fluorescence

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Doctoral dissertation

Changes in skin NADH fluorescence induced by exercise in highly trained athletes using Flow Mediated Skin Fluorescence

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Spis Treści

I. AUTOREFERAT W JĘZYKU POLSKIM

Podstawą rozprawy doktorskiej jest cykl publikacji pod wspólnym tytułem: Zmiany fluorescencji NADH w skórze pod wpływem wysiłku fizycznego u wysokowytrenowanych sportowców badane metodą Flow Mediated Skin Fluorescence, w którego skład wchodzą trzy publikacje przygotowane na podstawie badań wykonanych w ramach projektu naukowego nr ANG/ZK/2/2016 będącego częścią projektu POIR.01.01.01-00-0540/15 finansowanego z Programu Operacyjnego Inteligentny Rozwój 2014-2020 współfinansowanego ze środków Europejskiego Funduszu Rozwoju Regionalnego:

1. ***Flow-Mediated Skin Fluorescence method for non-invasive measurement of the NADH at 460 nm – a possibility to assess the mitochondrial function.*** Postępy Biologii Komórki 44 (4): 333–352, 2017. IF: 0.158, MNiSW: 20

2. ***The effect of exercise on the skin content of the reduced form of NAD and its response to transient ischemia and reperfusion in highly trained athletes.*** Frontiers in Physiology 10: 600, 2019. IF: 3.201, MNiSW:100

3. ***The effect of a 7-week training period on changes in skin NADH fluorescence in highly trained athletes.*** Applied Sciences 10: 5133, 2020. IF: 2.474, MNiSW: 70

1. Wstęp

Już w połowie ubiegłego stulecia Duysens i Amesz (1957) jako pierwsi wykonali badania NADH (redukowanej formy dinukleotydu nikotyamidoadeninowego) przy użyciu metody fluorescencyjnej (spektrofotometrii). Na podstawie dalszych badań kolejni autorzy uznali, ze pomiar fluorescencji NADH może być cennym źródłem informacji o funkcji mitochondriów (Chance i Baltscheffsky 1958, Chance i Jobsis 1959, Mayevsky i Chance 2007).

Dinukleotyd nikotynoamidoadeninowy (NAD) jest molekułą występującą niemal we wszystkich komórkach ludzkiego organizmu. NAD może występować w dwóch formach: utlenionej (NAD^+) i zredukowanej (NADH) (Dolle i wsp. 2010; White i Schenk 2012). Skutki jego niedoboru mogą być bardzo poważne, zalicza się do nich szereg chorób sercowo-

naczyniowych, a także metabolicznych (Braidy i wsp. 2018; Rajman i wsp. 2018). NADH jest syntetyzowany w cytoplazmie, mitochondriach i jądrze komórkowym, ale jego utlenianie zachodzi tylko w mitochondriach (Dolle i wsp. 2010; White i Schenk 2012). NAD^+/NADH będąc donorem i akceptorem jonów wodorowych uczestniczy w cyklu Krebsa (gdzie NAD^+ ulega redukcji do NADH), ale również w łańcuchu transportu elektronów (gdzie ma miejsce utlenianie NADH do NAD^+). Molekuła NAD biorąc udział w produkcji adenozyno-5′-trifosforanu (ATP), pełni kluczową rolę w pozyskiwaniu energii na poziomie komórkowym (Mayevsky i Chance 2007; White i Schenk 2012). Prawdopodobnie metabolizm NAD odbywa się w podobny sposób we wszystkich komórkach ludzkich: leukocytach, komórkach wątroby, mózgu jak i w komórkach skóry (Green 1997; Ament i Verkerke 2009; Mayevsky i Barbiro-Michaely 2009), dlatego jego monitorowanie może być źródłem wielu cennych informacji o stanie organizmu.

Dotychczas wykorzystywane metody oceny mitochondriów w większości wymagały inwazyjnego pobierania próbek za pomocą biopsji. Badania prowadzono in vitro, a koszty takich badań były bardzo wysokie (O'Donnell i wsp. 2004; Mayevsky i Rogatsky 2007; Marín-García 2013). Alternatywą może być pośrednia ocena funkcji mitochondrialnej za pomocą fluorescencji NADH (Mayevsky i Rogatsky 2007; Mayevsky i Barbiro-Michaely 2009). Metoda ta pozwala na nieinwazyjne badanie in vivo w czasie rzeczywistym. Historia monitorowania fluorescencji NADH w celu oceny funkcji mitochondrialnej sięga lat '50 XX wieku (Chance i Williams 1955; Duysens i Amesz 1957; Chance i Baltscheffsky 1958; Chance i Jobsis 1959; Chance i Thorell 1959). Mayevsky i Rogatsky (2007) w swoim przeglądzie przytaczają szereg prac wskakujących, że na podstawie oceny fluorymetrycznej NADH uzyskuje się informację głównie o NADH zlokalizowanym w mitochondriach, natomiast NADH zawarty w cytoplazmie nie ma na pomiar istotnego wpływu. Na podstawie obserwacji zmian poziomu fluorescencji NADH można wnioskować o funkcji mitochondrialnej, z uwagi na zadanie pełnione przez NAD^+/NADH w procesie oddychania komórkowego.

Badacze już dawno zainteresowali się zmianami równowagi NAD^+/NADH w efekcie zastosowania wysiłku fizycznego, jednak podawali sprzeczne informacje o kierunku tych zmian, co wynikało najczęściej z różnic metodologicznych (Graham i wsp. 1978; Sahlin 1985; Henriksson i wsp. 1986; Katz i Sahlin 1987; White i Schenk 2012). Należy zaznaczyć, iż ilość NAD jest w organizmie w danym momencie stała. Procentowy udział NAD^+/NADH zmienia się zależnie od pewnych czynników, takich jak dostępność tlenu (Mayevsky i Chance

2007), np. podczas zastosowania okluzji lub w efekcie intensywnych ćwiczeń. Natomiast w dłuższym czasie pula NAD może się zmieniać. Jego poziom moduluje dieta, zażywane leki, aktywność fizyczna. Poziom NAD maleje wraz z wiekiem (Kane i Sinclair 2018; Rajman i wsp. 2018).

Jak wiadomo, trening fizyczny wywołuje w organizmie szereg adaptacji. Zmiany te dotyczą nie tylko całych układów: mięśniowo-szkieletowego, oddechowego i in., ale również narządów, tkanek, komórek. Obejmują one również układ krwionośny, zarówno główne naczynia jak i naczynia mikrokrążenia (Green i wsp. 2017). Zmiany adaptacyjne zachodzą również w mitochondriach (Busquets-Cortés i wsp. 2017). Aktywność fizyczna wpływa także na gospodarkę biochemiczną (Ament i Verkerke 2009), w tym na zmiany równowagi NAD^+/NADH (O'Donnell i wsp. 2004). White i Schenk (2012) sugerują, że trening fizyczny, z uwagi na wzrost zapotrzebowania w trakcie wysiłku na ATP, stymuluje zwiększanie puli NAD. Pomimo iż powstał szereg bardzo dokładnych i zaawansowanych metod pozwalających na ocenę różnych aspektów działania mitochondriów, to jednak niewielu badaczy zgłębiło temat zmian zachodzących w nich pod wpływem treningu fizycznego. Prace, które powstały w tym obszarze dotyczą jedynie zmian w mitochondriach komórek mięśniowych (Phillips i wsp. 1996; Mayevsky i Chance 2007; Mayevsky i Rogatsky 2007; White i Schenk 2012). Również badania na temat wpływu wysiłku i treningu fizycznego na fluorescencję NADH prowadzone były w mięśniach szkieletowych (White i Schenk 2012) nie wiadomo jednak czy kierunek tych zmian jest taki sam w skórze.

W badaniach własnych postanowiono zbadać zmiany fluorescencji NADH w skórze zachodzące pod wpływem pojedynczego wysiłku a także treningu fizycznego. Wykorzystano nowatorską metodę Flow Mediated Skin Fluorescence (FMSF) pozwalającą na pośrednią ocenę funkcji mitochondrialnej z poziomu skóry, w sposób całkowicie nieinwazyjny. W trakcie badania obserwuje się w sposób ciągły (również w trakcie okluzji) zapis zmian fluorescencji NADH w czasie rzeczywistym.

2. Cel badań

Celem pracy było określenie zmian intensywności fluorescencji NADH w skórze pod wpływem pojedynczego wysiłku do odmowy (praca 2) oraz po 7 tygodniowym treningu w okresie przygotowawczym (praca 3) nową nieinwazyjną metodą Flow Mediated Skin Fluorescence u wysokowytrenowanych sportowców.

Postawiono następujące hipotezy badawcze:

- Wysiłek do odmowy wpłynie na poziom NADH w komórkach naskórka, a równowaga NAD^+/NADH przesunie się w kierunku NADH (praca 2 i 3).
- W efekcie treningu fizycznego w okresie przygotowawczym wzrośnie fluorescencja NADH mierzona w spoczynku i po maksymalnym wysiłku (praca 3).

3. Metody badawcze

Procedury badań

Badania prowadzone były w laboratorium Analizy Ruchu Człowieka w Zakładzie Lekkiej Atletyki i Przygotowania Motorycznego na Akademii Wychowania Fizycznego w Poznaniu. Wszyscy badani przechodzili przez ocenę stanu zdrowia, która kwalifikowała do uczestnictwa w testach. Badania wykonywane były w godzinach porannych. W laboratorium utrzymywano stałą temperaturę otoczenia (19-21^{0}C). Sportowcy w dniu badania mogli zjeść lekkie śniadanie, a przez 24 godziny poprzedzające test wysiłkowy nie mogli brać udziału w intensywnych sesjach treningowych. Testy rozpoczynano od pomiarów antropometrycznych oraz od badania ciśnienia tętniczego krwi za pomocą urządzenia Omron M3 (Omron, Japonia). Następnie wykonywano pierwszy pomiar fluorescencji NADH za pomocą urządzenia AngioExpert (Angionica, Łódź). Kolejno badany wykonywał test wysiłkowy do odmowy na bieżni mechanicznej (H/P Cosmos, Pulsar, Niemcy). Zawodnikom pobierano krew z opuszka palca w celu oznaczenia stężenia mleczanu w spoczynku i 2 minuty po zakończeniu testu. W 3–4 minucie po zakończeniu wysiłku ponownie badano ciśnienie krwi oraz fluorescencję NADH. Protokół badania był zgodny z Deklaracją Helsińską. Zgodę na ich przeprowadzenie wydała Komisja Bioetyczna przy Uniwersytecie Medycznym w Poznaniu, decyzja nr. 1017/16 z dnia 5 października 2016. Wszyscy sportowcy uczestniczyli w badaniach dobrowolnie i zostali poinformowani o możliwości wycofania się z nich na każdy etapie.

Pomiar fluorescencji NADH

Badanie fluorescencji NADH przeprowadzono przy użyciu urządzenia AngioExpert (Angionica, Łódź, Polska). Badana była intensywność fluorescencji o długości fali 460 nm.,

która jest charakterystyczna dla NADH, w odpowiedzi na aktywację światłem UV o długości fali 340 nm. (Mayevsky i Chance 2007; Mayevsky i Barbiro-Michaely 2009; Mayevsky i wsp. 2011). Fluorescencja odnotowywana podczas pomiaru pochodziła głównie z komórek naskórka (Dunaev i wsp. 2015). Wykorzystywane do badań urządzenie AngioExpert dokonuje ciągłej rejestracji sygnału, podczas pomiaru spoczynkowego (2 min), w trakcie okluzji tętniczej (200s.) oraz po przywróceniu krążenia w kończynie (3 min). W celu wywołania całkowitej okluzji tętniczej w przedramieniu w mankiecie sfigmomanometru (będącego częścią urządzenia) uzyskiwano ciśnienie o 50 mmHg wyższe od wartości ciśnienia skurczowego badanego zawodnika.

Przed każdym badaniem FMSF przeprowadzono pomiar ciśnienia tętniczego za pomocą ciśnieniomierza Omron M3 (Omron, Japonia). Pomiar za pomocą urządzenia AngioExpert (Angionica, Łódź, Polska) wykonany był dwukrotnie, bezpośrednio przed testem wysiłkowym oraz 3–4 minuty po jego zakończeniu.

Test wysiłkowy

Test wysiłkowy wykonywany był na bieżni mechanicznej (model 150/50 LC, H/P Cosmos Pulsar, Niemcy). W celu monitorowania parametrów krążeniowo-oddechowych badani byli wyposażani w pulsometr Polar (Polar H6 Bluetooth Smart; Polar Electro Oy, Finlandia) oraz maskę połączoną ze spirometrem MetaMax 3B-R2 (Cortex Medical, Niemcy). Test rozpoczynał się od 4 minutowej rozgrzewki, prędkość przesuwu taśmy wynosiła 6 km/h. Następnie prędkość wzrastała progresywnie o 2 km/h co 3 min. W trakcie całego testu kąt nachylenia bieżni był stały i wynosił 1%. Test trwał do momentu zgłoszenia przez badanego wyczerpania. Parametry oddechowe analizowane były za pomocą oprogramowania MetaSoft Studio 5.1.0 Software (Cortex Biophysik, Niemcy). Maksymalny pobór tlenu (VO_{2max}) określany był, w oparciu o spełnienie minimum trzech z poniższych kryteriów: VO_2 ustabilizowało się pomimo dalszego wzrostu obciążenia, osiągnięto tętno na poziomie przynajmniej 95% przewidywanego dla danego wieku, współczynnik wymiany oddechowej wynosił przynajmniej (RER) ≥1.1, stężenie mleczanów we krwi było na poziomie ≥9 mmol/l dla mężczyzn, oraz ≥7 mmol/l dla kobiet (Edvardsen i wsp. 2014). Wyznaczono również szczytowe wartości tętna (HR_{max}) dla każdego z zawodników.

Próbki krwi

Krew pobierano z opuszka palca dwukrotnie – bezpośrednio przed i 2 minuty po teście wysiłkowym. Do mikroprobówki za pomocą kapilary pobierano każdorazowo 20 µl pełnej krwi. Pomiar poziomu mleczanu wykonano używając urządzenia Biosen C-line (EKF Diagnostics, Wielka Brytania).

Pomiary antropometryczne

Pomiary antropometryczne (wysokość i masę ciała) wykonywano za pomocą cyfrowej stacji pomiarowej (Seca 285, SECA, Niemcy). Wskaźnik masy ciała (BMI) obliczono przez podzielenie masy ciała przez kwadrat wysokości ciała (kg/m^2).

4. Dyskusja i omówienie wyników

Publikacja 1

Flow-Mediated Skin Fluorescence method for non-invasive measurement of the NADH at 460 nm – a possibility to assess the mitochondrial function. Postępy Biologii Komórki 44 (4): 333–352, 2017. IF: 0.158 , MNiSW: 20

W przedstawionej pracy Greta Sibrecht i Olga Bugaj są wspólnie wyróżnionymi autorkami (pierwszy autor).

AngioExpert jest nowym urządzeniem medycznym przeznaczonym do nieinwazyjnego diagnozowania i monitorowania zaburzeń mikrokrążenia, regulacji metabolicznej (zmian poziomu NADH). Ze względu na to, w pierwszej publikacji przybliżono sposób i obszar działania urządzenia AngioExpert oraz metody Flow Mediated Skin Fluorescence.

Metoda opiera swoje działanie na zdolności cząsteczki NADH do fluorescencji o długości 460 nm., aktywowanej pod wpływem światła wzbudzania o długości 340 nm. Jak powszechnie wiadomo w mitochondriach zachodzi oddychanie komórkowe, w wyniku którego dochodzi do produkcji wysokoenergetycznej cząsteczki adenozynotrifosforanu (ATP). W procesie tym molekuła NAD, utleniając się do NAD^+ i redukując do NADH

przenosi jony wodorowe. W trakcie oddychania komórkowego NAD^+ redukuje się do NADH w reakcjach glikolizy w cytoplazmie i cyklu Krebsa w macierzy mitochondrialnej, natomiast NADH utlenia się do NAD^+ w łańcuchu transportu elektronów, na wewnętrznej błonie mitochondrialnej. Utlenianie NADH do NAD^+ zachodzi w obecności tlenu. W przypadku niedoboru tlenu utlenianie to może zachodzić w ograniczonym stopniu również w procesach anaerobowych, ale wydajność tych procesów jest znacznie niższa niż aerobowych. W efekcie NADH gromadzi się w organizmie.

NAD występuje w cytoplazmie, jądrze komórkowym oraz w mitochondriach (Stein i Imai 2012; White i Schenk 2012; Dolle i wsp. 2013). Ponieważ błona jądra komórkowego jest przepuszczana dla NAD poprzez specjalne pory, to stężenie NAD^+/NADH w jądrze i cytozolu jest podobne. Natomiast błona mitochondrialna jest nieprzepuszczalna dla NAD i aby cząsteczki NAD^+ i NADH mogły się utleniać i redukować konieczne jest działanie czółenek (jabłczanowo-asparaginianowych i glicerolo-3-foforanowych), które transportują elektrony niezbędne do zachodzenia tych reakcji (White i Schenk 2012). Niedawno odkryto również istnienie nierozpoznanego dotychczas transportera NAD (lub NADH) który umożliwia transport cząsteczek przez błonę mitochondrialną (Davila i wsp. 2018). Dzięki takim właściwościom monitorowanie aktywności NADH może stanowić cenne źródło informacji o funkcjonowaniu mitochondriów.

Możliwości monitorowania mitochondriów jest wiele, a każda z nich daje inne informacje. Pozwalają one na badanie ilości, struktury i gęstości mitochondriów w komórce. Jednakże większość metod wymaga inwazyjnego pobrania próbek, często nie dając możliwości obserwowania zmian in vivo. Alternatywą jest monitorowanie fluorescencji NADH. Metody oparte o fluorymetrię oceniają poziom NADH w jednostkach umownych, nie dając informacji o jego bezwzględnej ilości. Powalają jednak na ocenę dynamiki przemian NADH w odpowiedzi na różne bodźce i obserwację tych zmian w czasie rzeczywistym w sposób nieinwazyjny. Ocena fluorescencji NADH była od dawna uważana za dobrą metodę pośredniej oceny funkcji mitochondrialnej (Mayevsky i Chance 2007), jednakże do tej pory nie badano tej fluorescencji w komórkach skóry u ludzi.

Molekuła NADH jest fluoroforem co oznacza, że posiada zdolność do absorbcji fali o określonym spektrum długości, w odpowiedzi emitując falę o innej długości. W przypadku NADH długość fali absorbowanej wynosi 320-380 nm., a długość fali emitowanej 420-480 nm. (Chance i Baltscheffsky 1958; Zhu i wsp. 2015). Mayevsky i Chance (2007) uznali że

NADH w skórze najlepiej odpowiada fala emitowana o długości 460 nm. Na podstawie tych ustaleń powstały dwa nowoczesne urządzenia. Pierwsze z nich, CritiView, pozwala na monitorowanie szeregu parametrów, w tym fluorescencję NADH na salach intensywnej terapii, a urządzenie wprowadzane jest do cewki moczowej wraz z cewnikiem Foleya (Mayevsky i wsp. 2011). Drugie, wykorzystane w badaniach własnych to AngioExpert (Angionica, Łódź, Polska), który został zaprojektowany przez polskich naukowców z Łodzi. Umożliwia on nieinwazyjną ocenę fluorescencji NADH in vivo w czasie rzeczywistym. W trakcie badania metodą FMSF wykorzystaną w urządzeniu AngioExpert, wykonuje się okluzję tętnicza przedramienia (Katarzynska i wsp. 2019), co pozwala na obserwację reakcji komórek skóry w sytuacji niedoboru tlenu. Informacja ta może być bardzo przydatna np. w chorobach układu krążenia (Tarnawska i wsp. 2018). Istotne jest to, że sygnał rejestrowany jest z powierzchownych warstw skóry – głębokość penetracji falą świetlną osiąga maksymalnie 0,5 mm, natomiast większość sygnału pochodzi z głębokości ok. 0,1 mm. Z uwagi na to, iż skóra na tej głębokości nie jest ukrwiona, fluorescencja zbierana z tego poziomu jest w całości zależna od podaży substratów oraz tlenu z głębszych warstw skóry (Dunaev i wsp. 2015).

