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Changes in the viability and secretory activity of rat adipose derived stem cells in vitro culture stimulated by a pulsed electromagnetic field

Zmiany żywotności i aktywności sekrecyjnej szczurzych komórek macierzystych izolowanych z tkanki tłuszczowej w hodowli in vitro, stymulowanych pulsacyjnym polem elektromagnetycznym

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Pracę wykonano w Katedrze Patofizjologii UJ CM
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Kraków, 2019 rok

Spis treści

I. Wprowadzenie

I.1. *Odziaływanie pola elektromagnetycznego o różnej częstotliwości na komórki*

Częstotliwości pola elektromagnetycznego w przedziale od 0 do 300Hz są konwencjonalnie nazywane niskimi częstotliwościami. Emiterami takiego pola są linie wysokiego napięcia, stacje transformatorowe, silniki elektryczne samochodów czy stacje radiolokacyjne [1]. Liczne badania pokazały wpływ tych pól magnetycznych na systemy biologiczne [2]. Pulsacyjne pole elektromagnetyczne o bardzo niskiej częstotliwości (extremely low frequency pulsed electromagnetic field, ELF-PEMF) stymuluje apoptozę w proliferujących komórkach. Badanie prowadzone na komórkach ludzkiego kostniakomięsaka MG-63 w hodowli komórkowej eksponowane na ELF-PEMF (50Hz, 1mT) wykazały zahamowanie wzrostu komórkowego, indukcję apoptozy oraz zwiększoną syntezę reaktywnych form tlenu (ROS) [3]. Działanie ELF-PEMF znalazło praktyczne zastosowanie w leczeniu zaburzeń kostnych w ostatnich latach [4]. Długotrwałe działanie pulsacyjnego pola elektromagnetycznego może wywierać także negatywne skutki w postaci obniżonej ruchliwości plemników i spadku poziomu testosteronu [5]. Badania prowadzone na szczurach poddanych 45 dniowej ekspozycji na ELF-PEMF o parametrach 50Hz, 1mT, wykazały wzrost wskaźników stresu oksydacyjnego w ich wątrobie [6]. Natomiast krótsza 5 dniowa ekspozycja stałym polem magnetycznym (o parametrach 5kHz, 128 mT) powodowała indukcję apoptozy na hepatocytach dorosłych męskich szczurów [7]. Z kolei zastosowanie pulsacyjnego pola elektromagnetycznego o wysokiej częstotliwości (27,12MHz, 6±1V/m) wpłynęło na lepsze utlenowanie tkanek w mózgu zdrowego szczura oraz zwiększony przepływ krwi [8]. Działanie PEMF (o parametrach 25Hz i 10mT) na rany cukrzycowe u dorosłych samców szczura pokazało wzrost całkowitej grubości tkanki oraz przyspieszyło gojenie ran [9]. Eksperymenty prowadzone na ludzkich jednojądrzastych komórkach krwi obwodowej (PBMCs) z oddziaływaniem PEMF (o częstotliwości 50Hz, 5mT) wykazały obniżenie żywotności PBMCs pochodzących od pacjentów z chorobą Crohna jak również od zdrowych dawców. Efekt przeciwzapalny wywierany przez działanie PEMF manifestował się wzrostem sekrecji IL-10 w stymulowanej hodowli limfocytów [10]. Rezultaty naukowe autorstwa Wang P. et al. potwierdzają obserwacje, że użycie PEMF o częstotliwości 15 Hz, 3mT indukuje także apoptozę osteoklastów [11].

I.2. Biologiczne aspekty działania pulsacyjnego pola elektromagnetycznego (PEMF)

W ciągu ostatnich 30 lat nastąpił znaczny rozwój badań z wykorzystaniem pól elektromagnetycznych prowadzonych na liniach komórkowych, zwierzętach doświadczalnych wskazując na efekty biologiczne i wartość przedkliniczną [12]. Odpowiedź biologiczna na działanie pola elektromagnetycznego zależy od wrażliwości komórek, ich aktywności proliferacyjnej oraz parametrów samego pola elektromagnetycznego takich jak częstotliwość, intensywność, modulacja, polaryzacja, tryb (pulsacyjny, chwilowy, średni), moc a także całkowita pochłonięta energia [13]. Udowodniono, iż pole elektromagnetyczne oddziałuje na błony komórkowe wpływając na przewodnictwo sodu w stosunku do potasu, aktywność kanałów wapniowych a także na cykl komórkowy. Zjawiska fizjologiczne krótko bądź długoterminowe regulują mechanizmy komórkowe, które są uwarunkowane kinetyką kanałów sodowych, chlorkowych i wapniowych [14]. Badania z wykorzystaniem pola elektromagnetycznego pokazują wspomagający wpływ na leczenie przez osłabienie miejscowej reakcji zapalnej, w pooperacyjnym obrzęku stawów, a także w zniesieniu bólu [15]. Pacjenci z chorobą Parkinsona poddani przezczaszkowej stymulacji polem magnetycznym o częstotliwości 5Hz wykazywali poprawę funkcji poznawczych i autonomicznych, chodu oraz nastroju [16]. Zaobserwowano również, że terapia PEMF obniża markery stanu zapalnego i mogłaby stanowić potencjalną metodę terapeutyczną w leczeniu zapaleń tkanek miękkich [17]. Wyniki prac eksperymentalnych wykazały aktywację szlaków śmierci komórkowej w komórkach proliferujących pod wpływem działania PEMF o niskiej częstotliwości (50mV,50Hz) [18]. Badania analizujące wpływ pulsacyjnego pola magnetycznego (15Hz, 6mT) na stan mięśnia sercowego po zawale u szczurów, wykazują promowanie angiogenezy mięśnia sercowego i poprawę czynności narządu [19]. Praktyczne, potencjalne zastosowanie pola magnetycznego wynika z jego przeciwzapalnego, przeciwbólowego oraz pro-regeneracyjnego działania w badaniach eksperymentalnych [20].

I.3. *Magnetostymulacja jako praktyczne wykorzystanie działania pola elektromagnetycznego*

Pole magnetyczne wokół Ziemi ulega ciągłym zmianom oraz powolnym wahaniom. Pierwsza zmierzona częstotliwość rezonansowa Schumanna wynosiła 7,83Hz [21]. Od momentu odkrycia tego zjawiska w 1986r. elektromagnetyczna częstotliwość Ziemi uważana była za wartość niezmienną, natomiast aktualnie wynosi ona ok.16,5Hz. Organizm ludzki czy zwierzęcy wykazuje zdolności adaptacyjne do stałego magnetycznego oddziaływania środowiska [22]. W świecie roślin magneto-skaryfikacja jest stosowana w procesie pobudzania nasion roślin do intensywniejszego kiełkowania i wzrostu. Zastosowanie pól magnetycznych w medycynie, czyli tzw. "magnetoterapia" jest jedną z popularnych metod fizjoterapeutycznych wspomagających leczenie wielu schorzeń. W ostatnim dziesięcioleciu użyto do regulacji aktywności komórkowej pól elektromagnetycznych o różnych zakresach. Stymulacja polem magnetycznym stosowana do nieinwazyjnego modulowania ekspresji genów, wpływa na wewnątrzkomórkowe organelle oraz aktywuje organizmy in vivo [23]. Ostatnie doniesienia wykazują, że stymulacja elektromagnetyczna mogłaby stanowić nieinwazyjną metodę wczesnej diagnostyki chorób naczyń obwodowych i docelowo cukrzycowych owrzodzeń stopy [24]. U pacjentów z zaburzeniami erekcji użycie kombinacji przezczaszkowej magnetoterapii i elektrostymulacji przezbrzusznej przez okres 6 miesięcy powodowało zmniejszenie masy ciała, podniesienie poziomu testosteronu oraz poprawienie funkcji erekcji [25]. Badania prowadzone z wykorzystaniem terapeutycznej przezczaszkowej magnetoterapii i elektrycznej stymulacji u pacjentów z zaburzeniami erekcji oraz otyłości brzusznej powodowały poprawę metabolizmu lipidów i funkcji erekcji, także działały hipotensyjne i uspokajająco [26]. Połączenie magnetoterapii i hydroterapii w leczeniu blizn po głębokich ranach oparzeniowych u dzieci poprawiało wyniki leczenia po zastosowaniu 12 tygodniowej terapii w porównaniu do konwencjonalnego postępowania, poprzez zmniejszenie stopnia hiperplazji tkanek, bólu oraz odczuwania swędzenia blizn [27].

II. Cele pracy

Celem niniejszej rozprawy była analiza wpływu ekspozycji pulsacyjnego pola elektromagnetycznego (PEMF) o niskiej częstotliwości na żywotność i metabolizm komórek macierzystych pochodzących z tkanki tłuszczowej (ADSCs), izolowanych od szczurów utrzymywanych na diecie niskotłuszczowej i wysokotłuszczowej.

Cele szczegółowe pracy dotyczyły:

1. Określenia zmian w żywotności szczurzych komórek macierzystych izolowanych z tkanki tłuszczowej (ADSCs) pod wpływem działania PEMF oznaczonych za pomocą cytometrii przepływowej.

2. Oceny wybranych parametrów stanu zapalnego w supernatantach z hodowli szczurzych ADSCs eksponowanych na działanie PEMF oraz w surowicy zwierząt eksperymentalnych, przez pomiar poziomu wybranych cytokin prozapalnych: czynnika martwicy nowotworu –TNFα i interleukiny 6 –IL-6 oraz rezystyny.

3. Analizy wpływu PEMF na poziom wybranych adipokin (adiponektyna i leptyna) produkowanych przez hodowlę ADSCs pochodzących od szczurów bytujących na diecie niskotłuszczowej lub wysokotłuszczowej oraz w surowicy zwierząt eksperymentalnych.

Cele uzupełniające pracy stanowiły:

1. Analizę poziomu glikemii we krwi zwierząt doświadczalnych wszystkich grup badawczych różniących się kalorycznością przyjmowanej karmy bytowej.

2. Analizę pomiaru temperatury ciała zwierząt otrzymujących karmę standardową oraz o zwiększonej zawartości tłuszczu.

III. Wykaz publikacji składających się na dysertację

W skład przedstawionej pracy doktorskiej wchodzą cztery artykuły opublikowane w następujących czasopismach związanych z medycyną eksperymentalną:

1. *Changes in viability of rat adipose-derived stem cells isolated from abdominal/perinuclear adipose tissue stimulated with pulsed electromagnetic field. Baranowska A, Skowron B, Nowak B, Ciesielczyk K, Guzdek P, Gil K, Kaszuba-Zwoinska J. J Physiol Pharmacol. 2017 Apr;68(2):253-264.*
   praca oryginalna; punktacja IF =2.478; punktacja MNiSW= 25.

   Artykuł pierwszy, opublikowany w Journal of Physiology and Pharmacology skupia się na ocenie żywotności szczurzych komórek macierzystych pochodzących z tkanki tłuszczowej (ADSCs) w hodowli komórkowej poddanych ekspozycji na działanie pulsacyjnego pola elektromagnetycznego (PEMF).

2. *Low Grade Inflammation in Visceral and Subcutaneous Adipose Tissue Originated from Adipose Derived Stem Cells in Experimental Model of Obesity in Rats under Influence of Pulsed Electromagnetic Field Interaction. Baranowska Agnieszka, Skowron Beata, Ciesielczyk Katarzyna, Guzdek Piotr, Gil Krzysztof and Kaszuba-Zwoińska Jolanta. Journal of Animal Research and Nutrition, 2018, Vol.3 No.1:1-8.*
   praca oryginalna; punktacja IF =0; punktacja MNiSW=0.

   Artykuł drugi, opublikowany w Journal of Animal Research and Nutrition analizuje wybrane parametry procesu zapalnego w hodowli ADSCs stymulowanych PEMF w warunkach in vitro oraz w surowicy zwierząt. Przedstawia stężenie glukozy we krwi zwierząt utrzymywanych na diecie niskotłuszczowej oraz indukującej otyłość.

3. *Obesity related adipokines release in rat adipose derived stem cell cultures influenced by pulsed electromagnetic field. Baranowska A, Skowron B, Gil K, Kaszuba-Zwoińska J.Folia Med Cracov. Vol. LVIII, 2, 2018: 131–145.*
   praca oryginalna; punktacja IF =0; punktacja MNiSW= 10.

   Artykuł trzeci, opublikowany w Folia Medica Cracoviensia przedstawia zależność pomiędzy poziomem adipokin produkowanych przez ADSCs i stymulowanych PEMF,

a wpływem diety indukującej otyłość u zwierząt, od których izolowano ADSCs do hodowli.