W pracy przedstawiono szereg parametrów z których kluczowe zostały wykorzystane w późniejszych pracach.

Parametry mierzone (rycina a):

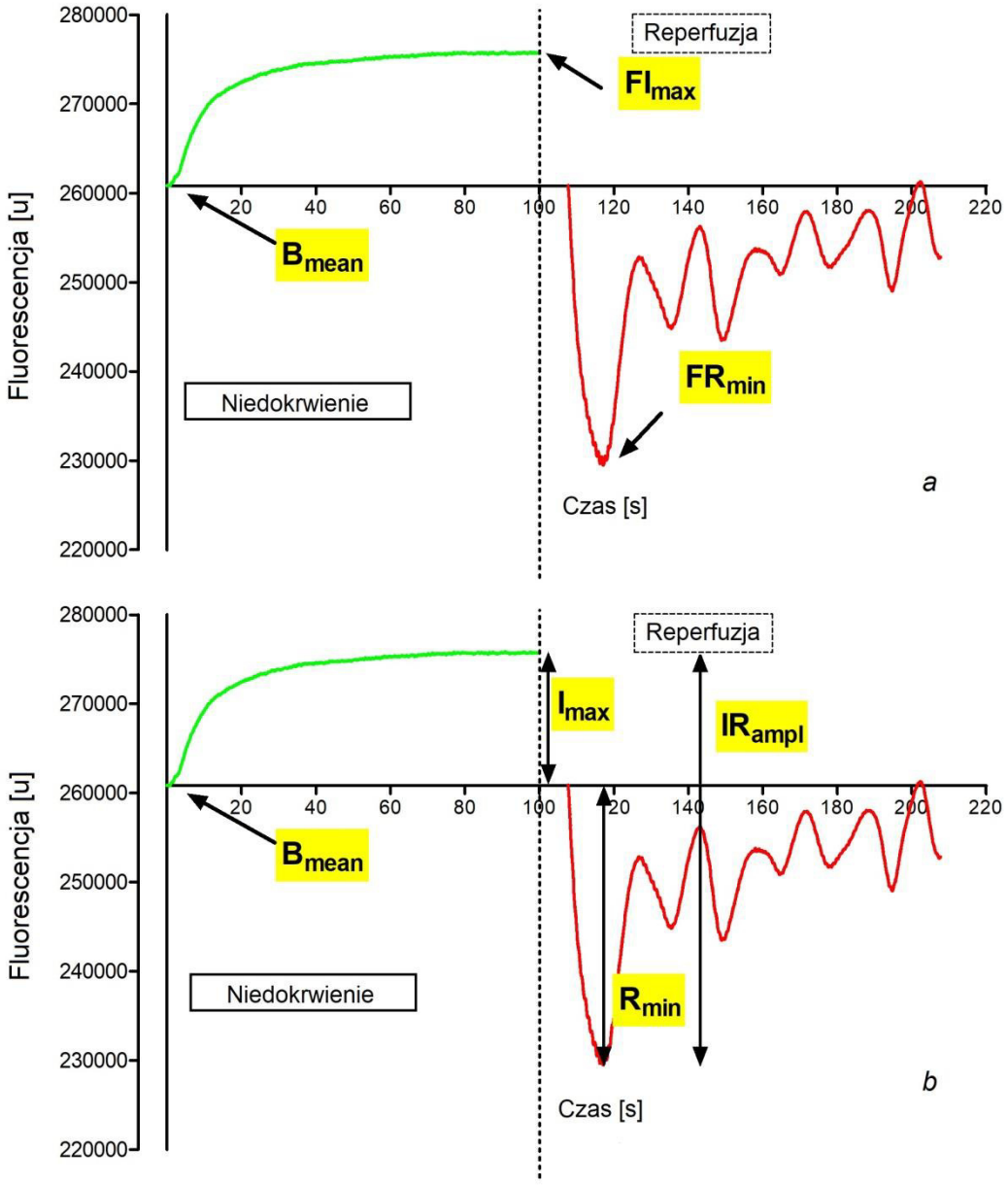
- B_{mean} [u] – średnia wartość fluorescencji o długości 460 nm. rejestrowana podczas spoczynku; wartość bazowa fluorescencji NADH,
- FI_{max} [u] – maksymalna wartość fluorescencji rejestrowana podczas okluzji tętniczej przedramienia
- FR_{min} [u] – najniższa wartość fluorescencji rejestrowana podczas reperfuzji

Parametry estymowane/liczone (rycina b):

- I_{max} [u] – maksymalny przyrost fluorescencji powyżej linii bazowej podczas okluzji tętniczej przedramienia, liczony jako różnica pomiędzy FI_{max} i B_{mean},
- R_{min} [u] – maksymalny spadek fluorescencji poniżej linii bazowej podczas reperfuzji, liczony jako różnica pomiędzy B_{mean} a FR_{min},

- IR_{ampl} [u] – maksymalny zakres zmian fluorescencji podczas niedokrwienia i reperfuzji, liczony jako suma I_{max} i R_{min},
- CI_{max} – udział NADH powstałego w trakcie okluzji do całej ilości NADH będącej w obrocie podczas niedokrwienia i reperfuzji, liczony jako iloraz I_{max} /IR_{ampl}.

W parametrach B_{mean}, FI_{max}, FR_{min}, I_{max}, R_{min}, IR_{ampl} [u] oznacza jednostki umowne.



Publikacja 2

The effect of exercise on the skin content of the reduced form of NAD and its response to transient ischemia and reperfusion in highly trained athletes. Frontiers in Physiology 10: 600, 2019. DOI: 10.3389/fphys.2019.00600. IF: 3.201, MNiSW:100

Wykorzystując metodę FMSF przebadano 121 wysoko wytrenowanych sportowców (94 mężczyzn i 27 kobiet) reprezentujących różne dyscypliny sportowe. Wśród badanych było 41 biegaczy długodystansowych, 27 triathlonistów, 25 zawodników taekwondo olimpijskiego, 9 wioślarzy, 8 futsalistów, oraz 6 sprinterów, 4 szermierzy i 1 tenisistka. Zawodnicy byli w wieku 16 – 40 lat, należeli do Kadry Narodowej lub też startowali w zawodach na poziomie międzynarodowym. Wszyscy zawodnicy byli badani w trakcie okresu przygotowawczego. Celem pracy było określenie zmian intensywności fluorescencji NADH w skórze pod wpływem pojedynczego wysiłku do odmowy.

W badaniach własnych prowadzonych nieinwazyjnie w skórze za pomocą oceny fluorescencji NADH wykazano, że wysiłek do wyczerpania powoduje zmianę ilości skórnego NADH, przesuwając całą krzywą obrazującą zmiany fluorescencji w kierunku wyższych wartości. Zmiany te obejmują zarówno wyniki pomiaru bazowego poziomu fluorescencji NADH, jak również fluorescencji badanej podczas niedokrwienia i reperfuzji. W stosunku do pomiaru wykonanego przed wysiłkiem zmiany obejmują wzrost w parametrach: B_{mean} ($p<0.001$), oznaczającego bazową fluorescencję przed wywołaniem niedokrwienia; FI_{max} ($p<0.001$, tylko u mężczyzn) wskazującego maksymalny wzrost fluorescencji; FR_{min} ($p<0.001$ u mężczyzn i $p<0.01$ u kobiet) obrazujący maksymalny spadek fluorescencji w trakcie reperfuzji po przywróceniu krążenia, a także R_{min} ($p<0.01$) parametr wskazujący na różnice między B_{mean} a FR_{min}, czyli spadek fluorescencji znormalizowany do bazy. Obniżeniu uległy wartości parametrów I_{max} ($p<0.001$), wskazującego na różnicę miedzy FI_{max} i B_{mean}, oraz CI_{max} ($p<0.001$), przedstawiający udział I_{max} w IR_{ampl}. Parametr IR_{ampl} obrazujący całą amplitudę zmian podczas niedokrwienia i reperfuzji (I_{max} + R_{min}) nie uległ zmianie po wysiłku.

Podsumowując, wysiłek fizyczny do wyczerpania, modyfikuje bazowy metabolizm NADH komórek skóry, oraz badany podczas niedokrwienia i reperfuzji. Bezpośrednio po wysiłku wartości fluorescencji NADH przesuwa się w kierunku wyższych wartości. Bezwzględna ilość NADH ulega podwyższeniu w trakcie niedokrwienia oraz reperfuzji

powysiłkowej w porównaniu do stanu spoczynkowego. Obserwowane zmiany w wartościach fluorescencji NADH podczas niedokrwienia i reperfuzji są silnie zależne od warunków metabolicznych. Warunki te istotnie modyfikowane są w wyniku ćwiczeń i utrzymują się przez kolejne kilka minut po jego zakończeniu. Nasilenie fluorescencji NADH w żywych komórkach skóry w wyniku zmian metabolizmu NADH wywołanych wysiłkiem fizycznym obejmuje nie same mięśnie, lecz wpływa również na inne komórki i narządy.

Publikacja 3

The effect of a 7-week training period on changes in skin NADH fluorescence in highly trained athletes. Applied Sciences 10: 5133, 2020. IF: 2.474, MNiSW: 70

Przebadano 41 sportowców w wieku od 18 do 35 lat. Wszyscy zawodnicy byli członkami Kadry Narodowej, lub startowali w zawodach na krajowym i międzynarodowym poziomie. Sportowcy reprezentowali następujące dyscypliny sportowe: triathlon na dystansie olimpijskim (pływanie 1,5 km, jazda na rowerze 40 km, bieg 10 km) (7 mężczyzn, 4 kobiety), biegi długodystansowe (na 5 km, 10 km i biegi maratońskie) (6 mężczyzn, 2 kobiety), taekwondo olimpijskie (6 mężczyzn, 1 kobieta), biegi sprinterskie (100 m, 200 m i sztafetowe 4 x 100 m) (6 mężczyzn, 1 kobieta), kajakarstwo (3 mężczyzn) i szermierkę (5 kobiet). Badania przeprowadzono dwukrotnie, na początku 7 tygodniowego okresu przygotowawczego rocznego cyklu przygotowań, oraz pod koniec tego okresu w celu określenia zmian intensywności fluorescencji NADH w skórze.

W mierzonych parametrach w pierwszym terminie badań (przez okresem 7 tygodniowego treningu) po ćwiczeniach wykazano wzrost w parametrze B_{mean} ($p<0.05$) (fluorescencji bazowej), natomiast w drugim terminie badań po ćwiczeniach wszystkie parametry wykazały wzrost: B_{mean} ($p<0.001$) , FI_{max} ($p<0.05$), FR_{min} ($p<0.001$). Odnotowano istotny wzrost poziomie $p<0.001$ we wszystkich parametrach mierzonych (B_{mean}, FI_{max}, FR_{min}) badanych zarówno przed jak i po wysiłkowo, w stosunku do wartości przed treningiem.

Odnotowano również zmiany pod wpływem zastosowanego treningu w parametrach kalkulowanych. Wartości parametru I_{max} wykazały spadek po ćwiczeniach w obu terminach na poziomie $p<0.001$. Natomiast wartość I_{max} wzrosła po treningu w spoczynku na poziomie $p<0.001$ oraz w badaniu po teście wysiłkowym na poziomie $p<0.01$. Parametr R_{min} wzrósł istotnie po wysiłku w obu terminach badań (w 1 terminie na poziomie $p<0.05$, oraz w drugim

terminie na poziomie $p<0.001$). Wartości R_{min} zarówno przed i powysiłkowe były wyższe w drugim terminie ($p<0.001$). IR_{ampl} czyli parametr obrazujący amplitudę zmian fluorescencji nie różnił się przed i po wysiłku w żadnym z terminów. Jednak po treningu wykazano istotny wzrost ($p<0.001$) zarówno w badaniu spoczynkowym jak i powysiłkowym w tym parametrze. Ostatni parametr CI_{max} wskazujący na udział I_{max} w całkowite amplitudzie zmian (IR_{ampl}) istotnie malał po wysiłku w obu terminach badań ($p<0.001$). Nie wykazano różnic po treningowych w tym parametrze.

Wyniki powyższych badań po raz pierwszy obrazują zmiany fluorescencji NADH w komórkach naskórka u wysoko wytrenowanych sportowców. Wykazano, że po treningu wzrósł poziom fluorescencji NADH. Wiadomo, że w wyniku treningu dochodzi do szeregu adaptacji, w tym adaptacji mitochondrialnych (Drake i wsp. 2017). Ocena fluorescencji NADH może być wykorzystywana do pośredniej oceny funkcji mitochondrialnej jak i ich statusu metabolicznego (Mayevsky i Barbiro-Michaely 2009). Ocena fluorescencji NADH nie daje jasnej odpowiedzi, jakie procesy metaboliczne nastąpiły w komórkach, pozwala jednak obserwować potreningowy wzrost fluorescencji NADH, sugerując tym samym wzrost puli NAD w odpowiedzi na zastosowany trening w okresie przygotowawczym u badanych zawodników.

Okres przygotowawczy charakteryzuje się przewagą treningów o charakterze wytrzymałościowym, niezależnie od uprawianej dyscypliny sportu. Taki rodzaj treningu skutkował u badanych przez nas sportowców podniesieniem poziomu fluorescencji NADH w parametrach mierzonych (B_{mean}, FI_{max}, FR_{min}), co prawdopodobnie wskazuje na zmiany adaptacyjne w mitochondriach skóry. Nasze wyniki wydają się spójne z wynikami wcześniejszych badań wykonywanych na mięśniach, w których autorzy obserwowali po treningu wzrost poziomu białek związanych z biogenezą mitochondrialną, oraz poprawę mitochondrialnej funkcji oddechowej (Yan i wsp. 2012; Busquets-Cortés i wsp. 2017; Granata i wsp. 2018).

5. Wnioski

Przeprowadzone badania pozwalają na sformułowanie następujących wniosków:

1. Wysiłek fizyczny do odmowy modyfikuje metabolizm NADH zarówno bazowy, jak i mierzony podczas niedokrwienia i reperfuzji w komórkach skóry przedramienia, przesuwając bazową fluorescencję NADH w kierunku wyższych wartości.

2. Trening w okresie przygotowawczym spowodował wzrost fluorescencji NADH w grupie sportowców wyczynowych, badanej przed i po wysiłku do odmowy, co może sugerować wzrost puli NAD w organizmie.

II. DISSERTATION SUMMARY

The doctoral dissertation is based on series of studies entitled: Changes in skin NADH fluorescence induced by exercise in highly trained athletes using Flow Mediated Skin Fluorescence, and consists of three publication supported by project ANG/ZK/2/2016 as part of the project from the European Union from the resources of the European Regional Development Fund under the Smart Growth Operational Program, Grant No. POIR.01.01.01-00-0540/15:

1. ***Flow-Mediated Skin Fluorescence method for non-invasive measurement of the NADH at 460 nm – a possibility to assess the mitochondrial function.*** Advances in Cell Biology 44 (4): 333–352, 2017. IF: 0.158 , MNiSW: 20

2. ***The effect of exercise on the skin content of the reduced form of NAD and its response to transient ischemia and reperfusion in highly trained athletes.*** Frontiers in Physiology 10: 600, 2019. IF: 3.201, MNiSW:100

3. ***The effect of a 7-week training period on changes in skin NADH fluorescence in highly trained athletes.*** Applied Sciences 10: 5133, 2020. IF: 2.474, MNiSW: 70

1. INTRODUCTION

In the middle of the 20th century Duysens and Amesz (1957) for the first time studied NADH (reduced form of nicotinamide adenine dinucleotide) using the fluorescence method (spectrophotometry). Other authors, based on later studies, found that the measurement of NADH fluorescence can be a valuable source of information about the mitochondrial function (Chance and Baltscheffsky 1958; Chance and Jobsis 1959; Mayevsky and Chance 2007).

Nicotinamide adenine dinucleotide (NAD) is a molecule present in practically all human cells. NAD occurs in two forms, an oxidized NAD^+, and reduced NADH (Dolle et al. 2010; White and Schenk 2012). The effect of its deficiency can be very serious, including a number of cardiovascular and metabolic diseases (Braidy et al. 2018; Rajman et al. 2018). NADH is synthetized in the cytosol, mitochondria and in the nucleus, but it is oxidized only in the mitochondria (Dolle et al. 2010; White and Schenk 2012). NAD^+/NADH as a donor and

acceptor of hydrogen ions, takes part in Krebs cycle (where NAD^+ is reduced to NADH) and in electron transport chain (where NADH is oxidized to NAD^+). The NAD molecule taking part in adenosine triphosphate (ATP) production, plays a key role in energy production at a cellular level (Mayevsky and Chance 2007; White and Schenk 2012). It seems that NAD metabolism is similar in all humans cells: leukocytes, hepatocytes, brain cells and in the skin cells (Green 1997; Ament and Verkerke 2009; Mayevsky and Barbiro-Michaely 2009), so monitoring it can offer a valuable source of information about the state of the human body.

So far, most methods of mitochondria study required invasive sampling with the help of biopsy. Tests were conducted in vitro, and the cost was high (O'Donnell et al. 2004; Mayevsky and Rogatsky 2007; Marín-García 2013). The alternative could be indirect evaluation of mitochondrial function using NADH fluorescence (Mayevsky and Rogatsky 2007; Mayevsky and Barbiro-Michaely 2009), a non-invasive and real-time method. The history of NADH fluorescence monitoring started in the 50s of the 20th century (Chance and Williams 1955; Duysens and Amesz 1957; Chance and Baltscheffsky 1958; Chance and Jobsis 1959; Chance and Thorell 1959). Mayevsky and Rogatsky (2007) in their review cite a number of papers showing that the NADH fluorescence method gives the information about NADH localized mainly in mitochondria, but cytoplasmic NADH does not have any important impact on the measurement results. NAD^+/NADH takes part in the process of cellular respiration, therefore the observation of NADH fluorescence changes, could be an indicator of a mitochondrial function.

Researchers for a long time have been interested in changes in NAD^+/NADH balance as a result of physical exercise, but they shared inconsistent information about the direction of those changes, what was usually caused by methodology differences (Graham et al. 1978; Sahlin 1985; Henriksson et al. 1986; Katz and Sahlin 1987; White and Schenk 2012). It is important to mention that the amount of NAD in the body remains constant in the short time frames. The NAD^+/NADH percentage changes depend on certain factors, like oxygen availability (Mayevsky and Chance 2007), e.g. as a result of occlusion or intensive physical exercise. However, in the long period of time the NAD pool can change. Its amount is modulated by diet, taken medicaments, physical activity. The NAD level decreases with age (Kane and Sinclair 2018; Rajman et al. 2018).