4. *Effect of the pulsed electromagnetic field on the release of inflammatory mediators from adipose-derived stem cells (ADSCs) in rats. Agnieszka Baranowska, Beata Skowron, Krzysztof Gil, Jolanta Kaszuba-Zwoińska, Folia Med Cracov. Vol. LVIII, 4, 2018: 21–34.*
   praca oryginalna; punktacja IF =0, punktacja MNiSW= 10.

Artykuł czwarty, opublikowany w Folia Medica Cracoviensia pokazuje różnice w stężeniu mediatorów procesu zapalnego w supernatantach z hodowli ADSCs, uwzględniając płeć badanych zwierząt oraz zmiany w temperaturze ciała zwierząt utrzymywanych na karmie niskotłuszczowej i wysokotłuszczowej.

IV. Metodologia badań składających się na dysertację

IV.1. Badana grupa zwierząt doświadczalnych

Badaniami objęto:

1. Oseski szczurów obu płci [WistarKrf: (Wi) Wu]
2. Osobniki dorosłe szczurów obu płci [WistarKrf: (Wi) Wu]

Zgodnie z celem projektu założono 8 grup eksperymentalnych:

- Grupę nr 1 i 3 stanowiły oseski płci żeńskiej i męskiej wraz z matkami utrzymywane na karmie niskotłuszczowej przez 21 dni doświadczenia
- Grupę nr 2 i 4 stanowiły oseski płci żeńskiej i męskiej wraz z matkami utrzymywane na karmie wysokotłuszczowej przez 21 dni doświadczenia
- Grupę nr 5 i 7 stanowiły osobniki dojrzałe płciowo płci żeńskiej i męskiej utrzymywane na karmie niskotłuszczowej przez 21 dni doświadczenia
- Grupę nr 6 i 8 stanowiły osobniki dojrzałe płciowo płci żeńskiej i męskiej utrzymywane na karmie wysokotłuszczowej przez 21 dni doświadczenia

Projekt uzyskał zgodę I Lokalnej Komisji Etycznej ds. Doświadczeń na Zwierzętach działającej przy Uniwersytecie Jagiellońskim w Krakowie nr 84/2014 z dnia 21 maja 2014 r.

IV.2. Procedury wdrożone do projektu

Procedura nr 1. Zastosowanie karmy niskotłuszczowej

- W czterech grupach eksperymentalnych zwierzęta otrzymywały ad libitum karmę (Labofeed B, Pasze Kcynia) zawierającą:
- 25% białka
- 8% tłuszczu
- 67% węglowodanów

Procedura nr 2. Zastosowanie karmy wysokotłuszczowej

- W czterech grupach eksperymentalnych zwierzęta otrzymywały ad libitum karmę (VERSELE-LAGA Opti Life Adult Active) zawierającą:
- 32% białka
- 22 % tłuszczu
- 40% węglowodanów
- 6% składniki mineralne

Procedura nr 3. Pomiar masy ciała

Pomiar masy ciała wykonany we wszystkich badanych grupach dwa razy w tygodniu w godzinach porannych (waga OHAUS NAWIGATOR 2100/0.1g)

Procedura 4. Pomiar temperatury ciała

Pomiar temperatury ciała wykonany dwa razy w tygodniu w godzinach porannych we wszystkich badanych grupach z wykorzystaniem termometru na podczerwień (Anima, Vivari) w przypadku osesków, natomiast u osobników dorosłych zastosowano termometr rektalny (Anima, Vivari).

Procedura nr 5. Autopsja zwierząt

W 21 dniu doświadczenia szczury ze wszystkich badanych grup zostały uśmiercone przez przedawkowanie środka anestetycznego – pentobarbitalu (Morbital, Biowet Puławy, sposób podania - dootrzewnowo, 200mg/kg m.c). W czasie sekcji została pobrana tkanka tłuszczowa okołonajądrzowa od samców natomiast od samic tkanka tłuszczowa brzuszna podskórna. Od zwierząt obu płci była pobierana krew z serca na heparynę z dodatkiem antyglikolitycznego fluorku sodu celem oznaczenia glikemii.

IV.3. Izolacja i hodowla szczurzych komórek macierzystych pochodzących z tkanki tłuszczowej (ADSCs)

Tkanka tłuszczowa była pobierana w warunkach aseptycznych od szczurów dojrzałych płciowo i od osesków, w oparciu o opublikowaną metodykę badań [28]. Wyizolowaną tkankę tłuszczową przemywano buforowanym roztworem soli fizjologicznej (PBS, Sigma-Aldrich, Niemcy), zawierającym 1% roztwór antybiotyku (penicyliny /streptomycyny-Sigma - Aldrich, Niemcy). Następnie poddawano trawieniu kolagenazą typu 1 (1 mg/ml; Gibco by Life Technologies USA) w temperaturze 37°C przez 1 godzinę. Aktywność enzymu była neutralizowana za pomocą medium hodowlanego (DMEM Sigma-Aldrich, Niemcy) zawierającego 10% bydlęcą surowicę płodową (FBS, Gibco by Life Technologies, USA) i 1% roztwór penicyliny/ streptomycyny. Następnie zawiesinę trawionej tkanki przesączono przez filtr (średnica porów 100µm, Fisher Scientific, USA), po czym wirowano przez 10min. przy 300g, uzyskując pelet komórek o wysokiej gęstości. Osad komórkowy zawieszano w DMEM suplementowanym 10% FBS i 1% roztworem penicyliny/streptomycyny. Komórki przenoszono do butelek hodowlanych T75 (Corning, Sigma-Aldrich, Niemcy), hodowlę inkubowano przez noc w cieplarce, w temp. 37°C, z 5%CO_2 i 90% wilgotnością. Nieadherentne komórki i erytrocyty były usuwane po 24 godzinach a komórki adherentne były przemyte roztworem PBS z dodatkiem antybiotyku. Następnie adherentne komórki (ADSCs) zawieszano w suplementowanym medium hodowlanym DMEM. Medium hodowlane było zmieniane, co 72h, aż do uzyskania konfluencji hodowli ADSCs. Gdy komórki osiągały 80-90% konfluencji, umieszczono je w roztworze trypsyny - EDTA (0,25% wag./obj., Sigma-Aldrich, Niemcy) na czas 10-15 min. w temp. 37°C. W celu zatrzymania aktywności trypsyny dodawano suplementowane medium hodowlane DMEM, wirowano komórki przy obrotach 416g przez 10 min. Pelet zawierający ADSCs zawieszano w suplementowanym medium DMEM. Komórki liczono w komorze hemocytometru Fuchs–Rosenthala a następnie zakładano z nich hodowlę na 96-dołkowej płytce hodowlanej (Corning, Sigma-Aldrich, Niemcy) w trzech powtórzeniach o gęstości wyjściowej komórek 0,25 x 10^6/ml i hodowano je w temp. 37°C, z 5% CO_2 i 90% wilgotnością do eksperymentu.