It is commonly known, physical training impacts a number of adaptations in the body. These changes affect not only the entire systems: musculoskeletal, respiratory etc., but also organs, tissues and cells. Changes take place also in circulatory system, major blood vessels

and microcirculation vessels as well (Green et al. 2017). The adaptation changes take place also in the mitochondria (Busquets-Cortés et al. 2017). Physical activity impacts biochemistry (Ament and Verkerke 2009), including changes in NAD^+/NADH balance (O'Donnell et al. 2004). White and Schenk (2012) suggest, physical training, due to increased demand of ATP during physical exercise, stimulates an increase in the NAD pool. Even though there are a lot of very accurate and advanced methods, which allow to evaluate various aspects of mitochondrial function, researchers did not conduct a lot of studies focusing on the changes occurring in mitochondria as a result of physical training. Papers mention mitochondrial changes only in muscular cells (Phillips et al. 1996; Mayevsky and Chance 2007; Mayevsky and Rogatsky 2007; White and Schenk 2012). Research on physical activity and training impact on NADH fluorescence were conducted also only in the skeletal muscles (White and Schenk 2012). However, it is not known if the direction of these changes is the same in the skin.

In my own research it was decided to examine NADH fluorescence changes in the skin impacted by physical exercise and training. The novel Flow Mediated Skin Fluorescence (FMSF) method was used, which enables an indirect evaluation of mitochondrial function at the skin level in a completely non-invasive way. During the examination NADH fluorescence changes are observed continuously (also during occlusion) and in real time.

2. Study Aim

The aim of this study was to evaluate the changes of NADH fluorescence in the skin, as a result of exercise to exhaustion (paper 2) and a 7-week training period in general preparation phase (paper 3) using a novel non-invasive Flow Mediated Skin Fluorescence method in highly trained athletes.

The following research hypotheses were made:

- Exercise to exhaustion impacts NADH fluorescence level in the epidermal cells, and the NAD^+/NADH balance will shift toward NADH (papers 2 and 3).
- NADH fluorescence evaluated at rest and after maximal exercise will increase as a result of training in the preparatory period (paper 3).

3. Research Methods

Research Procedures

The study was conducted in the Human Movement Laboratory of the Department of Athletics, Strength and Conditioning at the Poznan University of Physical Education. After arriving at the laboratory, the health status of each participant was evaluated, what qualified to participate in the tests. The examinations were conducted in the morning. At the laboratory constant temperature (19–21^{0}C) was maintained. Athletes at the test day could eat only a light breakfast, and 24 h before tests they couldn't take part in intensive exercise session. The tests were started with anthropometric measurements and blood pressure measurement with the use of the blood pressure monitor Omron M3 (Omron, Japan). Then the first NADH fluorescence measurement was conducted using AngioExpert device (Łódź, Poland), next participants underwent the treadmill exercise test to exhaustion (H/P Cosmos, Pulsar, Germany). Capillary blood samples were obtained from the fingertip from all athletes, at rest and 2 min after the exercise test to measure the level of lactate. 3–4 min after ending the exercise test, another blood pressure and NADH fluorescence measurements were taken. The study protocol was compliant with the Declaration of Helsinki. The study was approved by the Ethics Committee of the Poznan University of Medical Sciences in Poland (no. 1017/16 issued on the 5th October 2016). All athletes participated in this study voluntarily, and they were informed about a possibility to withdraw their consent at any time.

NADH Fluorescence measurement

NADH fluorescence measurement was conducted using AngioExpert (Angionica, Łódź, Poland) device. The fluorescence intensity at a wavelength of 460 nm (characteristic for NADH) was evaluated in response to activation by the light at the wavelength 340 nm (Mayevsky and Chance 2007; Mayevsky and Barbiro-Michaely 2009; Mayevsky et al. 2011). The fluorescence recorded during the measurement mainly came from epidermal cells (Dunaev et al. 2015). The used device AngioExpert continuously measures the fluorescence at rest (2 min), during controlled ischemia (200s), and during reperfusion after restoration of circulation in the limb (3 min). To induce a total occlusion of the brachial artery, an occlusion cuff (which is a part of the device) was inflated up to the pressure of 50 mmHg above the systolic blood pressure (SBP) of each athlete.

Before every FMSF measurement blood pressure measurement using blood pressure monitor Omron M3 (Omron, Japan) was conducted. The measurement using AngioExpert (Angionica, Łódź, Poland) device was conducted twice, immediately before exercise test and 3–4 minutes after its end.

Exercise test

The exercise test was conducted on a treadmill (model 150/50 LC, H/P Cosmos Pulsar, Germany). To monitor cardiopulmonary parameters the participants were equipped with the heart rate monitor Polar (Polar H6 Bluetooth Smart; Polar Electro Oy, Finland) and the mask connected to MetaMax 3B ergospirometer (Cortex Biophysik BmbH, Leipzig, Germany). The test started with a 4 min warm-up, at the speed of 6 km/h. Next, the treadmill speed increased progressively 2 km/h every 3 min. During the whole test the treadmill incline was set to the constant value of 1%. The test lasted until the athlete reported exhaustion. Respiratory parameters were analyzed using the MetaSoft Studio 5.1.0 Software (Cortex Biophysik, Germany). Maximal oxygen uptake (VO_{2max}) was considered to be reached if at least three of the following criteria were fulfilled: the oxygen uptake (VO_2) was stabilized despite the further load increase, HR reached at least 95% of the age-adjusted HR, respiratory exchange ratio (RER) was ≥ 1.1, blood lactate concentration was at the level ≥9 mmol/l for men and ≥7 mmol/l for women (Edvardsen et al. 2014). The maximal heart rate (HR_{max}) was defined for every athlete.

Blood Sampling

Capillary blood samples were collected from the fingertip twice, immediately before and 2 min after the exercise test. 20 µl of whole blood were drawn every time to a micro test tube using a capillary. To measure the level of lactate, Biosen C-line (EKF Diagnostics, UK) was used.

Anthropometric Measurements

The anthropometric measurements (height and body mass) were measured with a digital measuring station (Seca 285, SECA, Germany). Body mass index (BMI) was calculated as body weight divided by the square of body height (kg/m^2).

4. Results and their analysis

Publication 1

Flow-Mediated Skin Fluorescence method for non-invasive measurement of the NADH at 460 nm – a possibility to assess the mitochondrial function. ADVANCES IN CELL BIOLOGY 44 (4): 333–352, 2017. IF: 0.158, MNiSW: 20

In the following paper Greta Sibrecht and Olga Bugaj both are highlighted authors (first author).

AngioExpert is a new medical device intended for non-invasive diagnostics and monitoring of microcirculation disorders and metabolic regulation (changes in NADH fluorescence level). The mechanisms of working of AngioExpert device and Flow Mediated Skin Fluorescence method were presented in the first publication.

The method is using NADH molecule ability to fluorescence at the wavelength 460 nm, in response to activation by the light at the wavelength 340 nm. It is commonly known, in the mitochondria cellular respiration takes place, and as a result of this the high-energy adenosine triphosphate (ATP) molecule is produced. In this process NAD molecule oxidizes to NAD^+ and reduces to NADH, transferring hydrogen ions. During the cellular respiration NAD^+ is reduced to NADH in glycolysis in the cytoplasm, and in the citric acid cycle in mitochondrial matrix. NADH is oxidized to NAD^+ in electron transport chain, in the inner mitochondrial membrane. NADH oxidation to NAD^+ takes place when the oxygen is present. When there is a deficiency of oxygen, NADH oxidation to NAD^+ is possible at the limited level during anaerobic respiration, but the efficiency of this process is much lower than aerobic one. As a result, NADH is accumulating in the body.

NAD occurs in cytoplasm, nucleus and in the mitochondria (Stein and Imai 2012; White and Schenk 2012; Dolle et al. 2013). Nucleus membrane is permeable for NAD through special pores, so the concentration of NAD^+/NADH in the nucleus and in the cytosol is similar. In contrast, mitochondrial membrane is impermeable for NAD. NAD^+/NADH molecules need special shuttles (malate-aspartate and glycerophosphate shuttles), which transport electrons through the mitochondrial membrane, for processes of reduction and oxidation (White and Schenk 2012). Recent reports indicate that NAD can also pass through

mitochondrial membrane using an unrecognized NAD (or NADH) transporter (Davila et al. 2018). Therefore, monitoring NADH activity could be a valuable source of information about mitochondrial function.

There are many methods which are applied for studying mitochondria, each of them provides a little bit different information. They are useful to examine mitochondrial amount, structure and density in cells. However, most of them require invasive sampling, very often they don't allow to observe the changes in vivo. Another option is to monitor NADH fluorescence. Methods using fluorimetry evaluate NADH level in arbitrary units, so they don't provide information about its absolute value. However, they allow to evaluate dynamics of NADH changes in response to different stimuli and provide observation in real time in non-invasive way. Evaluation of NADH fluorescence since a long time has been considered a good method to indirectly evaluate mitochondrial function (Mayevsky and Chance 2007). However, the fluorescence has not been measured in human skin cells so far.

NADH molecule is a fluorophore, that means, it has an ability to absorb the wave at a certain spectrum of length and, as a response, it emits a wave at another length. For NADH, absorption wavelength is 320-380 nm, and emitted wavelength is 420-480 nm (Chance and Baltscheffsky 1958; Zhu et al. 2015). Mayevsky and Chance (2007) proofed that the most optimal wavelength for NADH measurement in the skin is 460 nm. Based on those facts, two novel devices were invented. The first one, CritiView, allows to monitor a number of parameters, including NADH fluorescence, in intensive care units. The device is placed into the urethra by Foley's catheter (Mayevsky et al. 2011). The second one, AngioExpert (Angionica, Łódź, Poland) – designed by Polish scientists from Łódź, was used in my own study. This device enables NADH fluorescence evaluation in vivo, non-invasive and in real time. During the examination with FMSF method, used in AngioExpert device, forearm arterial occlusion is made (Katarzynska et al. 2019). This occlusion allows to observe skin the cells reaction, under the oxygen deficit. This kind of information could be very useful for example in circulation diseases (Tarnawska et al. 2018). It is important, the recorded signal comes from the most superficial skin layers – the depth of wavelength penetration achieves maximal 0.5 mm depth. However, most of the signal comes from only 0.1 mm depth. On that depth the skin is not supplied with blood, so the fluorescence recorded during the measurement depends on supply of substrates and oxygen from deeper skin layer (Dunaev et al. 2015).

In the article a number of parameters were presented, and the most important of them were used in later studies.

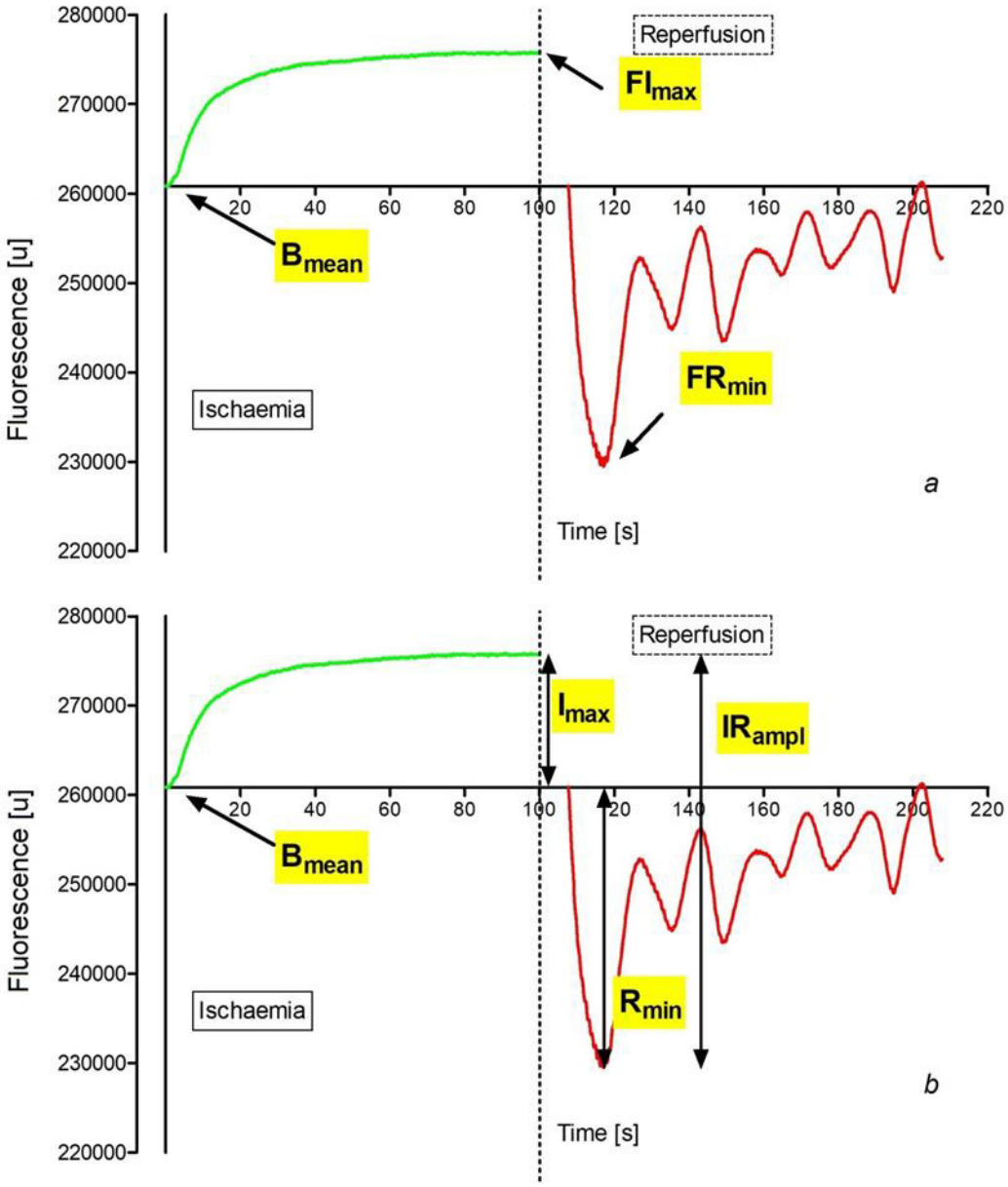
Measured parameters (Figure a):

- B_{mean} [u] – mean fluorescence value at 460 nm, recorded during rest; mean value of the basal NADH fluorescence,
- FI_{max} [u] – the maximal fluorescence value recorded during forearm ischaemia,
- FR_{min} [u] – the minimal fluorescence value recorded during reperfusion.

Calculated parameters (Figure b).

- I_{max} [u] – maximal increase in NADH fluorescence above the baseline during forearm ischaemia, the difference between FI_{max} and B_{mean},
- R_{min} [u] – maximal decrease in NADH fluorescence below the baseline during reperfusion, the difference between B_{mean} and FR_{min},
- IR_{ampl} [u] – the maximal range of the fluorescence change during ischaemia and reperfusion, the sum of I_{max} and R_{min},
- CI_{max} – the contribution of the NADH generated during occlusion to the total amount of NADH turned over during ischaemia and reperfusion, the contribution of I_{max} in IR_{ampl}.

In parameters: B_{mean}, FI_{max}, FR_{min}, I_{max}, R_{min}, IR_{ampl} arbitrary units [u] were used.



Publication 2

The effect of exercise on the skin content of the reduced form of NAD and its response to transient ischemia and reperfusion in highly trained athletes. Frontiers in Physiology 10: 600, 2019. DOI: 10.3389/fphys.2019.00600. IF: 3.201, MNiSW:100

Using FMSF method 121 highly trained athletes (94 men and 27 women) representing various sports disciplines were examined. 41 athletes represented long-distance running, 27 triathlon, 25 Olympic taekwondo, 9 rowing, 8 futsal, 6 sprint running, 4 fencing, and 1 tennis.

All participants were aged between 16 and 40 years, and they were members of Polish National Teams or participants of national and international sport competitions. All participants were examined during the preparatory period of the annual training cycle. The aim of the study was to examine the changes in NADH fluorescence intensity in the skin as the response to single exercise to exhaustion.

In my own study, using non-invasive NADH fluorescence measurement in the skin, it was shown that exercise to exhaustion modifies amount of NADH in the skin shifting up the entire fluorescence change curve. These changes include the baseline NADH fluorescence values, and the values recorded during ischemia and reperfusion as well. After the exercise there was a significant increase in B_{mean} ($p<0.001$), FI_{max} ($p<0.001$, only in men) showing the maximal fluorescence increase; FR_{min} ($p<0.001$ in men and $p<0.01$ in women) showing the maximal decrease in fluorescence after reperfusion, and R_{min} ($p<0.01$) showing difference between B_{mean} and FR_{min} (decrease in fluorescence normalized to the baseline). Reduction was seen in following parameters: I_{max} ($p<0.001$) (showing differences between FI_{max} and B_{mean}) and CI_{max} ($p<0.001$) (showing contribution of I_{max} in IR_{ampl}). IR_{ampl} parameter, which shows the maximal range of the fluorescence change during ischaemia and reperfusion ($I_{max} + R_{min}$), did not change after exercise.

In conclusion, exercise until exhaustion modifies NADH metabolism (basal and measured during ischemia and reperfusion) of skin cells. Immediately after exercise, there is a shift of the NADH fluorescence to higher values. The absolute NADH amount increases during post-exercise ischemia and reperfusion, compared to resting conditions. The observed changes in the NADH amount during ischemia and reperfusion strongly depend on metabolic conditions. These metabolic conditions are significantly modified by exercise and last for the next few minutes after its end. The intensification of NADH fluorescence in living skin cells, as a result of metabolic changes in NADH accompanying physical exercise extends beyond muscles and affects other cells and organs.

Publication 3

The effect of a 7-week training period on changes in skin NADH fluorescence in highly trained athletes. Applied Sciences 10: 5133, 2020. IF: 2.474, MNiSW: 70

Forty one highly trained athletes, aged between 18 to 35 years, took part in the study. All athletes were members of the Polish national team or they took part in national and international competitions. Athletes represented the following sport disciplines: triathlon at Olympic distance (1.5 km swim, 40 km bike ride, 10 km run) (7 men, 4 women), long-distance running (5 km, 10 km, and marathon) (6 men, 2 women), Olympic taekwondo (6 men, 1 woman), sprint (100 m, 200 m, and 4x100 m relay) (6 men, 1 woman), canoeing (3 men), and fencing (5 women). Athletes underwent the examinations twice: at the beginning of the 7-week general preparation phase and at the end of this phase, the aim was to evaluate the changes in NADH fluorescence in the skin.

In the measured parameters in the first phase of examination (before the 7-week training phase), after exercise, only B_{mean} parameter (basal fluorescence) significantly increased ($p<0.05$), however in the second phase of examination all measured parameters showed increase after exercise: B_{mean} ($p<0.001$), FI_{max} ($p<0.05$), FR_{min} ($p<0.001$). All measured parameters (B_{mean}, FI_{max}, FR_{min}), measured both before and after exercise, significantly increased at the level $p<0.001$ after training.

As a result of training, there were also noticed the changes in calculated parameters. The values of I_{max} significantly decreased after exercise in both pre- and post-training examinations at the level $p<0.001$. However, I_{max} values were higher after than before training, in both pre-exercise at the level $p<0.001$ and after treadmill test at the level $p<0.01$. R_{min} significantly increased after exercise compared to resting conditions in both examinations before (at the level $p<0.05$) and after training (at the level $p<0.001$). The pre- and post-exercise values of R_{min} were higher at the second term of examination ($p<0.001$). The IR_{ampl} parameter, which shows the amplitude of the fluorescence changes, did not significantly differ between resting and post-exercise conditions in both examinations. After training however, its both pre- and post-exercise values were significantly higher at the level ($p<0.001$). The last one, CI_{max} parameter, which shows the impact of I_{max} in whole amplitude of the changes (IR_{ampl}), was significantly lower after than before exercise in both examinations ($p<0.001$). There were no differences observed in this parameter before, when compared after training.

The changes in NADH fluorescence in epidermal cells in highly trained athletes have been investigated for the first time. A significant increase in NADH fluorescence after training was shown. It is known that physical training induces a number of adaptations including mitochondrial adaptations (Drake et al. 2017). The measurement of NADH

fluorescence may be used to indirectly evaluate the mitochondrial function and information about its metabolic status (Mayevsky and Barbiro-Michaely 2009). NADH fluorescence evaluation alone does not allow us to answer the question of what particular metabolic changes take place in the cells but lets us observe increase in the NADH fluorescence after training, what suggests increase in NAD pool in response to the training applied during the preparatory period in our athletes.

The preparatory period is characterized by predominance of endurance exercise, regardless of sport discipline. In athletes examined by us, the endurance-dominant training affected the increase in the NADH fluorescence in measured parameters (B_{mean}, FI_{max}, FR_{min}), which can indicate the adaptation changes in skin mitochondria. Our findings seem to be consistent with earlier studies, which were carried out using muscles. In those studies authors observed post-training increase in the level of proteins related to mitochondrial biogenesis and an improvement in mitochondrial respiratory function (Yan et al. 2012; Busquets-Cortés et al. 2017; Granata et al. 2018).

5. Conclusions

The conducted research let us draw following conclusions:

1. Exercise until exhaustion modifies the skin NADH metabolism at rest, during ischemia and reperfusion in the forearm skin cells, shifts up basal NADH fluorescence to higher values.

2. Physical training in preparatory period results in an increase in the skin NADH fluorescence levels in highly trained athletes, at rest and after exercise until exhaustion, what could suggest increase in the NAD pool in the body.

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Streszczenie

Celem badań było określenie zmian fluorescencji NADH badanej za pomocą metody Flow Mediated Skin Fluorescence u wysoko wytrenowanych sportowców różnych dyscyplin sportowych, pod wpływem pojedynczego wysiłku do odmowy (artykuł 2) oraz w wyniku 7 tygodniowego treningu sportowego (artykuł 3). Do badań wykorzystano nowo powstałe urządzenie medyczne AngioExpert przeznaczone do nieinwazyjnego diagnozowania i monitorowania zaburzeń mikrokrążenia oraz regulacji metabolicznej poprzez określenie zmian poziomu fluorescencji NADH. Założono, że: (a) metoda FMSF pozwala na ocenę zmian fluorescencji NADH w wyniku zadziałania okluzji i w trakcie reperfuzji, (b) pojedynczy intensywny wysiłek fizyczny zmienia obrót NADH, a także (c) że w wyniku treningu fizycznego dochodzi do zmiany intensywności fluorescencji badanej zarówno przed i jak po wysiłku fizycznym.

Fluorescencje NADH określano w spoczynku a także bezpośrednio (3–4 minuty) po wysiłku do odmowy na bieżni mechanicznej. Pomiary wykonano w dwóch terminach badań, na początku oraz po 7 tygodniach treningu w okresie przygotowawczym.

Krzywa fluorescencji NADH, rejestrowana zarówno w czasie niedokrwienia jak i reperfuzji badana po wykonaniu pojedynczego wysiłku do odmowy ulegała przesunięciu w kierunku wyższych wartości w stosunku do wartości rejestrowanych przed wysiłkiem. Również 7 tygodniowy trening w okresie przygotowawczym wywoływał podniesienie badanych wartości mierzonych fluorescencji NADH zarówno przed jak i po teście wysiłkowym.

Sugeruje się, że pomiar fluorescencji NADH obrazuje zmiany metaboliczne w mitochondriach skóry zachodzące pod wpływem pojedynczego wysiłku do odmowy a także w wyniku zastosowanego 7 tygodniowego treningu w okresie przygotowawczym. Ponieważ adaptacje zachodzące pod wpływem treningu dotyczą całego ciała, dlatego zmiany w metabolizmie skóry mogą zapewniać łatwo dostępną informację o uogólnionych zmianach zachodzących w mitochondriach całego organizmu.

Abstract

The aim of the study was to identify the changes in NADH fluorescence, measured using Flow Mediated Skin Fluorescence method under the conditions of single exercise until exhaustion (publication 2) and 7-week physical training (publication 3). The research was carried out on a group of highly trained athletes representing various sport disciplines. The AngioExpert, a novel medical device for non-invasive evaluation of microcirculation disorders and metabolic regulation by tracking changes in NADH fluorescence level, was used. It was assumed that: FMSF method lets evaluate (a) changes in NADH fluorescence under the influence of occlusion and during the reperfusion, (b) a single intensive exercise changes NADH turnover, and (c) physical training result changes in fluorescence intensity evaluated both before and after exercise.

NADH fluorescence was evaluated at rest and immediately (3–4 minutes) after exercise to exhaustion on a treadmill. Measurements were conducted in two terms, at the beginning and after 7-week training in the preparatory period.

The NADH fluorescence curve evaluated during ischemia and reperfusion, recorded after exercise until exhaustion, shifted up to higher values, compared with measurement at rest. Also 7-week training in preparatory period impacted an increase of measured parameters of FMSF fluorescence, evaluated before and after exercise test.

It is suggested, NADH fluorescence measurement shows metabolic changes in skin mitochondria, which take place under the influence of single exercise until exhaustion, and also as a result of applied 7-week training in preparatory period. Adaptations occurring under the impact of training affect the whole body, so the changes in skin metabolism could provide easily available information about mitochondrial changes in whole body.

Załączniki

- Oświadczenia współautorów
- Publikacja nr 1
- Publikacja nr 2
- Publikacja nr 3

22.10.2019 r. Poznań

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Zakład Lekkiej Atletyki i Przygotowania Motorycznego

OŚWIADCZENIE

Mój udział w powstawaniu niżej wymienionej pracy polegał na: analizie piśmiennictwa, napisaniu manuskryptu.

Flow-Mediated Skin Fluorescence method for non-invasive measurement of the NADH at 460 nm – a possibility to assess the mitochondrial function

Postępy Biologii Komórki 44 (4): 333–352, 2017. Olga Bugaj

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Mój udział w powstawaniu niżej wymienionej pracy polegał na: zaprojektowaniu badań, rekrutowaniu i badaniu uczestników, analizie i interpretacji danych, przeprowadzeniu analizy piśmiennictwa oraz przygotowaniu manuskryptu.

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Mój udział w powstawaniu niżej wymienionej pracy polegał na: zaprojektowaniu badań, ustaleniu metodologii, przeprowadzeniu i walidacji badań, analizowaniu i zarządzaniu danymi, wizualizacji danych oraz napisaniu oryginalnej wersji manuskryptu:

The Effect of a 7-Week Training Period on Changes in Skin NADH Fluorescence in Highly Trained Athletes.

Applied Sciences 10: 5133, 2020. DOI: 10.3390/app10155133 Olga Bugaj

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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

*Both authors have equal contribution to this work and therefore should be considered as first authors

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)

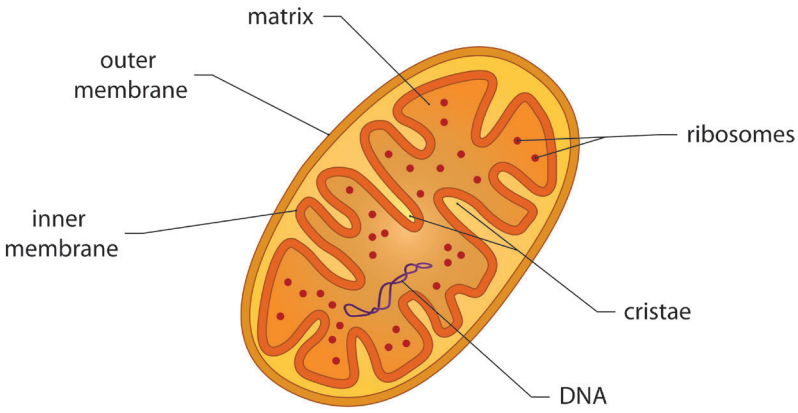

FIGURE 1. The structure of the mitochondrion

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

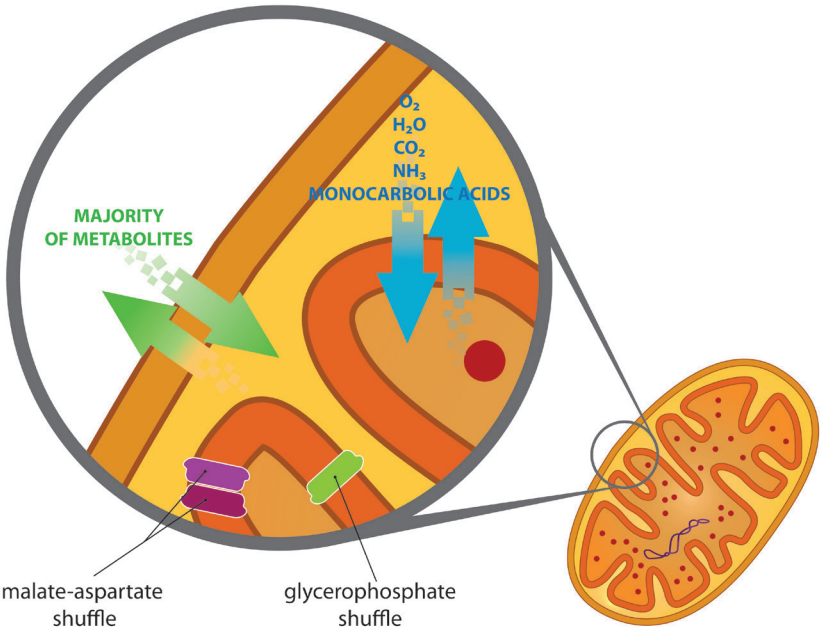

FIGURE 2. The permeability of the mitochondrial membranes and transporting shuttle

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.

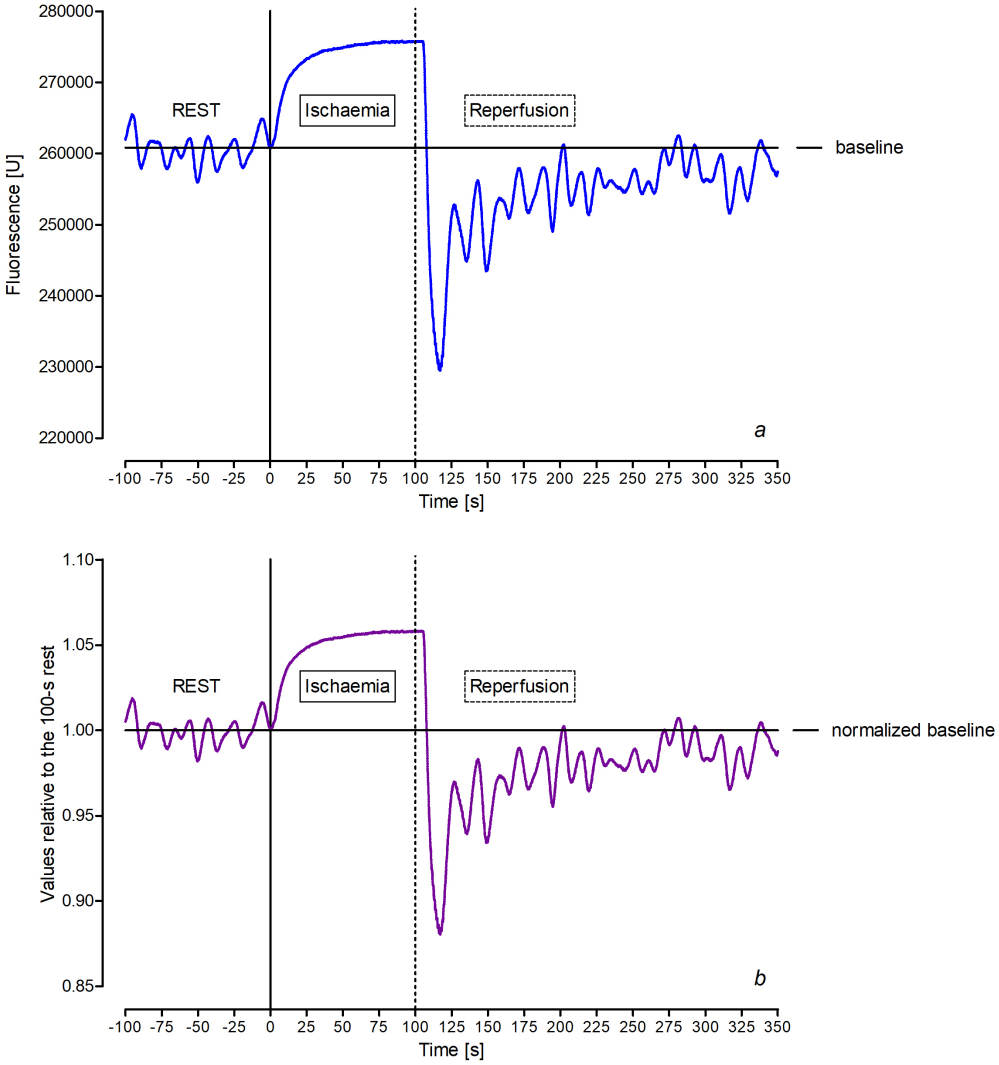

FIGURE 3. An example of typical epidermal fluorescence at a wavelength of 460 nm recorded in the forearm of a 47-year-old healthy man during rest in a seated position, then during 100-second ischaemia and then following reperfusion. Panel a shows fluorescence in its absolute units (u) whereas panel b 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

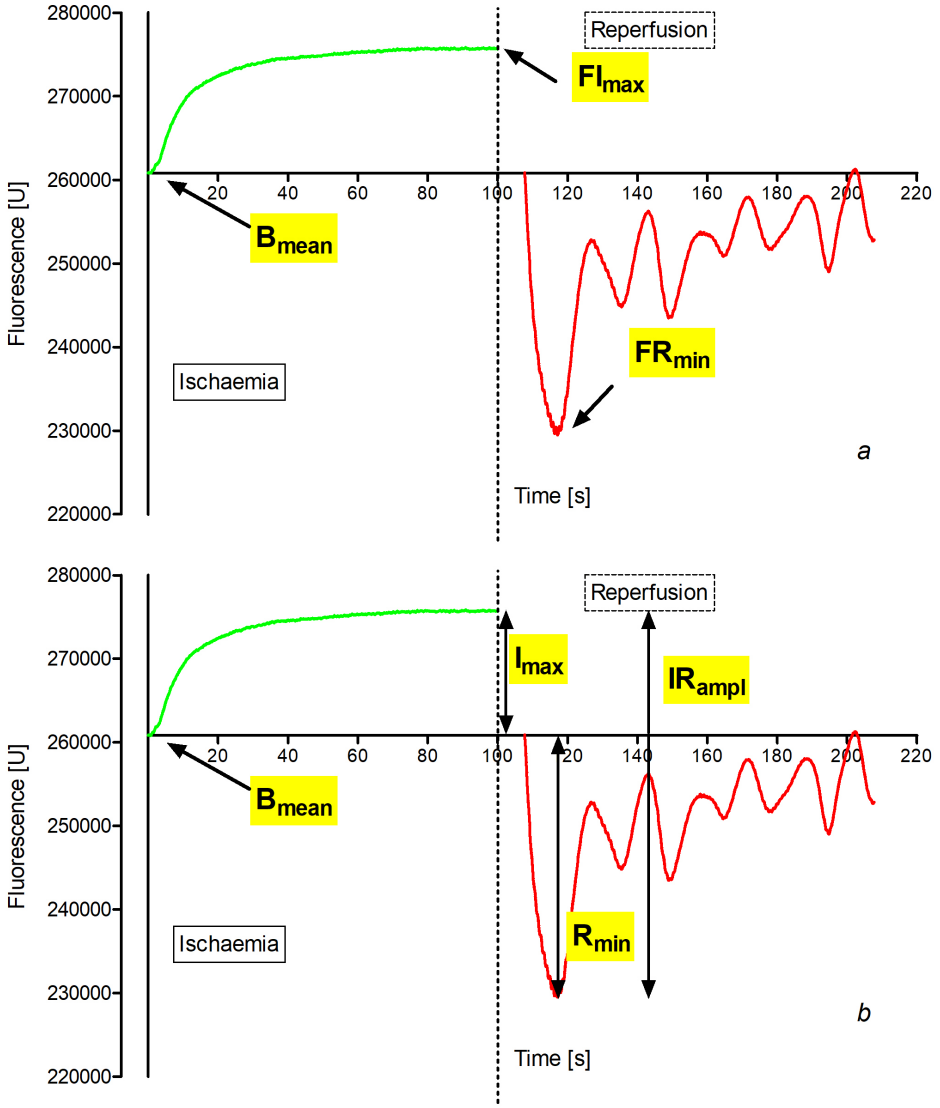

FIGURE 4. The same signal as in Figure 3 with a focus on the ischaemia and reperfusion phases. Panel a shows the three basic points in the signal, i.e. the B_{mean}, FI_{max} and FR_{min} which are starting points for the computation of other parameters shown in panel b and Figure 5, and explained in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

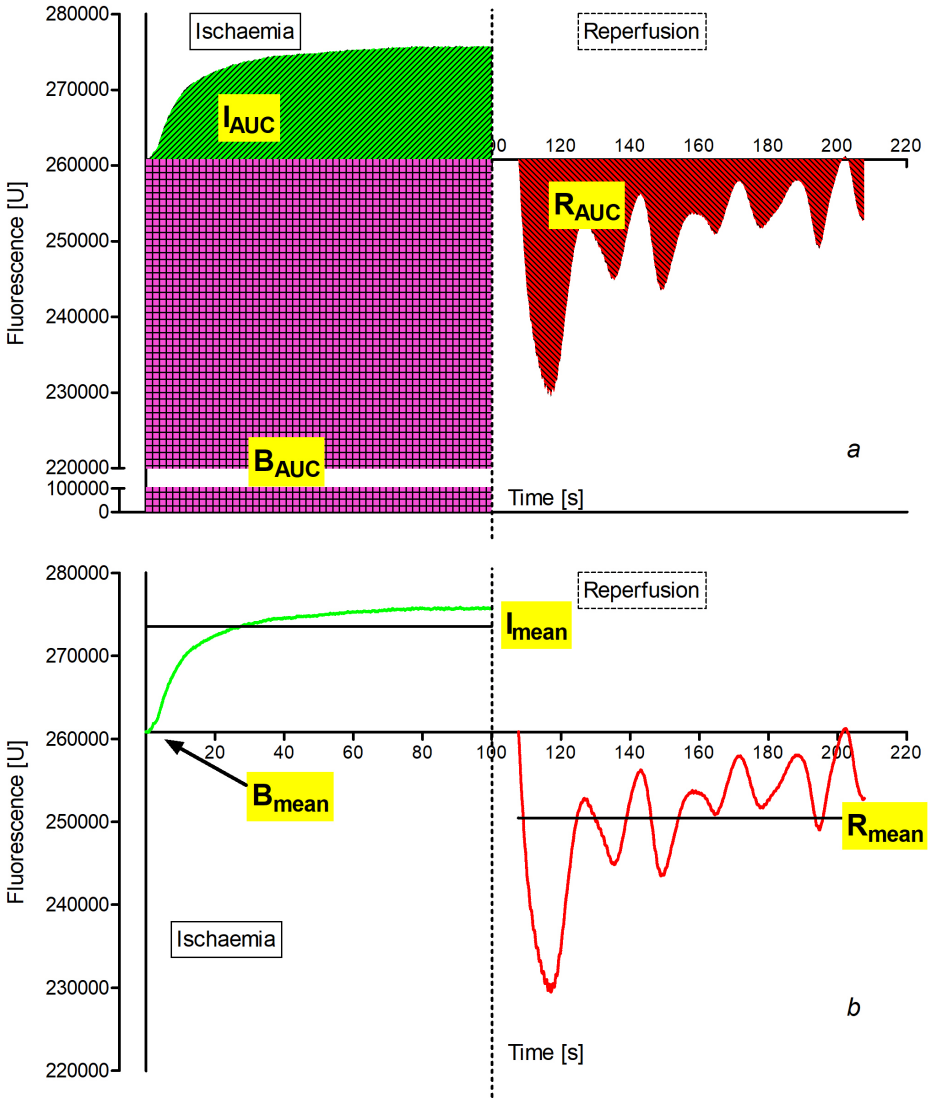

FIGURE 5. The same signal as in Figure 3 with a focus on the ischaemia and reperfusion phases. Panel a shows the three areas defined for the baseline (B_{AUC}), ischaemia (I_{AUC}), reperfusion (R_{AUC}). Panel b presents the positioning of the mean fluorescence during ischaemia (I_{mean}) and reperfusion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