IV.4. Ekspozycja ADSCs na działanie PEMF

Pierwsza stymulacja PEMF miała miejsce po 24 godz. trwania hodowli komórkowej ADSCs. Generator PEMF (zaprojektowany przez Instytut Technologii Elektronowej, Kraków, Polska) był umieszczany wewnątrz inkubatora do hodowli komórkowej, emitował pulsacyjne pole elektromagnetyczne o częstotliwości 7Hz i indukcji magnetycznej 30mT. Płytkę z hodowlą komórkową ADSCs umieszczono na czas ekspozycji polem elektromagnetycznym w kieszeni generatora. Ekspozycję na PEMF stosowano trzy razy przez 4 godziny z 24-godzinnymi przerwami. Próbki kontrolne znajdowały się w tym samym inkubatorze, ale w odległości 35 cm od generatora.

IV.5. Ocena parametrów śmierci komórkowej ADSCs za pomocą cytometrii przepływowej

Dwadzieścia cztery godziny po ostatniej ekspozycji na działanie PEMF, ADSCs były zbierane z płytek hodowlanych przez trypsynizację, a następnie odmywane trzykrotnie zimnym PBS (Sigma-Aldrich, Niemcy) i barwione aneksyną V wyznakowaną allofikocyjaniną (AnV-APC, BD, Pharmingen TM, USA) do analizy parametru wczesnej apoptozy. Jodek propydyny (PI, BD, Pharmingen TM, USA) zastosowano do oceny procentowej ilości komórek martwych w populacji. Komórki barwiące się aneksyną V (AnV+) były komórkami wczesno apoptotycznymi, komórki barwiące się aneksyną V i jodkiem propidyny (AnV+PI+) analizowano, jako późnoapoptotyczne i nekrotyczne a komórki dodatnie pod względem PI (PI+) jako nekrotyczne. Dla oznaczenia parametrów żywotności komórek, ADSCs przemywano dwukrotnie zimnym PBS i zawieszono w buforze wiążącym (BD, Pharmingen TM, USA) o gęstości 1×10^6 komórek/ml. Następnie 100µl zawiesiny komórkowej przenoszono do 5 ml próbki do analizy cytometrycznej i dodawano 5µl AnV-APC potem inkubowano komórki przez 15 minut w temperaturze pokojowej, w ciemności. Po tym czasie do komórek dodawano 400µl buforu wiążącego, a następnie komórki umieszczano na lodzie do momentu analizy w urządzeniu FACS CALIBUR (Becton Dickinson, San Jose, CA). Program Cell-Quest był wykorzystany do procentowej analizy barwionych poszczególnych populacji komórek ADSCs. Kontrole do ustawienia kompensacji urządzenia obejmowały komórki: niebarwione (AnV-APC-, PI-) i znakowane pojedynczo aneksyną V i jodkiem propydyny (AnV-APC i PI).

Fluorescencje pochodzącą od AnV-APC mierzono na kanale 4 (fluorescencja FL-4) natomiast fluorescencje od PI mierzono na kanale 3 (fluorescencja FL-3). Dla każdej próbki zbierano, co najmniej 10 000 zdarzeń (komórek).

IV.6. Ilościowy pomiar cytokin i adipokin techniką ELISA

Analiza immunoenzymatyczna była wykorzystywana do oznaczeń ilościowych w supernatantach hodowlanych ADSCs stężenia czynników odpowiedzialnych za regulację metabolizmu (leptyna, adiponektyna) oraz mediatorów procesu zapalnego (TNFα i IL-6 oraz rezystyny). Adipokiny i mediatory zapalenia były również oznaczane ilościowo w surowicy zwierząt wszystkich badanych grup doświadczalnych.

IV.7. Analiza temperatury ciała zwierząt doświadczalnych oraz poziomu glikemii.

Pomiar stężenia glukozy we krwi i temperaturę ciała zwierząt doświadczalnych porównywano u zwierząt obu płci, w różnym wieku, utrzymywanych na karmie niskotłuszczowej i wysokotłuszczowej.

IV.8. Analiza statystyczna wyników

Wyniki przedstawiono, jako średnie (±) odchylenie standardowe (S.D). Analizę statystyczną wyników przeprowadzone za pomocą testu t-Studenta. Znamienność statystyczną uznano dla $P \leq 0,05$.

V. Omówienie wyników

V.1. *Rezultaty naukowe*

V.1.1. PEMF a żywotność ADSCs

1. Ekspozycja szczurzych komórek macierzystych izolowanych z tkanki tłuszczowej (ADSCs) na pulsacyjne pole elektromagnetyczne (PEMF) powodowała statystycznie istotne zwiększenie ilości komórek będących we wczesnej apoptozie uzyskanych z grupy dorosłych samic szczura na diecie wysokotłuszczowej (HF): procent komórek wczesnoapoptotycznych nieeksponowanych na PEMF (8.84± 5.30 %) vs. procent komórek wczesnoapoptotycznych eksponowanych na PEMF (17.48 ± 10.52 %).

2. Działanie PEMF powodowało znaczne różnice w żywotności ADSCs izolowanych od osesków płci żeńskiej utrzymywanych na diecie niskotłuszczowej (LF) w postaci zmniejszonej liczby komórek ulegających nekrozie (1.13 ± 0.59 %) w porównaniu do komórek nieeksponowanych na PEMF (6.97 ± 0.64 %). Natomiast w przypadku osesków płci męskiej na tej samej diecie ekspozycja komórek na PEMF zwiększyła populację komórek późnoapoptotycznych (12.35 ± 4.66%) w stosunku do komórek nieeksponowanych na PEMF (9.87± 3,47%).