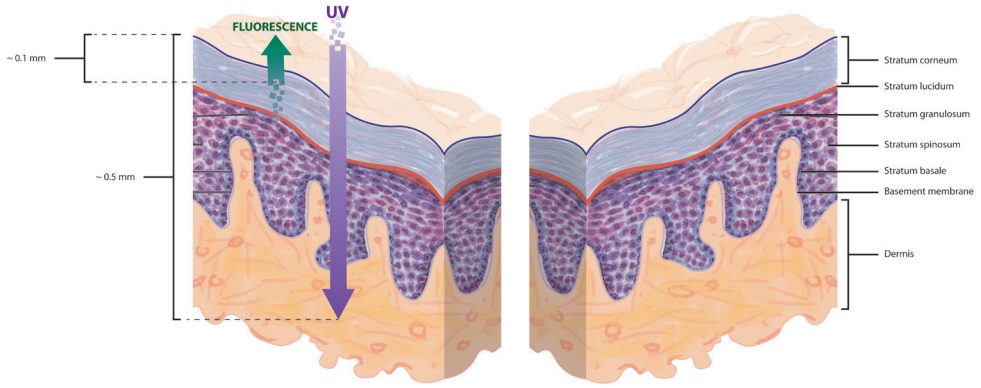

FIGURE 6. The layers of the epidermis with the depth of ultraviolet (UV) light penetration exciting NADH to emit fluorescence light. Of note, whereas the UV penetration reaches 0.5 mm, the fluorescence comes from the outermost layer of the depth of less than 0.1 mm. At this depth there are no capillary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

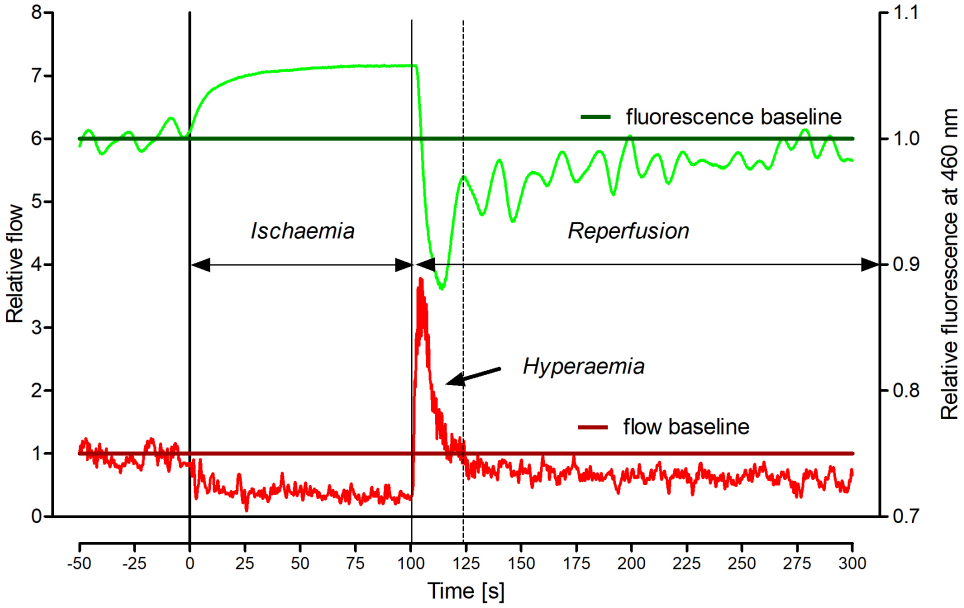

FIGURE 7. Simultaneous recordings of the blood flow in the microcirculation by the Laser Doppler Flow method and cell NADH amount by the fluorescence at 460 nm. The microcirculatory flow was measured by the Laser Doppler Perfusion Monitor (PeriFlux System 5000, Perimed, Sweeden – the device made available, 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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The Effect of Exercise on the Skin Content of the Reduced Form of NAD and Its Response to Transient Ischemia and Reperfusion in Highly Trained Athletes

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Reduced nicotinamide adenine dinucleotide (NADH) is synthesized in the cellular nucleus, cytoplasm and mitochondria but oxidized into NAD^+ almost exclusively in mitochondria. Activation of human skin by the 340 nm ultraviolet light triggers natural fluorescence at the light length of 460 nm, which intensity is proportional to the skin NADH content. This phenomenon is used by the Flow Mediated Skin Fluorescence (FMSF) which measures changes in the skin NADH content during transient ischemia and reperfusion. We examined the effects of exercise to exhaustion on the skin changes of NADH in response to 200 s forearm ischemia and reperfusion in 121 highly trained athletes (94 men and 27 women, long-distance running, triathlon, taekwondo, rowing, futsal, sprint running, fencing, and tennis). We found that exercise until exhaustion changes the skin content of NADH, modifies NADH turnover at rest, during ischemia and reperfusion in the most superficial living skin cells. Compared to the pre-exercise, there were significant increases in: mean fluorescence recorded during rest as the baseline value (B_{mean}) ($p < 0.001$), the maximal fluorescence that increased above the baseline during controlled forearm ischemia (FI_{max}) ($p < 0.001$, only in men), the minimal fluorescence after decreasing below the baseline during reperfusion (FR_{min}) ($p < 0.001$ men; $p < 0.01$ women) and the difference between B_{mean} and FR_{min} (R_{min}) ($p < 0.01$), and reductions in the difference between FI_{max} and B_{mean} (I_{max}) ($p < 0.001$) and I_{max}/IR_{ampl} ratio (CI_{max}) ($p < 0.001$) after the incremental exercise test. There was no statistical difference between pre- and post-exercise the maximal range of the fluorescence change during ischemia and reperfusion (IR_{ampl}). In conclusion, exercise to exhaustion modifies the skin NADH content at rest, during ischemia and reperfusion as well as the magnitude of changes in the NADH caused by ischemia and reperfusion. Our findings suggest that metabolic changes in the skin NADH accompanying exercise extend beyond muscles and affect other cells and organs.

Keywords: NADH, FMSF, incremental exercise test, athletes, mitochondrion

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is a cellular coenzyme present in practically all living cells. The two forms of NAD, an oxidized NAD^+, and reduced NADH can be found in the cell cytosol, nucleus and mitochondria (Dolle et al., 2010; White and Schenk, 2012). Both are crucial in the transfer of electrons between different molecules in reactions catalyzed by oxidoreductases (Mayevsky and Chance, 2007). One of the primary functions of the NAD^+/NADH coenzymes is their involvement in energy production, which mostly takes places in mitochondria. Therefore, the measurement of these coenzymes is used as a marker of the mitochondrial activity (Mayevsky and Chance, 2007). NAD^+/NADH are involved in several steps of the citric acid cycle, the transfer of energy and protons between this cycle and oxidative phosphorylation, and the production of adenosine triphosphate (ATP) which is the most essential energy particle (Mayevsky and Chance, 2007; White and Schenk, 2012). NAD has an important function in redox reactions and also serves as a cofactor for many enzymes such as mitochondrial sirtuins and NAD glycohydrolases (Dolle et al., 2013).

The nuclear membrane is permeable for NAD^+ and NADH through special pores, so the concentration of NAD^+/NADH is comparable between the nucleus and cytosol. (White and Schenk, 2012). Recent reports indicate that NAD can also penetrate the mitochondrium using an unrecognized NAD (or NADH) transporter (Davila et al., 2018).

While NADH is produced in the nucleus, cytosol, and mitochondria, it is oxidized to NAD^+ mainly within the mitochondrial electron transport chain (White and Schenk, 2012). It is assumed that mitochondrial NAD^+/NADH metabolism is similar in all living human cells, e.g., circulating leucocytes, myocytes, liver, brain or skin cells, and comparably affected by blood flow, oxygen and nutrient delivery, as well as inner and external factors or changing conditions, such as physical activity (Green, 1997; Ament and Verkerke, 2009; Mayevsky and Barbiro-Michaely, 2009; White and Schenk, 2012).

It should be noted that the total NAD pool in the body is not constant over a longer time. It is affected by physical activity, diet, and NAD boosters. In addition, NAD pool decreases with aging, as does the sirtuins (NAD-dependent deacetylases) activity that controls almost all cell functions (Kane and Sinclair, 2018; Rajman et al., 2018). The effects of depleted NAD pool may be serious and include several cardiovascular and metabolic disorders (Braidy et al., 2018, Rajman et al., 2018). NAD pool can be reduced due to various factors, e.g., DNA damage, free radicals or excessive ultraviolet radiation. This results in higher activation of poly (ADP-ribose) polymerase (PARP) and increased NAD^+ turnover and depletion. A chronic immune activation and intensified production of inflammatory cytokines can also occur, leading to increased activity of CD38 protein [a catalyst in cyclic metabolism of ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP)] and, back again, to a decrease in NAD^+ levels (Braidy et al., 2018).

By contrast, an increased activity of NAD^+ dependent sirtuins (1−7) has several health benefits related to deceleration of certain aging processes. Sirtuins are located in cell nucleus (SIRT1, 6 – 7), cytoplasm (SIRT1 – 2) and mitochondrium (SIRT 3 – 5). Depending on the location, they support such processes as angiogenesis stimulation or protection against vascular endothelial dysfunction and ischemia-reperfusion damage (Kane and Sinclair, 2018). The intake of NAD^+ precursors can positively affect cognition function, improve the function of vascular endothelium, act cardioprotective or improve insulin sensitivity and fat free acids oxidation (Rajman et al., 2018).

Initially, studies on the relationship between NADH and physical activity were performed with the use of biopsy samples from working animal muscle (Chance and Connelly, 1957; Edington, 1970; Edington and McCafferty, 1973; Duboc et al., 1988) and in humans (Graham et al., 1978; Sahlin, 1985; Katz and Sahlin, 1987; Sahlin et al., 1987; Graham and Saltin, 1989). Similarly, NADH in skin cells was first studied in animal skin samples (Pappajohn et al., 1972; Palero et al., 2011) and then in humans (Mayevsky and Barbiro-Michaely, 2009; Balu et al., 2013).

Metabolic changes accompanying intensive physical activity, e.g., reduction in pH due to an increase in lactate production (and thus H^+), modify homeostasis of the whole body, its cells, and organelles, including mitochondria (Green, 1997; Ament and Verkerke, 2009; Kane, 2014). It has been shown that high-intensity exercise modifies the NAD^+/NADH balance (White and Schenk, 2012). Oxygen is critical for proper mitochondrial energy production. Therefore, a reduction in oxygen content during hypoxia/anoxia observed in the course of ischemia slows down or even stops mitochondrial function, the oxidation of NADH to NAD^+ and thus the generation of ATP particles (Mayevsky and Rogatsky, 2007). The restoration of oxygen delivery during reperfusion may recover this process. It is unknown, however, whether the alterations in the NAD^+/NADH balance during ischemia and reperfusion may be affected by metabolic changes induced by intensive exercise.

To explore this problem, we applied the Flow Mediated Skin Fluorescence (FMSF) method which measures the 460 nm fluorescence of NADH in the skin at rest, during controlled ischemia and reperfusion (Piotrowski et al., 2016). This method is based on an optical property of NADH which absorbs light waves in the range of 320 – 380 nm and then emits back the fluorescent light at a longer length of 420 – 480 nm (Mayevsky and Rogatsky, 2007). The intensity of this fluorescence is proportional to the amount of generated NADH. Therefore, changes in fluorescence at a specific light range are monitored to measure the amount of NADH in solutions, cells, tissues, and organs (e.g., brain, liver or skin) (Mayevsky and Rogatsky, 2007). The FMSF is a fluorescence-based method used for the quantification of NADH in a completely non-invasive way and in real time. We hypothesize that intensive exercise to exhaustion influences the contents of NADH in skin epidermal cells, and shifts the NAD^+/NADH balance toward NADH. Therefore, in this study, we aimed to examine the continuous changes in mitochondrial NADH content at rest, during the controlled 200 s forearm ischemia and the following reperfusion before and immediately after exercise to exhaustion in healthy athletes. Additionally, we analyzed the effects of exercise to exhaustion on the NADH

skin content at rest, during ischemia and reperfusion separately for men and women.

MATERIALS AND METHODS

Ethics Statement

The study was designed and performed in agreement with the Declaration of Helsinki. The study protocol and all forms were approved by the Ethics Committee of the Poznań University of Medical Sciences in Poland (no. 1017/16 issued on the 5th October 2016). All athletes participated in this study voluntarily and gave their informed consent.

Participants

From a group of potential candidates, we excluded athletes who had recent infection or injury with accompanying clinical signs and symptoms such as fever, cough, swelling, or pain. Athletes who were regularly taking any medications for chronic disease not limiting their physical activity (e.g., bronchodilators for asthma, antihistamines for allergy) were also excluded. Only athletes without any medication (except for hormonal contraception) were included.

In Poland, all athletes participating in competitions are required to have a current health certificate every six months; they undergo regular obligatory medical assessment by a physician specializing in sports medicine. Only athletes with a valid health certificate were enrolled.

We recruited 121 healthy highly trained athletes (94 men and 27 women) fulfilling the following inclusion criteria: age in the range between 16 and 40 years, members of Polish National Teams in selected sports disciplines or participants of national and international sports competitions, training at least once a day for at least six days a week. To recruit individuals representing different characteristics of regular training (aerobic, anaerobic, mixed), we selected athletes from the following sports disciplines: long-distance running (n = 41), triathlon (n = 27), taekwondo (n = 25), rowing (n = 9), futsal (n = 8), sprint running (n = 6), fencing (n = 4), and tennis (n = 1). All participants were examined during the preparatory period of the annual training cycle, i.e., not at the peak of their performance.

Study Design

The study was conducted in the Human Movement Laboratory of the Department of Athletics Strength and Conditioning, at the Poznań University of Physical Education (Poland). After arriving at the laboratory, we evaluated the health status of each participant. Medical history was obtained and physical examination performed, including brachial blood pressure with the use of the blood pressure monitor Omron M3 (Omron, Japan) in the seated position. Blood pressure was measured twice, at rest before the cardiopulmonary exercise test (CPET) and 2 – 3 min after its completion. The following blood pressure parameters were collected for further analysis: systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse pressure (PP).

The majority of participants underwent the treadmill CPET at least once previously. Nevertheless, all athletes were informed about specific requirements and potential risks related to this test. Each participant was asked to avoid any intensive training in the last 48 h preceding the CPET, drinking alcohol for 24 h and coffee or caffeine containing drinks or supplements for 12 h. On the CPET day, participants were allowed to eat a light breakfast. Before the CPET, each participant spent 30 min in the air-conditioned laboratory for acclimatization to its environment. The temperature was kept constant within a range of 19–21°C. Before the CPET and 3–4 min after its completion, the flow-mediated skin fluorescence (FMSF) by the AngioExpert device (Angionika, Poland) was measured in each athlete. **Figure 1** summarizes the measuring procedure of FMSF.

Incremental Cardiopulmonary Exercise Testing

The incremental CPET was performed on a treadmill model 150/50 LC (pluto®) with the following sizes of the running surface: length 1500 mm and width 500 mm, (H/P Cosmos Pulsar, Germany). Before the test, all athletes were equipped with a chest strap heart rate monitor (Polar H6 Bluetooth Smart; Polar Electro Oy, Finland) and a properly sized face mask (Cortex Medical, Germany) connected to the MetaMax 3B-R2 mobile spiroergometry device. The CPET started at a speed of 6 km/h for the 4 min warm-up, and then the treadmill speed increased by 2 km/h every 3 min. The treadmill's incline was set at a constant value of 1%. The CPET was stopped when the athlete reached voluntary exhaustion. After stopping the treadmill, the athlete stood for at least 2 min during recovery and finally was disconnected from the face mask and the Polar chest heart rate monitor. All parameters were measured continuously and then averaged for each breath by the MetaSoft Studio 5.1.0 Software (Cortex Biophysik, Germany). For this study, we used only data on heart rate (HR), estimated maximal heart rate according to the formula "207- 0.7 × age" (Gellish et al., 2007) and the peak or maximal oxygen consumption (VO_2max) normalized to body mass. VO_2max was measured only when all of the following three criteria were fulfilled: VO_2 reached plateau despite a further increase in speed of the treadmill, HR reached at least 95% of age-adjusted HR estimate and respiratory exchange

FIGURE 1 | The measuring procedures using the Flow Mediated Skin Fluorescence before and after the cardiopulmonary exercise test.

ratio $\geq$ 1.1 (Edvardsen et al., 2014). In the remaining cases, only the VO_2 peak value was used for the description of the intensity of exercise.

NADH Fluorescence

The AngioExpert device (AngioExpert, Angionica, Poland) (Piotrowski et al., 2016) was employed to evaluate the 460 nm fluorescence of the skin in response to activation by the 340 nm UV light (Mayevsky and Chance, 1973; Friedli et al., 1982; Mayevsky and Chance, 2007; Mayevsky and Barbiro-Michaely, 2009; Mayevsky et al., 2011; Sibrecht et al., 2017). The wavelength of 340 nm emitted in the FMSF method is only specific to NADH, thus no other substance in the skin can be excited. The 460 nm fluorescence in response to activation takes place mainly in epidermal cells (Dunaev et al., 2015), because the wavelength of the emitted light penetrates and is absorbed up to 0.5 mm deep. The AngioExpert continuously measured the 460 nm fluorescence from the most superficial skin cells in the forearm at rest, then during controlled ischemia triggered by total occlusion of the brachial artery by the brachial blood pressure cuff, and, finally, during reperfusion after deflation of the blood pressure cuff (Piotrowski et al., 2016; Sibrecht et al., 2017) (**Figure 2**).

To quantify the FMSF response, we used the following parameters (**Figure 2**) (Sibrecht et al., 2017):

- B_{mean} [kFU] – mean fluorescence at 460 nm recorded before ischemia as the baseline value;
- FI_{max} [kFU] – the maximal fluorescence that increased above the baseline during controlled forearm ischemia;
- FR_{min} [kFU] – the minimal fluorescence after decreasing below the baseline during reperfusion;
- I_{max} [kFU] – the difference between FI_{max} and B_{mean};
- R_{min} [kFU] – the difference between B_{mean} and FR_{min};
- IR_{ampl} [kFU] – the maximal range of the fluorescence change during ischemia and reperfusion;
- CI_{max} – I_{max}/IR_{ampl} ratio.