3. W przypadku ADSCs izolowanych od osesków obu płci bytujących na diecie HF działanie PEMF wywołało statystycznie istotne zmiany w zakresie parametrów wczesnej apoptozy. U osesków męskich nastąpił procentowy spadek komórek wczesnoapoptotycznych poddanych działaniu PEMF (3.36 ± 1.48%) w stosunku do komórek nieeksponowanych na PEMF (4.62±1.93%), podobną tendencję obserwowano także u osesków żeńskich (8.74 ± 3.01 %) vs. (10.92 ± 3.11%).

4. ADSCs izolowane od osesków żeńskich bytujących na diecie HF i traktowane PEMF wykazały statystycznie mniejszy odsetek komórek będących w późnym stadium apoptozy (12.15 ± 2.44 %) w stosunku do komórek nieeksponowanych na PEMF (13.66 ± 1.23%). Po ekspozycji na PEMF ilość komórek nekrotycznych u tych zwierząt była niższa (0.56 ± 0.20 %) niż komórek niepoddanych działaniu PEMF (0.74 ± 0.12 %).

5. W przypadku ADSCs pochodzących od dorosłych osobników płci męskiej utrzymywanych na diecie LF po ekspozycji na PEMF zaobserwowano statystycznie istotny spadek w populacji komórek wczesnoapoptotycznych (1.91±2.21%) w porównaniu do komórek nieeksponowanych na PEMF (5.41 ± 3.83%), podobny efekt obserwowano w populacji komórek późnoapoptotycznych po działaniu PEMF (4.16 ± 5.54 %) vs. (10.67 ± 6.71 %).

V.1.2. PEMF a aktywność sekrecyjna ADSCs

1. ADSCs izolowane od osesków płci żeńskiej utrzymywanych na diecie LF poddane działaniu PEMF wykazywały obniżoną produkcję TNF -α po ekspozycji na PEMF (2,61 ± 1,43 pg/ml) w porównaniu do komórek nieeksponowanych (14,29 ± 8,42 pg/ml). Natomiast odwrotna zależność była obserwowana w hodowli ADSCs pochodzącej od osesków płci męskiej utrzymywanych na tej samej diecie, pod wpływem PEMF nastąpił istotny wzrost uwalnianego przez komórki TNF-α (5,72±1,89 pg/ml) w porównaniu do ADSCs nieeksponowanych na działanie PEMF (0,2 ± 0,01 pg/ml).

2. Poziom IL-6 w supernatantach hodowli komórkowej ADSCs izolowanych od osesków płci żeńskiej utrzymywanych na diecie LF eksponowanych na działanie PEMF był statystycznie istotnie wyższy (41,69 ± 3,44 pg/ml) niż w grupie nieeksponowanej na PEMF (19,86± 0,42 pg/ml). Natomiast ADSCs pochodzące od osesków płci męskiej utrzymywanej na tej samej karmie, pod wpływem PEMF wytwarzały mniej IL-6 (30,89 ± 15,73 pg/ml) w porównaniu do komórek nieeksponowanych na PEMF (55,13 ± 15,73 pg/ml).

3. Poziom rezystyny w hodowli komórkowej ADSCs pochodzących od osesków płci męskiej utrzymywanych na diecie LF pod wpływem PEMF wzrastał (16,57±0,69ng/ml) w stosunku do komórek nieeksponowanych (14,97± 0,006 ng/ml).

4. Ekspozycja na PEMF indukowała wzrost stężenia TNF-α w supernatantach hodowli komórkowej ADSCs izolowanych od osesków obu płci na diecie HF: poziom TNF-

α w hodowli ADSCs od samic wynosił: 4,57 ± 1,57 pg/ml w stosunku do komórek nieeksponowanych na działanie PEMF: 2,09 ± 0,89 pg/ml; poziom TNF-α w hodowli ADSCs od samców wynosił: 5,34 ± 1,62 pg/ml w stosunku do komórek nieeksponowanych na działanie PEMF 1,85 ± 1,83 pg/ml.

5. Działanie PEMF na hodowlę ADSCs izolowanych od osesków płci żeńskiej utrzymywanej na diecie HF powodowało statystycznie istotny spadek ilości IL-6 (21,99 ± 2,23 pg/ml) w porównaniu ADSCs do nieeksponowanych na działanie PEMF (34,65±4,28 pg/ml).

6. Hodowle ADSCs uzyskane od dorosłych osobników obu płci utrzymywanych na diecie LF pod wpływem PEMF wytwarzały więcej TNF-α: ADSCs pochodzące od samic na PEMF (6,85 ± 1,72 pg/ml) w porównaniu do hodowli ADSCs nieeksponowanej na działanie PEMF (1,05± 0,90 pg/ml), ADSCs od samców eksponowane (5,82 ± 2,48 pg/ml) vs hodowla nieeksponowana na działanie PEMF (3,49 ± 0,25 pg/ml).

7. Działanie PEMF indukowało istotne zmiany w poziomie IL-6 w hodowli komórkowej ADSCs pochodzących od dorosłych samic utrzymywanych na diecie LF; komórki produkowały mniej IL-6 (29,21 ± 6,35 pg/ml) niż komórki nieeksponowane na PEMF (39,79 ± 1,98 pg/ml); natomiast w przypadku ADSCs izolowanych od dorosłych samców na utrzymywanych na diecie LF po ekspozycji PEMF efekt był przeciwny, nastąpił wzrost stężenia IL-6 (41,56 ± 8,56 pg/ml) vs. komórki nieeksponowane na PEMF (34,32 ± 6,39 pg/ml).

8. W hodowli komórkowej ADSCs pochodzącej od dorosłych samic utrzymywanych na diecie LF po ekspozycji na PEMF, zaobserwowano podniesienie poziomu rezystyny (16,89 ± 0,54 ng/ml) vs. hodowla nieeksponowana na działanie PEMF (14,21 ± 1,13 ng/ml). Podobny wynik uzyskano w hodowli ADSCs od dorosłych samców utrzymywanych na diecie HF, poziom rezystyny w hodowli ADSCs po stymulacji PEMF podniósł się (17,00 ± 0,27 ng/ml) w stosunku do hodowli nieeksponowanej na PEMF(15,29 ± 0,36 ng/ml).

9. Poziom rezystyny w hodowli ADSCs izolowanych od dorosłych samic utrzymywanych na diecie LF pod wpływem działania PEMF był wyższy (16,89±0,54 ng/ml) w porównaniu do hodowli ADSCs nieeksponowanej na działanie PEMF (14,21±1,13 ng/ml). Analogiczny trend występował w przypadku

hodowli ADSCs izolowanych od dorosłych samców utrzymywanych na diecie HF – obserwowano wzrost poziomu rezystyny pod wpływem PEMF (17,00±0,27 ng/ml) w stosunku do komórek nieeksponowanych (15,29±0,36 ng/ml).

10. Znaczący wzrost stężenia TNF-α zanotowano w hodowli komórkowej ADSCs izolowanych od dorosłych samic i samców utrzymywanych na diecie HF: ADSCs od samic eksponowane na PEMF (70,22 ± 28,48 pg/ml) a ADSCs nieeksponowane (5,76 ± 2,23 pg/ml); ADSCs od samców pod wpływem PEMF wynosił: 70,23±35,84 względem kontroli 7,61±1,14. Podobna zależność wystąpiła w hodowli ADSCs otrzymywanych od dorosłych samców utrzymywanych na diecie HF; poziom IL-6 po ekspozycji na PEMF wynosił : 41,12 ± 3,49 pg/ml a w komórkach hodowli nieeksponowanej wynosił: 28,84 ±0,20 pg/ml.

11. Ekspozycja na PEMF hodowli komórkowej ADSCs pochodzących od osesków płci żeńskiej utrzymywanej na diecie LF powodowała wzrost ilości uwalnianej adiponektyny: (5,28±0,38pg/ml) w porównaniu z hodowlą ADSCs nieeksponowaną na PEMF (0,19 ± 0,01 pg/ml). Hodowla komórkowa ADSCs pochodząca od osesków płci żeńskiej na diecie LF poddana PEMF uwalniała mniejsze ilości leptyny (2,20 ± 0,70 pg/ml) w porównaniu z komórkami nieeksponowanymi (7,79 ± 6,68 pg/ml).

12. Poziom adiponektyny w hodowli ADSCs pochodzącej od osesków płci żeńskiej utrzymywanej na diecie HF obniżył się statystycznie istotnie po ekspozycji PEMF: (2,20 ± 0,70 pg/ml) vs komórki nieeksponowane na PEMF (7,79 ± 6,68 pg/ml). ADSCs izolowane od osesków obu płci utrzymywanych na diecie HF poddane działaniu PEMF produkowały więcej leptyny (u samic:5,68 ±0,14 pg/ml, u samców: 7,65 ± 2,02 pg/ml) niż komórki nieeksponowane (u samic: 4,41 ± 0,42 pg/ml, u samców 2,78 ± 0,83 pg/ml).

13. ADSCs wyizolowano od dorosłych osobników płci żeńskiej utrzymywanych na diecie LF po stymulacji PEMF produkowały mniej adiponektyny (0,173± 0,008 pg/ml) i leptyny (0,36 ± 0,25 pg/ml) vs. do komórek nieeksponowanych na działanie PEMF (adiponektyna 0,77± 0,15 pg/ml; leptyna 1,62 ± 0,49 pg/ml). Odwrotny efekt zaobserwowano w hodowli ADSCs izolowanej od dorosłych osobników płci męskiej utrzymywanej na diecie LF gdzie, poziom leptyny był

wyższy (3,97± 0,011pg/ml) po zadziałaniu PEMF w stosunku do komórek nieeksponowanych (1,32± 0,84 pg/ml).

14. W hodowli ADSCs uzyskanej od dorosłych samic utrzymywanych na karmie HF po ekspozycji PEMF stężenie adiponektyny było wyższe niż w przypadku komórek nieeksponowanych na PEMF (0,77±0,008 vs. 0,17± 0,40 pg/ml).W hodowli ADSCs pochodzącej od dorosłych osobników obu płci utrzymywanej na diecie HF po ekspozycji na PEMF poziom leptyny zmienił się znacząco: u samic nastąpił spadek ilości adipokiny (0,35 ± 0,25 pg/ml) w stosunku do komórek nieeksponowanych na działanie PEMF(0,61 ± 0,10 pg/ml) natomiast u samców zanotowano wzrost poziomu leptyny (3,27 ± 0,41 pg/ml) w porównaniu do komórek nieeksponowanych (0,68 ± 0,41 pg/ml).

V.1.3. Dieta, wiek, płeć a glikemia i parametry stanu zapalnego

1. Dieta zawierające tłuszcze nasycone predysponuje do rozwoju otyłości i przewlekłego stanu zapalnego a także nowotworów [29]. Stężenie IL-6 w surowicy u męskiego potomstwa gryzoni karmionych dieta wysokotłuszczową było istotnie zwiększone w 21. dniu życia co jest spójne z naszymi wynikami [30].Wprowadzenie diety HF na etapie rozwoju zarodkowego i laktacji skutkowało u osesków obu płci wyższym poziomem cytokin prozapalnych w surowicy. Poziom IL-6 w surowicy u osesków obu płci utrzymywanych na diecie HF był wyższy niż u osesków na diecie LF (samice na diecie HF: 115,28 pg/ml vs. samice na diecie LF: 32,61 pg/ml, samce na HF:129,86 pg/ml vs. samce na LF:13,42 pg/ml).

2. Otyłość jest chorobą gdzie występuję przewlekły stan zapalny, któremu towarzyszy niekorzystna dla organizmu sekrecja cytokin prozapalnych, w tym IL-6, TNF-α. Powyższe mediatory stanu zapalnego pełnią kluczową role w schorzeniach związanych z otyłością takich jak cukrzyca, zespół metaboliczny i choroby sercowo-naczyniowe [31]. W surowicy uzyskanej od osesków płci żeńskiej na diecie HF zmierzono wyższe wartości stężenia TNF-α (13,25± 0,15 pg/ml) niż w surowicy zwierząt na diecie LF (6,97 ± 1,25 pg/ml). Stężenie w surowicy TNF-α i IL-6 w grupie dorosłych samic utrzymywanych na diecie HF było wyższe niż u zwierząt tej samej płci utrzymywanych na diecie LF. Poziom TNF-α na diecie HF wynosił: 32,50±2,51pg/ml w porównaniu do poziomu TNF-α na diecie LF: 3,40±0,32 pg/ml.

Stężenie IL-6 na diecie HF wynosiło: 87,56±27,22 pg/ml w stosunku do stężenia IL-6 na diecie LF 22,94±0,88pg/ml. W grupie dorosłych samców utrzymywanych na diecie HF poziom TNF-α (48,87± 8,85 pg/ml) i IL-6 (45,43± 11,49 pg/ml) był wyższy niż zwierząt utrzymywanych na diecie LF (TNF-α: 4,11± 0,09 pg/ml;IL-6: 20,02± 4,69 pg/ml).