For this study, we continuously measured and recorded skin fluorescence for 2 min before ischemia, then for 200 s of ischemia and finally for 3 min during post-ischemic reperfusion. To obtain total net forearm ischemia, we used a brachial blood pressure cuff placed on the ipsilateral arm and inflated the cuff to 50 mmHg of pressure above the systolic blood pressure of each participant. Such pressure is necessary for temporal, short-lasting compression of the artery and complete cessation of blood flow to the forearm and hand below. The transient forearm ischemia is usually obtained by this procedure in many physiological and clinical studies, for example during testing the flow-mediated arterial vasodilation (Harris et al., 2010; Pahkala et al., 2011; Bailey et al., 2017). The measurements were performed in laboratory conditions in a constant environment according to a strict procedure as described elsewhere (Piotrowski et al., 2016). To avoid any environmental interferences, during the measurement all subjects kept their studied forearm motionless in a special curved form with the fluorescence sensor placed at its bottom. The forearm covered completely the fluorescent sensor to prevent any transmission of external light which might interact with the sensor and fluorescence measurement. Since the fluorescence is monitored and measured continuously with the sampling rate of 25 Hz, the shown signal is a continuous line. Any unexpected sudden departure from this continuity other than caused by a controlled ischemia and reperfusion was considered as an artifact and either not taken to further analysis or instantly repeated, if possible. The repetition was allowed only if the artifacts were present at rest, before any ischemia.

Statistical Analysis

Continuous data distribution was analyzed using the Kolmogorov–Smirnoff test. Due to normal data distribution, results are presented as mean values and standard deviations (SD). The comparisons between pre- and post-exercise results were made with the paired *t*-test- first for all athletes and then separately for men and women. Only $p < 0.05$ was considered statistically significant. All statistical analyses were made using MedCalc Statistical Software version 18.2.1 (MedCalc Software bvba, Ostend, Belgium[1]; 2018).

RESULTS

Baseline Characteristics

The mean age of all participants was around 23 years, their BMI, resting systolic and diastolic blood pressures were normal. The average peak heart rate obtained during the CPET was nearly 189 beats/minute, which corresponded to almost 95% of the estimated maximal HR indicating a very intense (maximal) exercise. **Table 1** summarizes baseline data all studied athletes.

Separate clinical characteristics for participating men and women are shown in **Table 2**.

[1]http://www.medcalc.org

TABLE 1 | Clinical characteristics of studied athletes.

Parameter:	Mean	SD
Age [years]	23.41	5.32
Training experience [years]	8.15	3.44
BMI [kg/m^2]	22.29	2.55
SBP at rest [mmHg]	125.88	12.18
DBP at rest [mmHg]	72.20	7.87
PP at rest [mmHg]	53.68	12.26
SBP after CEPT [mmHg]	146.08	22.34
DBP after CEPT [mmHg]	74.42	8.21
PP after CEPT [mmHg]	71.66	24.53
Predicted maximal HR [beats/minute]	190.61	5.32
Peak HR during CPET [beats/minute]	188.88	9.77
Achieved percentage of the predicted maximal HR [%]	99.09	9.53
VO_2max [ml/min/kg]	60.61	8.03

BMI, body mass index; CPET, cardiopulmonary exercise test; DBP, diastolic blood pressure; HR, heart rate; PP, pulse pressure; SBP, systolic blood pressure; VO_2max, maximal oxygen uptake.

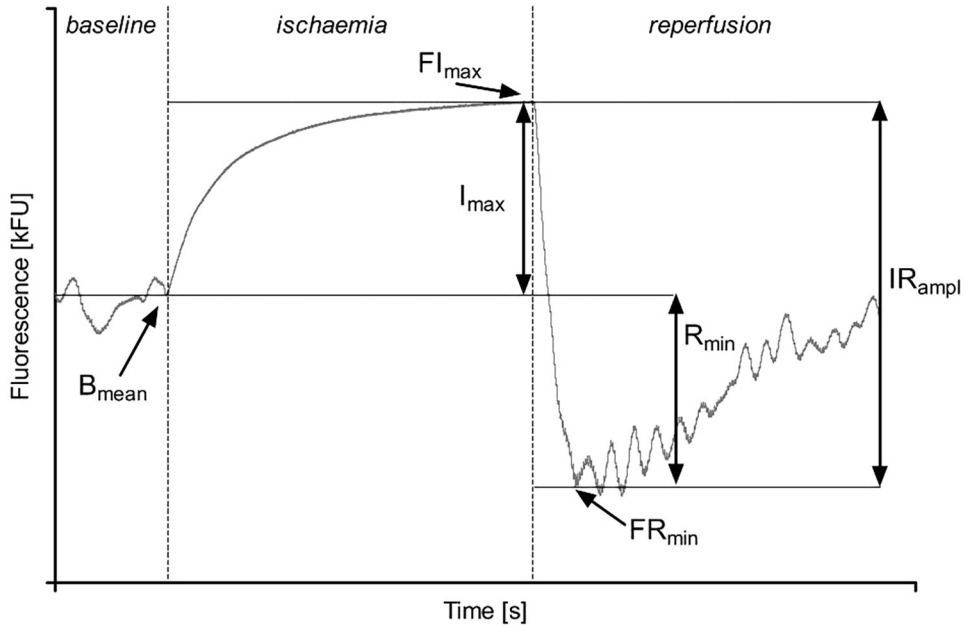

FIGURE 2 | Parameters describing the Flow Mediated Skin Fluorescence: B_{mean} – mean of the basal fluorescence; FI_{max} – maximal fluorescence value during ischemia; FR_{min} – the first minimal fluorescence value during reperfusion; I_{max} – the net increase of fluorescence over the baseline value during ischemia; IR_{ampl} – the amplitude of fluorescence change during ischemia and reperfusion; R_{min} – the net reduction of fluorescence below the baseline value.

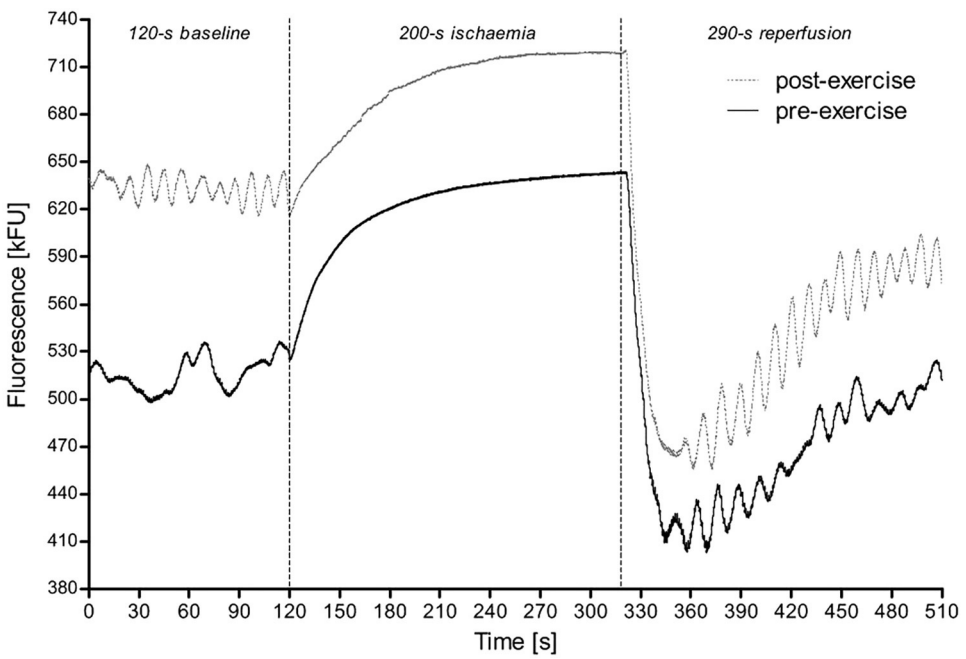

FIGURE 3 | A sample of the FMSF measurement from a 28-year-old female athlete (triathlete) before and after exercise test until exhaustion. The first 2 min correspond to the time before ischemia, which is then followed by the transient 200 s ischemia (gradual increase in the fluorescence) with subsequent reperfusion (sudden drop in the fluorescence). Of note, although in this figure the 290 s reperfusion is shown, for the study we used only the values recorded during the first 180 s.

TABLE 2 | Clinical characteristics of male and female athletes.

	Men		Women	
Parameter:	**Mean**	**SD**	**Mean**	**SD**
Age [years]	23.88	5.69	21.74	3.35
Training experience [years]	7.71	2.73	7.56	1.48
BMI [kg/m^2]	22.64	2.57	21.06	2.07
SBP at rest [mmHg]	128.91	11.21	115.30	9.25
DBP at rest [mmHg]	73.01	7.50	69.37	8.63
PP at rest [mmHg]	55.9	12.29	45.93	8.55
SBP after CEPT [mmHg]	148.98	23.32	136.00	14.91
DBP after CEPT [mmHg]	74.18	8.36	75.26	7.76
PP after CEPT [mmHg]	74.8	25.24	60.74	18.43
Predicted maximal HR [beats/minute]	190.28	3.98	191.78	2.34
Peak HR during CPET [beats/minute]	188.96	9.11	190.77	10.37
Achieved percentage of the predicted maximal HR [%]	98.29	11.27	95.84	19.89
VO_2max [ml/min/kg]	62.25	7.28	54.73	7.96

BMI, body mass index; CPET, cardiopulmonary exercise test; DBP, diastolic blood pressure; HR, heart rate; PP, pulse pressure; SBP, systolic blood pressure; VO_2max, maximal oxygen uptake.

Effects of Exercise on FMSF

Figure 3 shows an example of the typical 460 nm fluorescence at baseline, during ischemia and the following reperfusion before and after CPET to exhaustion.

Results of the comparison of the FMSF performed before and after the CPET are shown in **Table 3** for all athletes and in **Table 4** separately for sportsmen and sportswomen.

In all athletes (**Table 3**), compared to the pre-exercise rest, after the CPET there were significant increases in B_{mean}, $\mathrm{FI_{max}}$, $\mathrm{FR_{min}}$ and R_{min}, and reductions in I_{max}, $\mathrm{CI_{max}}$. No statistical differences between pre- and post-exercise were observed for $\mathrm{IR_{ampl}}$.

TABLE 3 | Comparison of the FMSF results acquired before (pre-exercise) and after (post-exercise) the CPET till exhaustion in all studied athletes.

	Pre-exercise		Post-exercise		
Parameter	**Mean**	**SD**	**Mean**	**SD**	***P*-value**
B_{mean} [kFU]	404.19	214.31	455.30	256.76	< 0.001
$\mathrm{FI_{max}}$ [kFU]	480.98	258.19	514.46	282.99	< 0.001
$\mathrm{FR_{min}}$ [kFU]	321.22	168.90	359.07	196.50	< 0.001
I_{max} [kFU]	76.79	47.64	59.16	36.33	< 0.001
$\mathrm{IR_{ampl}}$ [kFU]	159.76	94.49	155.40	95.46	0.261
R_{min} [kFU]	82.97	50.22	96.24	65.98	0.001
$\mathrm{CI_{max}}$	0.48	0.08	0.40	0.11	< 0.001

Averaged data are presented as the mean and standard deviation (SD), and the results of the paired t-test as p-value. B_{mean}, mean of the basal fluorescence; CI_{max} – I_{max}/IR_{ampl} ratio; FI_{max} – maximal fluorescence value during ischemia; FR_{min} – the first minimal fluorescence value during reperfusion; I_{max} – the net increase of fluorescence over the baseline value during ischemia; IR_{ampl} – the amplitude of fluorescence change between ischemia and reperfusion; R_{min} – the net reduction of fluorescence below the baseline value.

Both in male and female athletes exercise to exhaustion cause similar changes in the FMSF response. After the CPET there were significant increases in B_{mean}, $\mathrm{FR_{min}}$ and R_{min}, and reductions in I_{max}, $\mathrm{CI_{max}}$. However, the value of $\mathrm{FI_{max}}$ increased significantly only in men but not in women. In both groups, no statistical differences were found for $\mathrm{IR_{ampl}}$.

In general, these findings on the skin fluorescence at 460 nm translate to a significant increase in the skin NADH content before and during ischemia, and the following reperfusion after the CPET both in male and female athletes. Additionally, the relative (compared to pre-ischemic baseline) increase in the skin NADH during ischemia (I_{max}) was significantly lower, and its reduction during reperfusion (R_{min}) was more substantial after the CPET. Compared to pre-exercise, the contribution of the NADH change during ischemia to the maximal change of its content during both ischemia and reperfusion ($\mathrm{CI_{max}}$) was also reduced after the CPET. Changes in I_{max}, R_{min}, and $\mathrm{CI_{max}}$ were similar in male and female athletes.

DISCUSSION

We have found that exercise until exhaustion caused a significant elevation of the 460 nm skin fluorescence at baseline (B_{mean}), during controlled ischemia ($\mathrm{FI_{max}}$) and reperfusion ($\mathrm{FR_{min}}$) compared to the pre-exercise values. However, the observed post-exercise increase of the 460 nm fluorescence during ischemia (I_{max}) was smaller than before exercise. In contrast, the magnitude of the fluorescence drop during the reperfusion (R_{min}) was larger after exercise than before exercise. Additionally, the relative contribution of the ischemia-induced increase in fluorescence to the whole change in fluorescence during ischemia and reperfusion ($\mathrm{CI_{max}}$) was reduced after exercise. Except $\mathrm{FI_{max}}$, all descriptors of the FMSF changed, similarly, in male and female athletes. The lack of significant increase (borderline $p = 0.0583$) in $\mathrm{FI_{max}}$ in women appears to be caused by a much smaller size group of studied female athletes. Altogether, our findings suggest that exercise to exhaustion causes a significant increment in the NADH content in mitochondria. However, the ischemia-related rise in NADH is smaller whereas the reperfusion-related oxidation of NADH is more intense after than before exercise. These effects of exercise appear to be similar both in sportsmen and sportswomen.

There is some balance between the reduced (NADH) and oxidized (NAD^+) forms of NAD. Although the 460 nm skin fluorescence measures only NADH, its values also reflect indirectly NAD^+ as the total amount of NAD (i.e., combined amount of NADH and NAD^+) seems to be rather stable at a relatively short period (a few minutes) necessary to perform the CPET. In other words, the NADH amount changes at the cost of the NAD^+ content, and vice versa.

Previous animal and human studies showed that the amounts of NADH and NAD^+ and the NAD^+/NADH ratio change during ischemia and reperfusion. Palero et al. (2011) studied the effects of hypoxia on NADH in mice keratinocytes. They observed that NADH accumulated in the complex I of the electron transport chain and, in contrast to common knowledge, keratinocytes

TABLE 4 | Comparison of the FMSF results acquired before (pre-exercise) and after (post-exercise) the CPET till exhaustion in male and female athletes.

Parameter	Men					Women				
	Pre-exercise		Post-exercise		*P*-value	Pre-exercise		Post-exercise		*P*-value
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
B_{mean} [kFU]	366.06	198.17	415.34	241.96	< 0.0001	536.95	218.97	594.42	262.69	0.0003
FI_{max} [kFU]	437.77	242.09	473.59	273.02	0.0003	631.43	260.33	656.79	275.53	0.0583
FR_{min} [kFU]	289.41	154.65	327.37	181.78	< 0.0001	431.97	172.41	469.39	209.13	0.0026
I_{max} [kFU]	71.71	47.21	58.24	38.70	< 0.001	94.46	45.66	62.36	26.87	0.0003
IR_{ampl} [kFU]	148.36	92.69	146.21	98.57	0.5933	199.46	91.49	187.38	77.03	0.2510
R_{min} [kFU]	76.65	49.00	87.97	65.70	0.0028	104.99	49.06	125.02	59.49	0.0025
CI_{max}	0.48	0.08	0.42	0.11	< 0.0001	0.47	0.05	0.34	0.09	< 0.0001

Averaged data are presented as mean and standard deviation (SD), and the results of the paired t-test as p-value. B_{mean}, mean of the basal fluorescence; CI_{max}, I_{max}/IR_{ampl} ratio; FI_{max} – maximal fluorescence value during ischemia; FR_{min} – the first minimal fluorescence value during reperfusion; I_{max} – the net increase of fluorescence over the baseline value during ischemia; IR_{mpl} – the amplitude of fluorescence change between ischemia and reperfusion; R_{min} – the net reduction of fluorescence below the baseline value.

metabolism is not only anaerobic since it strongly depends on the oxygen delivery by blood in the underneath skin layers. Balu et al. (2013) used a clinical multiphoton microscope, applied arterial occlusion to human keratinocytes and found that, during ischemia, the NADH content increased in the cells near the basal layer of the skin (although not in the keratinocytes closest to the skin surface). Our study shows similar results obtained in the living human skin in real time using a completely non-invasive physiological model. Because the fluorescence emitted by skin derives only from the most superficial zone of depth up to 0.1 mm (Balu et al., 2013), it means that skin cells at this depth have a vivid metabolism of NADH, and react in a dynamic way to ischemia-triggered hypoxia and then to re-oxygenation during reperfusion.

Other studies have shown that the amount of NADH and/or NAD^+ also changes during exercise (White and Schenk, 2012). Depending on the intensity, an exercise may be accompanied by an aerobic, mixed aerobic-anaerobic or purely anaerobic metabolism. During aerobic or oxygen-dependent metabolism, various nutrients are turned into energy in mitochondria. When oxygen supplies are lower than needed, anaerobic processes start in the cytoplasm and with time predominate over aerobic energy production.

With progressing hypoxia until anoxia, the cellular metabolism switches to entirely anaerobic processes which are accompanied by various changes in the intra- and extracellular milieu (White and Schenk, 2012). The most classic consequences within the cells and between the intra- and extracellular spaces are changes in the concentration of H^+, lactate, ammonium, adenosine, and ions such as sodium, potassium, calcium, magnesium, and chlorides. Many metabolic effects of ischemia resemble those observed during anaerobic exercise. In both conditions, when oxygen is depleted, the NAD^+ particles are partially restored from NADH in the cytosol by reduction of pyruvate acid to lactic acid, with an accumulation of the latter as the by-product (Robergs et al., 2004; Finsterer, 2012; Kane, 2014). However, this way of energy generation is not as efficient as NADH oxidation in the mitochondrial electron transport chain during the aerobic effort – less ATP is made at the cost of higher nutrient usage and due to the accumulation of lactate.

Exercise until exhaustion alters the NAD^+/NADH ratio and, most probably, the rate of NADH oxidation to NAD^+. However, data on NAD^+ during exercise are sparse (White and Schenk, 2012). Whereas some researchers observed an exercise-induced increase in NAD^+ (Chance and Connelly, 1957; Jobsis and Stainsby, 1968), others found no change or reduction in its amount (Duboc et al., 1988; White and Schenk, 2012). Duboc et al. (1988) reported that it is NADH that increases during exercise. Other authors noticed that untrained animals had a higher concentration of mitochondrial NAD^+ than trained ones (Edington, 1970; Edington and McCafferty, 1973). Sahlin (1985) studied NADH in human muscle during short-lasting intensive exercise and found that at least 95% of the cellular NADH comes from mitochondria. This author also observed that NADH increases within 2 min after exercise initiation and it remains elevated during and immediately after the exercise completion. Additionally, 10 min is required for the NADH to return to baseline level after the exercise. Next, Sahlin et al. (1987) studied NADH in muscle samples taken after the exercise at the level of 40, 75, and 100% of VO_2max. The light exercise at the intensity of 40% VO_2max was accompanied by a decrease in the NADH content. In contrast, more intense exercise at the level of 75 and 100% VO_2max caused a significant increase in the NADH content. Graham et al. found that the NAD^+ content in human muscles decreased after moderate (75% of VO_2max) and high intensity (100% of VO_2max) exercise (Graham et al., 1978; Graham and Saltin, 1989). Stabilization of metabolic processes requires some time and depends on several factors, e.g., the active recovery (Menzies et al., 2010) and the level of physical performance (Ravier et al., 2006). Few minutes after intensive exercise is not enough for full metabolic recovery, including the complete removal of an excess of H^+ or full restoration of the aerobic metabolism. It is plausible that one of the main limiting factors for a rapid scavenging of the accumulated NADH during exercise is the system of the malate-aspartate shuttle (O'Donnell et al., 2004; White and Schenk, 2012; Satrústegui and Bak, 2015) that transfers H^+ from cytosol to the mitochondrial matrix

and enables NAD oxidation and reduction. It appears that the turnover rate of the mitochondrial shuttles is rather constant, or even reduced under specific conditions, and thus some time is required to transfer the excess of H^+ from cytosol into mitochondria (O'Donnell et al., 2004). Simultaneously, intact NAD can penetrate the mitochondrium using a still unrecognized NAD or NADH transporter (Davila et al., 2018). We assume, however, that this transporter has a limited capacity.