4. Miejscem syntezy rezystyny u gryzoni są adipocyty białej tkanki tłuszczowej. W badaniach naukowych prowadzonych na gryzoniach udowodniono zwiększony poziom rezystyny u zwierząt otrzymujących karmę o wysokiej zawartość tłuszczu i węglowodanów [32,33]. Rezystyna jest mediatorem oporności na insulinę [34]. Nasze badania potwierdziły tą zależności u osobników młodych i dorosłych utrzymywanych na diecie HF. Poziom rezystyny w surowicach osesków obu płci był wyższy gdy zwierzęta były utrzymywane na diecie HF (u samic 14,90 ± 1,04 ng/ml; u samców 14,05 ±1,01 ng/ml) niż w przypadku stosowania diety LF (samice 12,27± 2,48 ng/ml; samce 12,91 ± 0,65). Stężenie rezystyny uzyskane w surowicy dorosłych zwierząt obu płci utrzymywanych na diecie HF było wyższe niż u zwierząt utrzymywanych na diecie LF. U samic na diecie HF poziom tej adipokiny wynosił, 13,89±0,59 ng/ml natomiast na diecie LF wynosił odpowiednio: 11,72± 1,035 ng/ml. Podobna zależność wystąpiła u samców na diecie HF gdzie poziom rezystyny wyniósł 13,33± 0,99ng/ml w porównaniu do zwierząt na diecie LF, gdzie poziom rezystyny osiągnął wartość 11,28± 1,25ng/ml.

5. Adipokiny takie jak: adiponektyna i leptyna wykazują przeciwstawne działanie: leptyna zwiększa poziom cytokin pro zapalnych takich jak TNF-α i IL-6 skojarzonych z insulinoopornoscią i cukrzycą typu 2, natomiast adiponektyna wykazuje działanie przeciw zapalne, przeciw miażdżycowe oraz poprawia insulinowrazliwość tkanek [35]. Poziom leptyny zmierzony w surowicy osesków płci żeńskiej utrzymywanych na diecie LF kształtował się na niższym poziomie i wynosił: 1214,76±718,81pg/ml w stosunku do wartości leptyny w surowicy zwierząt tej samej płci utrzymywanych na diecie HF (5954,36±2468,92 pg/ml). Podobna zależność wystąpiła u osesków płci męskiej gdzie poziom leptyny na diecie LF wynosił: 269,94 ± 2972,01pg/ml natomiast na diecie HF kształtował się w zakresie: 5808,77±2468,92pg/ml.

6. Leptyna będąca hormonem sytości, hamuje przyjmowanie pokarmu, działa poprzez neurony w podwzgórzu. W sposób dodatni koreluje z ilością tkanki tłuszczowej w ustroju, depozyty podskórnej tkanki tłuszczowej uwalniają do krążenia ogólnego

więcej leptyny niż adipocyty z trzewnego depozytu tkanki tłuszczowej [36]. Wartości leptyny w surowicy dorosłych osobników płci żeńskiej utrzymywanych na diecie HF były wyższe (7015,27± 1518,5 pg/ml) niż w grupie zwierząt utrzymywanych na diecie LF (2619,51± 837,72pg/ml), podobna zależność zaobserwowana u osobników płci męskiej utrzymywanych na diecie HF (35471,02± 21706,97 pg/ml) w stosunku do osobników utrzymywanych na diecie LF (6295,99± 5868,62 pg/ml).

7. Fizjologiczne poziom adiponektyny jest wyższy u samic niż u samców ze względu na większą sekrecję z podskórnej tkanki tłuszczowej niż z trzewnej oraz działanie testosteronu w okresie okołoporodowym [37]. Rezultatem stosowania diety HF były wyższe wartości adiponektyny w grupie osesków płci żeńskiej (15278,2± 829,04 pg/ml) niż u tych samych zwierząt utrzymywanych na diecie LF (9437,71± 4409,45 pg/ml).W grupie dorosłych osobników płci żeńskiej i męskiej utrzymywanych na diecie HF występowało niższe stężenie adiponektyny (samice 16421,4±1722,57pg/ml, samce 15462,36±1949,80pg/ml) w porównywaniu do wartości uzyskanych w surowicy zwierząt utrzymywanych na diecie LF (samice25297,54± 2405,94 pg/ml, samce 24205,88± 2223,15 pg/ml).

8. W modelach opartych na gryzoniach zarówno u potomstwa narażonego na dietę wysokotłuszczowa ze strony matki w czasie ciąży i laktacji jak i u osobników dorosłych utrzymywanych na diecie HF, stężenie glukozy we krwi było podwyższone [38,39]. Nasze wyniki były zgodne z powyższymi doniesieniami: utrzymywanie zwierząt w różnym wieku i płci na karmie HF powodowało zmiany w poziomie glikemii. Grupa osesków obu płci prezentowała hiperglikemię w porównaniu do zwierząt utrzymywanych na diecie LF: samice na diecie HF (170,75± 36,06mg/dl) vs samice na diecie LF (131,25±11,09 mg/dl); samce na diecie HF (170,75 ±21,00mg/dl) vs samce na diecie LF (148,75±9,09mg/dl). Podobna tendencja utrzymała się u osobników dorosłych na diecie HF: samice na diecie HF (168,75±23,55 md/dl) vs samice na diecie LF (139,25±20,33 mg/dl); samce na diecie HF (258,62±50,51 md/dl) vs samice na diecie LF (168,75±35,23 md/dl). Poziom glikemii u dorosłych samców szczura utrzymywanych na diecie HF przekraczał 200 mg/ml co jest kryterium diagnostycznym cukrzycy typu II.

9. Długoterminowe spożycie karmy wysokotłuszczowej przez dorosłe szczurze osobniki płci męskiej skutkowało obniżoną temperatura ciała [40]. Otyłość zaburza prawidłową temperaturę ciała zwierząt w czasie ciąży, poprzez spadek powyższego

parametru [41]. Analiza temperatury ciała zwierząt doświadczalnych w różnym wieku, odmiennej płci utrzymywanych na dwóch różniących się kalorycznością karmach (dieta LF i HF) wskazuje że dieta indukująca otyłość odpowiada za obniżenie temperatury ciała szczurów doświadczalnych. Temperatura ciała dorosłych szczurów utrzymywanych na diecie LF wynosiła odpowiednio u samic 37,8ºC a, u samców 37,93 ºC. Temperatura ciała dorosłych zwierząt utrzymywanych na diecie HF wynosiła u samic 35,81ºC a u samców na 35,53 ºC.W grupie osesków temperatura ciała samic na diecie LF wyniosła: 35,11 ºC a samców 34,56ºC. Natomiast w grupie osesków utrzymywanych na diecie HF temperatura ciała wyniosła odpowiednio u samic 33,66 ºC a u samców 33,13 º C.