So far, NADH metabolism has been studied either during ischemia/reperfusion (hypoxia/normoxia) or in various exercise models, mostly with the use of invasive methods. To the best of our knowledge, none of the studies has ever shown the combined effect of exercise and ischemia-reperfusion on NADH, additionally using a completely non-invasive approach for measurement of NADH. In our study, the absolute fluorescence values of B_{mean}, FI_{max}, and FR_{min} were significantly higher after exercise than before it. This increase in fluorescence might be the net effect of the accumulation of the pre-exercise and newly produced NADH during the maximal exercise, particularly during the anaerobic part of the effort. The additional potential explanation is that the altered post-exercise metabolic conditions slowed the function of the MAS and also other shuttles, transporting H^+ to electron transport chain. The MAS activity can also be attenuated and even stopped by an increase of Ca^{2+} concentration in the cytoplasm (O'Donnell et al., 2004; Contreras and Satrústegui, 2009; Satrústegui and Bak, 2015), i.e., a common consequence of an anaerobic effort.

Post-exercise reduction of the I_{max} below the pre-exercise level is an interesting finding. The I_{max} corresponds to the amount of NADH that is directly generated during skin ischemia. We speculate that an excess of H^+ and electrons generated during the anaerobic part of the exercise saturated a substantial part of the NAD^+ and turned it into NADH (an increase of B_{mean} and FI_{max}). However, since the total amount of NAD in the form of NADH and NAD^+ is more or less constant in the cells in a short time frame, then less NAD^+ particles become available for the reduction to NADH during the post-exercise ischemia. It might also be possible that there are some intracellular protective mechanisms preventing an endless intracellular accumulation of NADH as in our model of addictive effects of exercise and ischemia. At the same time, an increase of the R_{min} value suggests that in the early phase of post-exercise reperfusion more NADH is oxidized to NAD^+ or some mechanisms promoting more rapid regeneration of NAD^+ are activated. Further, it appears that after exercise the limitation of the NADH increase during ischemia and the enhancement of the NADH oxidation to NAD^+ during reperfusion are comparable to those observed at rest. It shows that the responses of the NAD^+/NADH to ischemia and reperfusion are set at the specific individual range different for each person. Additionally, it suggests that there is a relative resistance of cells to produce an unlimited amount of NADH during such a dramatic metabolic challenge as maximal exercise.

Since the 460 nm fluorescence of the skin increases after exercise, it is the NADH that raises during and after maximal exercise. However, we measured the skin and not muscle NADH metabolism. Due to a significant contribution of skin in thermoregulation, it is plausible that the NADH changes in the skin might be not the same as in the muscles. With increasing intensity and duration of exercise, skin blood flow becomes relatively reduced as most of the blood is redirected toward working muscles, however, immediately after the exercise, skin blood vessels dilatation takes place. Despite a sudden influx of blood to the skin, and connected oxygen supply, other metabolic processes do not allow for quick restoration of homeostasis. It is possible that during exercise to exhaustion, there is some overlap and additive effect of metabolic changes in the muscles caused by anaerobic processes and relative skin ischemia due to some constriction of skin arteries. Nevertheless, it is assumed that mitochondrial function is generally the same in different types of cells, and thus changes observed in the mitochondria of skin cells should be, at least to some extent, similar to changes in mitochondria of the working muscles myocytes. In our study, we did not take into consideration the effect of factors acting over a longer period of time, such as sirtuins, PARP and CD38, because the measurement using FMSF method was of short duration and, consequently, the total NAD pool remained unchanged. We also did not investigate the effect of exercise training on change in NADH, but only the response to a single exercise bout (test until exhaustion).

Limitations of the study must be recognized. To study the NADH metabolism we used the continuous measurement of the autofluorescence by skin cells activated by the ultraviolet light. The application of the 460 nm fluorescence as a way of measuring NADH has been known for years. The group of Mayevsky made a series of seminal studies on the practical use of measuring the NADH content in skin cells or superficial cells of other organs (Mayevsky and Chance, 2007; Mayevsky et al., 2011) in humans. The FMSF method is further, although very specific, development of this approach, which has been proposed by Piotrowski et al. (2016). In the FMSF method, the 460 nm skin fluorescence is not only measured at rest but also during a dynamic metabolic challenge caused by the transient and controlled ischemia, and the following reperfusion. A potential limitation is also a heterogenic group of athletes. All of them are elite athletes at the national or international level representing different sports and adapted to different training types: endurance, speed, strength, speed-power, etc. However, we used the identical CPET test with the same goal – to stop it after exhaustion which is very individual and subjective. In this way, we were able to collect a wide range of VO_2max, resting and peak values of heart rate in healthy and physically active people. Next, our study was somewhat more exploratory than explanatory. We had a single and simple aim – to see whether maximal exercise-induced alterations in cell metabolism may influence the NADH metabolism of skin cells during ischemia and reperfusion. We were more interested in the combined effect of both provocations. Our data show some relevant alterations in the NADH during both processes and suggest that non-invasive FMSF method and the model of skin cells can be applied in the studies on physical exercise. It should also be mentioned that one of the limitations of the 460 nm fluorescence method is skin color of studied athletes. Although our participants came from the Caucasian ethnic group, there were significant differences in skin coloration which, even in the same person, can be modified

for example by sunbathing or tattooing. To limit these effects, we used paired statistical tests to compare the same parameters before and after exercise, and in this way, each person was compared with himself or herself. No measurement of NADH in the blood appears to be yet another limitation of our study. However, NADH is mainly present intracellularly, and it is an unstable and highly reactant particle (Singhal and Zhang, 2006). Moreover, NADH is degraded to nicotinamide by the extracellular NAD(P) nucleosidases (Johnson, 1984; O'Reilly and Niven, 2003). Nicotinamide is a final end-product of degradation of NADH and NAD^+ but also NADPH and $NADP^+$, so measurement of its changes is not specific for NADH only. There is also another technical problem – during complete brachial artery occlusion used for the controlled ischemia, there is no blood flow what makes a collection of blood samples impossible. The blood can be sampled only before ischemia and during the post-ischemic reperfusion but, as we show, the NADH concentration decreases within a couple of seconds after the restoration of the blood flow, i.e., before the blood might be even sampled. Nevertheless, we believe that studying the NADH concentration in the blood before and after ischemia at different conditions like rest and post-exercise deserves further exploration. Although all studied participants were elite athletes, this group was quite heterogenic and representing different sorts of sports. However, we did not find any significant differences between the included sports disciplines and obtained results. Since the number of athletes representing different sports disciplines varied from 1 to 41, we cannot exclude that there are some potential differences, which might be revealed if larger groups of athletes were studied. Finally, we have not measured the skin NAD^+ content as this form of NAD does not emit the fluorescent light at the length of 460 nm. Any conclusions on NAD^+ come from the assumption that the total amount of NAD in its reduced (NADH) and oxidized (NAD^+) forms is rather stable over short time. If so, then any change in the NADH is at the cost of NAD^+ and vice versa. In consequence, although NAD^+ was not measured, some conclusions on its changes may be drawn from studying the alterations of the NADH content.

Perspectives or Practical Applications

There are some instant potential consequences of our findings. First, the exercise-induced changes in NADH reflect mitochondrial function and a significant part of energy metabolism – these aspects are of great importance in the sports physiology. Second, it is highly probable that the observed post-exercise changes in the skin NADH content reflect metabolic alterations in the myocytes, and, if so, we assume that the FMSF might be used as a non-invasive and an *in vivo* approximate model of the working muscles. In this way, studying the effects of training on NADH metabolism appears to be simpler and more available now. Third, the anaerobic muscle metabolism during exercise to exhaustion is similar to tissue hypoxia which can be observed in different clinical conditions like chronic heart failure, cardiogenic shock, anemia, acute limb ischemia or high mountains environment. Any pharmacological or non-pharmacological intervention designed to improve oxygen delivery or tissue tolerance to hypoxia might be thus easier to study with the FMSF method. Similarly, any intervention designed to change the total amount of NAD content might be easily studied with this method. We are, however, aware, that these consequences are only some speculations as we have not tested them. Therefore they require future studies.

CONCLUSION

This is the first study which non-invasively evaluated the NADH skin content in human superficial skin cells in highly trained athletes. Until now methods used to evaluate NADH level, and therefore mitochondrial function, were not easily accessible. Metabolic changes, elicited by exercise to exhaustion, modify the skin NADH metabolism at rest, during ischemia and reperfusion in the most superficial living skin cells. Immediately after exercise, there is a shift of the baseline fluorescence of NADH in the skin cells toward higher values. The absolute NADH amount increases during post-exercise ischemia and reperfusion, compared to resting condition. However, compared to resting conditions, the relative rise in the NADH is significantly lower during ischemia, whereas the relative reduction in the NADH during reperfusion increases. These changes in the NADH metabolism during ischemia and reperfusion before and after exercise to exhaustion appear to be similar in male and female athletes.

The observed alterations in the NADH amount and its balance with NAD^+ during ischemia and reperfusion are strongly dependent on metabolic conditions, which are significantly modified by exercise to exhaustion, and last for the next few minutes after it. The intensification of NADH fluorescence in living skin cells suggests that metabolic changes in NADH accompanying exercise extend beyond muscles and affect other cells and organs.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of the Poznań University of Medical Sciences in Poland with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Poznań University of Medical Sciences in Poland (no. 1017/16 issued on the 5th October 2016).

AUTHOR CONTRIBUTIONS

OB designed the study, recruited and tested participants, analyzed and interpreted data, performed literature research, prepared the manuscript. JZ contributed to the study conception and design, recruited and tested participants, analyzed and interpreted data, performed literature research, prepared and revised the manuscript. KK contributed to the study conception and design, recruited and tested participants, analyzed and interpreted data, revised the manuscript. AK recruited and tested participants, revised the manuscript. DW tested participants,

contributed to data analysis and interpretation. PG contributed to the study conception and design, performed data and statistical analysis and interpreted results, performed literature research, prepared and revised the manuscript.

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applied sciences

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Article

The Effect of a 7-Week Training Period on Changes in Skin NADH Fluorescence in Highly Trained Athletes

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Abstract: The study aimed to evaluate the changes of nicotinamide adenine dinucleotide (NADH) fluorescence in the reduced form in the superficial skin layer, resulting from a 7-week training period in highly trained competitive athletes (n = 41). The newly, non-invasive flow mediated skin fluorescence (FMSF) method was implemented to indirectly evaluate the mitochondrial activity by NADH fluorescence. The FMSF measurements were taken before and after an exercise treadmill test until exhaustion. We found that athletes showed higher post-training values in basal NADH fluorescence (pre-exercise: 41% increase; post-exercise: 49% increase). Maximum NADH fluorescence was also higher after training both pre- (42% increase) and post-exercise (47% increase). Similar changes have been revealed before and after exercise for minimal NADH fluorescence (before exercise: 39% increase; after exercise: 47% increase). In conclusion, physical training results in an increase in the skin NADH fluorescence levels at rest and after exercise in athletes.

Keywords: nicotinamide adenine dinucleotide; training; athletes; mitochondrion

1. Introduction

Skin microcirculatory function and efficiency of blood supply to the skin can impact mitochondrial activity and the changes of nicotinamide adenine dinucleotide (NADH) fluorescence in the reduced form [1]. Mitochondrial function can be indirectly evaluated by NADH fluorescence [1] that has been measured in animals [2,3] and humans [1,4] at rest and under various conditions (e.g., ischemia and temperature changes). Bugaj et al. [5] were the first to describe the time course of NADH changes in the skin in athletes at rest and after exercise. In their study, a new method of evaluating NADH fluorescence—flow mediated skin fluorescence (FMSF)—was utilized. The FMSF method is based on the ability of NADH to autofluorescence. The fluorescence measured using the FMSF method reflects the dynamics of in vivo changes in NADH levels in most superficial layers of the skin [5–7]. Bugaj et al. [5] have shown that exercise to exhaustion induces changes in skin NADH fluorescence, in other words, the values recorded after exercise were higher than those before exercise (increase in: basal NADH fluorescence 13%, maximal NADH fluorescence 7% and minimal NADH fluorescence 12%).

Nicotinamide adenine dinucleotide (NAD) is synthesized in the cytosol, mitochondria, and nucleus. This molecule is active in the cytoplasm during glycolysis and in the mitochondria during oxidative

phosphorylation when adenosine-5′-triphosphate (ATP) is produced [8]. NAD occurs in two forms: oxidized NAD^+ and reduced NADH. NAD takes part in many biological reactions including electron transport. The reduction of NAD^+ to NADH occurs almost exclusively in the mitochondria at the final stage of cellular respiration [9,10].

In the human body, there is a pool of NAD that takes reduced (NAD^+) and oxidized (NADH) forms, transforming into each other [8]. Importantly, the NAD pool is only constant for relatively short periods [8,11]. In the long term, the NAD amount changes depending on several factors such as age, diet, physical activity, medicaments, boosters, time of the day, etc. [11]. NAD^+ metabolism is complex and includes many NAD^+-consuming pathways as well as de novo and salvage pathways [8].

Mayevsky and Barbiro-Michaely [1] have claimed that the monitoring of the NADH level in tissue provides important information about the mitochondrial metabolic state (energy production, amount of intracellular oxygen). In addition, changes in the NAD^+/NADH ratio reflect cellular respiration processes in mitochondria, thus indirectly represent their function [1,5]. Studies on changes in NADH in response to physical exercise were performed on animal and human skeletal muscle samples, but not in the skin [8,9,12]. Early reports including animals did not provide a clear answer as to how NADH levels were modified by exercise [13,14]. Subsequent human research had shown that intensive exercise, unlike light exercise, shifted the NAD^+/NADH balance toward NADH [8,15]. Only Koltai et al. [16] have examined the influence of endurance training on changes in NAD^+ level in rat muscles and showed that training resulted in an increase in NAD^+ biosynthesis.

Studies on skeletal muscle mitochondria are valuable, but usually invasive due to the use of the biopsy technique [17,18] and expensive if transmission electron microscopy is used [19]. However, it has been suggested that physical exercise brings beneficial changes not only in skeletal muscle mitochondria, but also in skin mitochondria [20]. It has been demonstrated that physical exercise results in several beneficial mitochondrial adaptations [19,21–25]. Various changes were extensively studied in skeletal muscle mitochondria [19,21,25–27], while only one study dealt with the changes in the skin [20]. However, we do not know whether training only affects muscle mitochondria, or the adaptations also take place in skin mitochondria that are easily accessible to study because they lie superficially.

To the best of our knowledge, there is a lack of studies describing the effect of physical training on changes in NADH fluorescence in the skin. The novel, noninvasive, and cheap flow mediated skin fluorescence method can be a source of valuable information about the mitochondrial activity. Therefore, the study aimed to evaluate the changes in NADH fluorescence in the superficial skin layer resulting from a 7-week training period in highly trained competitive athletes. We hypothesize that physical training results in an increase in the NADH fluorescence levels in athletes.

2. Materials and Methods

2.1. Subjects

Forty-one highly trained athletes (28 men, 13 women), ages ranging from 18 to 35 years, participated in the study. They were members of the Polish national team or athletes taking part in national and international competitions. They represented the following sport disciplines: triathlon (Olympic distance: 1.5 km swim, 40 km bike ride, 10 km run) (seven men, four women); long-distance running (5 km, 10 km, and marathon) (six men, two women); Olympic taekwondo (six men, one woman); sprint (100 m, 200 m, and 4 × 100 m relay) (six men, one woman); canoeing (three men); and fencing (five women). Before starting the study, each participant was informed about the aim and procedures, potential risks, and the possibility to withdraw at any time without giving any reason. All athletes gave their written consent to participate in the examinations and fulfilled a questionnaire on their health status and potential contraindications. All athletes had valid health certificates issued by a physician who specialized in sports medicine, thus were eligible for training and competition. Exclusion criteria were illness symptoms, injuries, and taking drugs (temporarily or chronically). Only the data of those athletes who were present at both examinations was analyzed. The study was conducted in accordance

with the Declaration of Helsinki. The Ethics Committee of the Poznan University of Medical Sciences in Poland approved the study protocol (approval no. 1017/16 issued on 5 October 2016).

2.2. Training Characteristics

All participants attended training sessions at least six times a week. During the whole 7-week period under study (general preparation phase of the one-year cycle), the athletes had on average 57 training sessions of a total duration of 71.2 h. The average duration of a single session was 84 min.

2.3. Study Design

The study was conducted in the Human Movement Laboratory of the Department of Athletics, Strength and Conditioning at the Poznan University of Physical Education (Poznań, Poland). Athletes arrived at the laboratory in the morning. During all measurements, the constant temperature was maintained (20-21 °C) by an air conditioning system. On the day of the examination, the participants could only eat a light breakfast. It was also recommended for them to avoid coffee or tea for 12 h, alcohol for 24 h, and hard exercise for 48 h before each examination. After arriving, athletes changed into their lightweight sports clothing (without watches and wristbands potentially affecting blood flow) and acclimatized to the laboratory conditions for at least 30 min. During this time, they completed the required questionnaires, and height and weight measurements were performed.

Athletes underwent the examinations twice: at the beginning of the general preparation phase and after seven weeks, at the end of this phase. Each time, the same procedure was applied: (1) initial resting blood pressure measurement; (2) resting NADH fluorescence measurement; (3) blood draw, (4) incremental exercise test; (5) second blood draw; (6) post-exercise blood pressure measurement; and (7) post-exercise NADH fluorescence measurement (3 min after the end of the test).

2.4. Incremental Exercise Test

The exercise test was conducted on the H/P Cosmos treadmill (h/p/cosmos sports & medical GmbH, Nussdorf – Traunstein, Germany). All participants were familiar with the treadmill test because they regularly (2-3 times a year) participated in similar tests. The purpose of this examination was to assess maximal oxygen uptake (VO_2max) and peak heart rate (HR).

Respiratory gases were collected and analyzed using the MetaMax 3B ergospirometer (Cortex Biophysik BmbH, Leipzig, Germany) and the MetaSoft Studio 5.1.0 software (Cortex Biophysik BmbH, Leipzig, Germany). The exercise protocol started with a 4-min warm-up at the treadmill speed of 6 km/h. Then, the treadmill accelerated by 2 km/h every 3 min. The treadmill inclination was 1% throughout the whole test. The test terminated if the athlete signaled his/her voluntary exhaustion by raising one hand. Maximal oxygen uptake was considered to be reached if the oxygen uptake (VO_2) was stabilized despite the further increase in treadmill speed. All participants were highly trained, so during the test, all of them reached a plateau in VO_2 uptake. We also checked three additional conditions to confirm reached maximal oxygen uptake: (i) HR reached at least 95% of the age-adjusted HR; (ii) cutoff blood lactate concentration ≥ 9 mmol/L for man and ≥7 mmol/L for women; and (iii) respiratory exchange ratio was ≥1.1 [28]. Heart rate was measured using the Polar H6 Bluetooth Smart monitor (Polar Electro Oy, Kempele, Finland) attached to a chest strap.

2.5. Lactic Acid Measurements

Capillary blood samples were obtained from the fingertip at rest and 2 min after the exercise test. A total of 20 µL of whole blood was drawn to a micro test tube using a capillary. Biosen C-line (EKF Diagnostics, Cardiff, UK) was used to measure the level of lactate.

2.6. Anthropometric Measure

Anthropometric measurements were performed according to standardized procedures. Body mass (kg) and height (cm) were measured with a digital measuring station Seca 285 (SECA, Hamburg, Germany). Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2).

2.7. Nicotinamide Adenine Dinucleotide Fluorescence

NADH fluorescence was measured using the AngioExpert device (Angionica, Łódź, Poland, 2016) based on the flow mediated skin fluorescence (FMSF) method. FMSF enables recording of the changes in NADH fluorescence as a function of time in response to ischemia and reperfusion in forearm skin cells. During the measurement, AngioExpert emits light at the wavelength of 460 nm [6,7]. NADH molecules have autofluorescence capability at a wavelength of 460 nm [9]. The changes in fluorescence intensity observed during the examination are produced in the most superficial skin cells (epidermis) [6,29], which is due to very shallow skin penetration by excitation light at the wavelength of 340 nm. About 90% of the recorded signal comes from the skin depth up to 0.5 mm. The activated skin region is not directly supplied with blood, but is supplied with oxygen by deeper blood vessels [6,7,29].

During the examination, each participant sat on a chair with his/her arm resting on the measuring device. Immediately before examination, systolic (SBP) and diastolic (DBP) blood pressure was measured using the Omron 3 (Omron, Kyoto, Japan) device. At the start of the FMSF examination, basal fluorescence was registered for 2 min. Then, an occlusion cuff was inflated up to the pressure of 50 mmHg above the SBP for 200 s. After this time, blood flow in the forearm was restored (cuff deflated) and the changes in NAD fluorescence were recorded for a further 3 min [7].

The following parameters related to NAD fluorescence were measured or calculated (Figure 1):

- B_{mean}—Basal fluorescence at the wavelength of 460 nm, recorded at rest at the beginning of the measurement;
- FI_{max}—The maximal increase in fluorescence above the baseline observed during forearm ischemia;
- FR_{min}—The maximal drop in fluorescence below the baseline observed during reperfusion;
- I_{max}—The relative increase in fluorescence = the difference between I_{max} and B_{mean};
- R_{min}—The relative drop in fluorescence = the difference between B_{mean} and FR_{mean};
- IR_{ampl}—The maximal range of changes in fluorescence = the sum of R_{min} and I_{max}; and
- CI_{max}—The relative (percentage) contribution of I_{max} to IR_{ampl} [7].

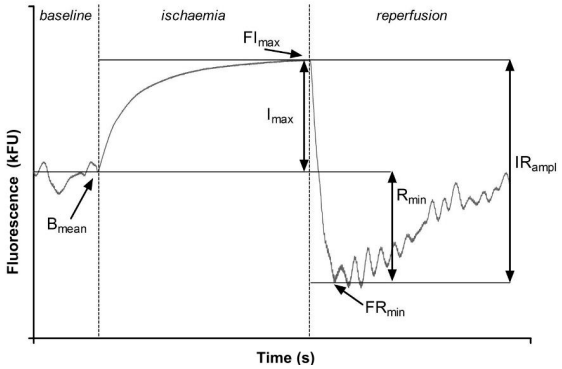

Figure 1. Parameters describing the Flow Mediated Skin Fluorescence. B_{mean}—Mean value of the basal fluorescence; FI_{max}—Maximal fluorescence during ischemia; FR_{min}—The first minimal fluorescence value during reperfusion; I_{max}—The net increase in fluorescence over the baseline during ischemia; IR_{ampl}—The amplitude of fluorescence change during ischemia and reperfusion; R_{min}—The net reduction in fluorescence below the baseline. Reprinted from Bugaj et al. [5].

The second measurement was made according to the same methodology, 3 min after the end of the treadmill test. A sample measurement of the NADH fluorescence from a 23-year-old male sprinter before and after training was shown in Figure 2.

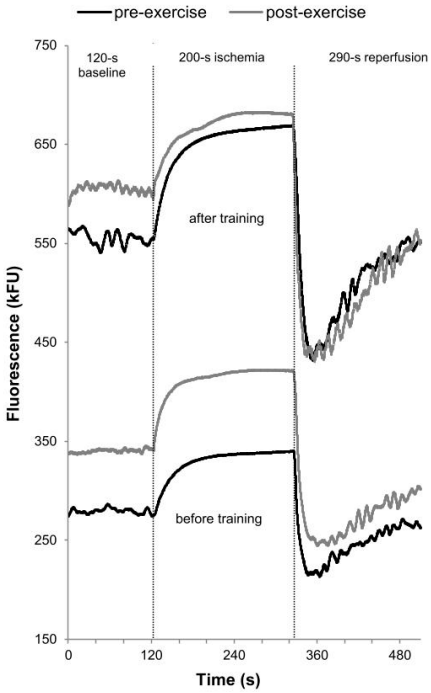

Figure 2. A sample Flow Mediated Skin Fluorescence measurement in a 23-year-old male sprinter. Changes in nicotinamide adenine dinucleotide fluorescence are shown before and after 7-weeks of training, at rest, and after cardiopulmonary exercise test until exhaustion. The first 2 min serve to determine the baseline fluorescence level. This was followed by a 200-s ischemia (increase in fluorescence) and a 290-s reperfusion (decrease in fluorescence).

3. Results

3.1. Basic Characteristics

The resting DBP, SBP, and BMI were within normal ranges. Other descriptive characteristics are presented in Table 1.

Table 1. Basic characteristics of the studied athletes.

Parameter	Before Training	After Training
Age (years)	22.4 ± 4	–
Training experience (years)	8 ± 2.3	–
Height (cm)	178.1 ± 7.3	178.1 ± 7.3
Weight (kg)	69.1 ± 10.3	69 ± 10.3
BMI (kg/m^2)	21.6 ± 2.3	21.6 ± 2.3
SBP_{rest} (mmHg)	127.6 ± 14.3	119.3 ± 10.8 ***
DBP_{rest} (mmHg)	69.9 ± 7.3	72.9 ± 9.3 *
SBP_{exerc} (mmHg)	148 ± 18.3	139.2 ± 16.3 **
DBP_{exerc} (mmHg)	74.5 ± 8.1	78.2 ± 8.1 *
VO_2max (mL/min/kg)	58.8 ± 8.6	59.5 ± 8.6
HR_{peak} (beats/min)	191.7 ± 8	191.9 ± 8.9
LA_{rest} (mmol/L)	1.2 ± 0.5	1.0 ± 0.3 **
LA_{max} (mmol/L)	9.9 ± 1.5	10.2 ± 1.9

Averaged data are presented as mean ± standard deviation (SD), and results of the *t*-test for dependent samples, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different pre-training. BMI−body mass index; SBP−systolic blood pressure; DBP−diastolic blood pressure; rest−before cardiopulmonary exercise test; exerc−after cardiopulmonary exercise test; VO_2max (mL/min/kg)−maximal oxygen uptake; HR_{peak}−peak heart rate; LA_{rest}−resting lactate concentration; LA_{max}−maximal lactate concentration.

3.2. Measured Parameters

The values of the measured parameters are shown in Figure 3. At the first examination (before the training period), only B_{mean} significantly increased between the pre- (410.8) and post-exercise (449.3) measurements. At the second examination (after the training period), the values of all measured parameters significantly increased between resting and post-exercise condition. B_{mean} increased from 579.5 to 671.9, 16%; FI_{max} increased from 685.8 to 742.4, 8% and FR_{min} from 459.1 to 520, 13%. All measured parameters (both resting and post-exercise) significantly increased between the first and second examination.

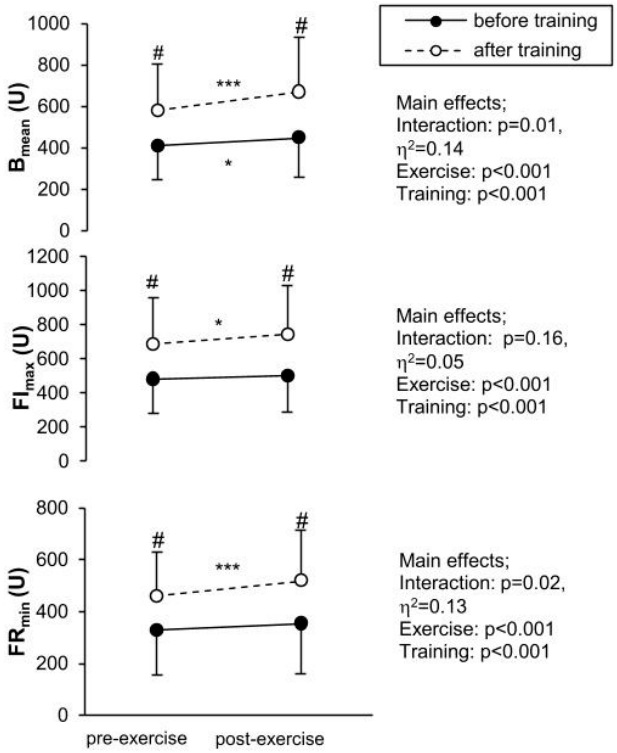

Figure 3. Measured parameters. Flow Mediated Skin Fluorescence parameters in athletes (N = 41) in two examinations, before and after the cardiopulmonary exercise test until exhaustion. B_{mean}—Changes in the mean value of the basal fluorescence; FI_{max}—Changes in maximal fluorescence during ischemia; FR_{min}—Changes in the first minimal fluorescence value during reperfusion. Values are means (SD). A two-way analysis of variance (relation between exercise and training), post-hoc Scheffe test, significant differences between pre- and post-exercise: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; significant differences between before and after training $^{\#}$ $p < 0.001$, ‡ $p < 0.01$, § $p < 0.05$.

3.3. Calculated Parameters

The values of the calculated parameters are presented in Figure 4. I_{max} significantly decreased after exercise in both pre- (from 72.6 to 53.9, 26% decrease) and post-training (from 106.3 to 70.6, 34% decrease) examinations. I_{max} values were higher after than before training pre- (from 72.6 to 106.3, 46% increase) and post-exercise (from 53.9 to 70.6, 31% increase).

R_{min} significantly increased after exercise compared to resting conditions in both examinations before (from 80.3 to 94.7, 18% increase) and after training (from 120.4 to 151.9, 26% increase). The pre- and post-exercise values of R_{min} were higher after than before training (pre-exercise 50% and post-exercise 60%).

The IR_{ampl} parameter did not significantly differ between resting and post-exercise conditions in both examinations. Its pre- and post-exercise values were significantly higher after than before the training period (pre-exercise from 152.9 to 226.7, 48% increase; post-exercise from 148.6 to 222.4, 50% increase).

The values of CI_{max} were significantly lower after than before exercise in both examinations (before training decreased from 0.5 to 0.4; after training decreased from 0.5 to 0.3). There were no differences observed before when compared to after training.

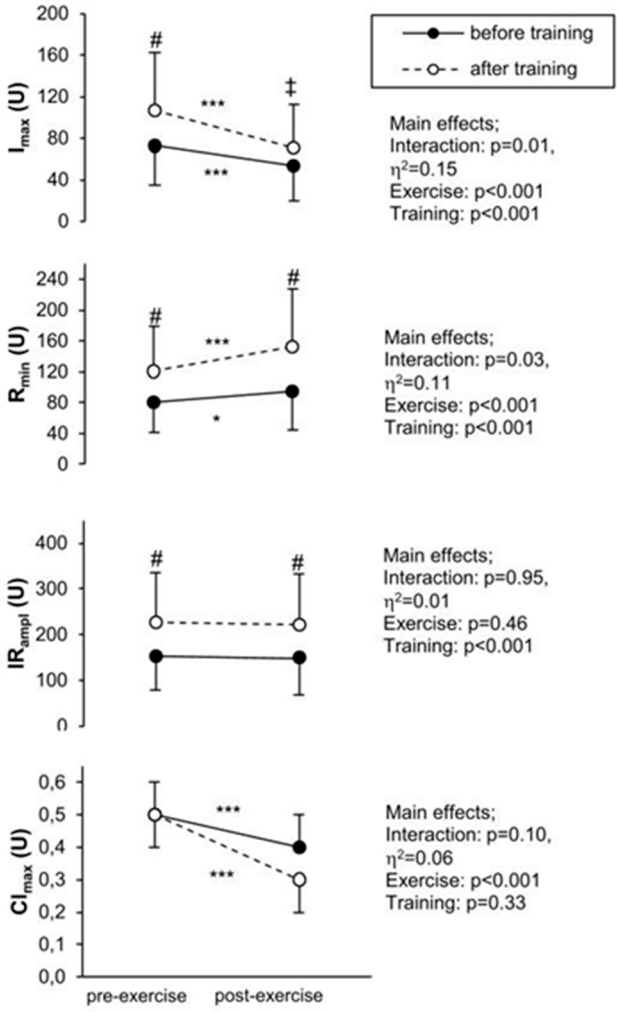

Figure 4. Calculated parameters. Flow Mediated Skin Fluorescence parameters in athletes (N = 41) in two examinations, before and after the cardiopulmonary exercise test. I_{max}—Changes in the net increase in fluorescence over the baseline during ischemia; IR_{ampl}—Changes in the amplitude of fluorescence change during ischemia and reperfusion; R_{min}—Changes in the net reduction in fluorescence below the baseline; CI_{max} – Changes in I_{max}/IR_{ampl} ratio. Values are means (SD). A two-way analysis of variance (relation between exercise and training), post-hoc Scheffe test, significant differences between pre- and post-exercise: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; significant differences between before and after training $^{\#}$ $p < 0.001$, ‡ $p < 0.01$, § $p < 0.05$.

4. Discussion

In this study, for the first time, the changes in NADH fluorescence in epidermal cells have been investigated in highly trained athletes before and after a training period. The main and novel finding is a significant increase in NADH fluorescence after training.

4.1. The Effect of Training

In our study, an increase in NADH fluorescence after a 7-week training period in highly trained athletes was observed. It is widely known that physical training induces several adaptations including mitochondrial adaptations [22]. The measurement of NADH fluorescence may be used

to indirectly evaluate the mitochondrial function and information about its metabolic status [1,5]. However, the changes in NADH fluorescence alone do not allow us to answer the question of what particular metabolic changes took place. It is known that NAD^+ and NADH are in balance (i.e., the more NAD^+, the less NADH and vice versa [8]). Therefore, the higher post-training NADH fluorescence shown in our study may indicate increased NAD turnover.

Our participants represented different sport disciplines, but the study was only conducted in the general preparation period of the annual training cycle. The main goal of this period, regardless of sports discipline, was the development of endurance capacity. VO_2max did not change after training in our athletes, which is in line with other reports [30,31] that also did not observe such changes in highly trained athletes in an annual training cycle. However, we assume that the changes occurred at the cellular respiration level. The endurance-dominant training in all athletes significantly affected the increase in the NADH fluorescence, which can be reflected by the changes in mitochondrial functions as shown in measured NADH parameters (B_{mean}, FI_{max}, FR_{min}). The post-training increase in B_{mean}, FI_{max}, and FR_{min} suggests a training-induced increase in the total NAD pool. However, there is a lack of research on training-induced changes in skin mitochondria. We can only compare our findings with those obtained from muscle mitochondria. To the best of our knowledge, the only research on training-related changes in NAD levels was performed on rat muscles. It has been found that NAD levels increased in response to endurance training [16]. There is a lack of studies on NAD changes in trained humans. The training-related changes in mitochondria have been widely described in human muscles [19,21,22,25,32]. The training-related changes in the mitochondria are probably connected with the improvement in mitochondrial biogenesis and the removal of dysfunctional mitochondria [21,22,25,32]. After training, an increase was observed in the levels of proteins related to mitochondrial biogenesis [21,25] and an improvement in mitochondrial respiratory function [19]. It is suggested that the profile of the mitochondrial changes depends on training intensity and volume. Training volume seems to affect mitochondrial content, whereas training intensity is correlated with the improvement in mitochondrial respiration [19]. It must be remembered that exercise does not necessarily imply exactly the same metabolic changes in muscle and skin mitochondria. However, intense physical activity affects mitochondrial activity and induces an increase in NADH fluorescence, which we have shown in our previous study [5]. Therefore, the observed increase in NADH fluorescence after 7-weeks of training may indirectly indicate adaptive changes in skin mitochondria.

4.2. Exercise Response

In our recent paper [5], we showed that a single bout of exercise until exhaustion induced a significant increase in skin NADH fluorescence. The results of this study are in line with our previous observations. We found that the I_{max} parameter, related to fluorescence intensity, decreased after exercise and that the R_{min} parameter increased after exercise. The likely explanation is that with limited aerobic metabolism, NADH is accumulated and the NAD^+ amount decreases because anaerobic metabolism does not allow for restoring NAD^+ from NADH to a sufficient extent [33–35].

However, some authors [36] suggest that the decrease in NADH fluorescence intensity during reperfusion not only shows the change in mitochondrial function, but also in microcirculatory and endothelial functions related to the efficiency of blood supply to the skin. Both the skin blood vessels' thermoregulatory [37–39] and endothelial [40] functions improved after training. Our study supports this view and suggests improvements in exercise tolerance based on NADH fluorescence measurement.

4.3. Practical Application

The FMSF method might be useful to evaluate metabolic adaptations related to mitochondrial function and/or microcirculatory function as the effect of training (training efficiency). This might also be used to observe the recovery after exercise when returning to the resting NADH values.

5. Conclusions

Athletes showed significant changes in NADH fluorescence in skin cells after a 7-week training period. We found that they achieved higher post-training values in basal NADH fluorescence (B_{mean}) (pre-exercise 41% increase and post-exercise 49% increase). Additionally, the maximal increase in fluorescence during occlusion (FI_{max}) and the maximal drop in fluorescence after reperfusion (FR_{min}) were higher at rest and post-exercise after training (FI_{max} 42% at rest, and 47% post-exercise, FR_{min} (39% at rest, and 47% post-exercise). In conclusion, physical training results in an increase in the skin NADH fluorescence levels at rest and after exercise in highly trained athletes. We suggest that the measurements can reflect the training-induced changes in the metabolic status of the skin mitochondria.

Author Contributions: Conceptualization, O.B. and J.Z.; Methodology, J.Z., K.K., and O.B.; Software, J.Z.; Validation, J.Z. and O.B.; Formal analysis, J.Z., D.W., and O.B.; Investigation, O.B., J.Z., D.W., K.K., A.K., and P.K.; Resources, K.K. and J.Z.; Data curation, J.Z. and O.B.; Writing—original draft preparation, O.B.; Writing—review and editing, K.K., J.Z., P.K., and A.K.; Visualization, O.B., J.Z., and A.K.; Supervision, J.Z. and K.K.; Project administration, J.Z.; Funding acquisition, J.Z. All authors have read and agreed to the published version of the manuscript.

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