VI. Wnioski

1. Hodowla ADSCs izolowana od dorosłych samic szczurów, utrzymywanych na diecie HF wykazywała wzrost ilości komórek wczesnoapoptotycznych po ekspozycji na PEMF. Komórki ADSCs, które uległy programowej śmierci, jaką jest apoptoza, nie będą się różnicowały się w adipocyty, tym samym ograniczając ilość tkanki tłuszczowej.

2. Działanie PEMF na hodowlę ADSCs otrzymaną od osesków płci żeńskiej, utrzymywanych na diecie HF wywołało spadek wytwarzanej prozapalnej cytokiny - IL-6, powodując zahamowanie aktywności zapalnej komórek ADSCs.

3. Poziom adiponektyny w populacji komórek ADSCs pochodzących od osesków płci żeńskiej utrzymywanych na diecie HF pod wpływem PEMF wzrastał. Powyższy efekt jest korzystny ze względu na ujemną korelację adiponektyny w przebiegu wielu chorób metabolicznych wynikających z otyłości.

4. Poziom cytokin prozapalnych (IL-6 i TNF-α) oznaczanych w surowicy osesków i zwierząt dorosłych obu płci utrzymywanych na diecie HF był wyższy niż u tych osobników utrzymywanych na diecie LF. Dieta niskotłuszczowa ograniczała prozapalną aktywność adipocytów. Natomiast wartości temperatury ciała zwierząt bytujących na diecie HF był niższe niż u zwierząt bytujących na diecie LF, prawdopodobnie w wyniku zmian

metabolicznych zachodzących w tkance tłuszczowej, co wymaga dalszych badań eksperymentalnych.

VI .1. *Aspekty praktyczne pracy*

Według wiedzy autorki do tej pory w literaturze naukowej brakuje wyników badań eksperymentalnych, które przedstawiałyby wpływ pulsacyjnego pola elektromagnetycznego na aktywność biologiczną i żywotność szczurzych komórek macierzystych izolowanych z tkanki tłuszczowej (ADSCs). Komórki macierzyste pochodzące z tkanki tłuszczowej (ASCs) są źródłem wyjściowym do różnicowania w kierunku adipocytów, osteoblastów, chondrocytów, miocytów czy neurocytów. Najnowsze prace z odziaływaniem pulsacyjnego pola elektromagnetycznego o niskiej częstotliwości (1mT, 50Hz) na ludzkie komórki macierzyste pochodzące z tkanki tłuszczowej (hASCs) przedstawiają proliferację komórek na wczesnym etapie i indukcję różnicowania osteogennego [42].

Realizacja projektu badawczego opierała się na zwierzęcym modelu otyłości z udziałem szczurów. Zwierzęta doświadczalne były źródłem do izolacji komórek ADSCs, różniły się wiekiem (oseski i osobniki dorosłe), płcią (szczury męskie i żeńskie) oraz kalorycznością spożywanej karmy (niskotłuszczowa - LF i wysokotłuszczowa – HF). Oprócz eksperymentów z hodowlą ADSCs stymulowanych PEMF o następujących parametrach: częstotliwość 7 Hz, indukcja magnetyczna 30 mT, 4 h dziennie, 3 krotnie w odstępach 24 godzin. Równolegle mierzono poziom adipokin i mediatorów zapalenia w surowicach zwierząt wykorzystanych do izolacji ADSCs. We wszystkich eksperymentalnych grupach zwierząt wykonywano także pomiar glikemii i temperatury ciała. Pomiar parametrów fizjologicznych i patofizjologicznych z poszczególnych grup badawczych pozwolił na ocenę efektów indukowanych pulsacyjnym polem elektromagnetycznym o niskiej częstotliwości w hodowli ADSCs. Komórki były izolowane od zwierząt w różnym wieku, odmiennej płci, utrzymywanych na karmie niskotłuszczowej i wysokotłuszczowej.

Działanie PEMF powodowało indukcję apoptozy w komórkach, zmianą profilu produkowanych adipokin i cytokin, co w przyszłości mogłoby przyczynić się do

praktycznego wykorzystania PEMF, jako czynnika terapeutycznego w zaburzeniach metabolicznych i procesie zapalanym. W przyszłości planowane są badania nad wpływem ekspozycji PEMF na proces różnicowania komórek ADSCs w kierunku adipocytów.

VII. Streszczenie w języku polskim

Otyłość u dzieci i dorosłych jest obecnie głównym problemem zdrowotnym, który stanowi wyzwanie dla zdrowia publicznego. Wzrost otyłości w dzisiejszych czasach próbuje się tłumaczyć m.in.: względami ewolucyjnymi. Nadmierna konsumpcja produktów wysokotłuszczowych i bogatych w cukry proste połączona z brakiem aktywności fizycznej jest niezgodna z hipotezą oszczędnego genotypu (Neel J. 1962). Hipoteza ta zakłada, że nasi przodkowie żyjący w warunkach przewlekłego niedoboru pożywienia posiadali genotypy determinujące do gromadzenia energii w postaci zapasów. Te korzystne dawniej genotypy przetrwały do czasów współczesnych, kiedy to organizm przebywa w środowisku bez ograniczonego dostępu do pożywienia, co może prowadzić do nadmiernego gromadzenia energii w postaci tłuszczu trzewnego. Właściwa masa ciała jest rezultatem utrzymania homeostazy wynikającej ze zbilansowanego przypływu i wydatkowania energii. Brak równowagi na korzyść dostarczania energii może być spowodowany przez czynniki genetyczne i środowiskowe, prowadząc do rozwoju otyłości. Udział czynników genetycznych podkreślają badania populacyjne, wykazujące że otyli rodzice częściej niż rodzice z prawidłową masą ciała posiadają otyłe dzieci. Postęp techniczny spowodował zmianę trybu życia na bardziej wygodny i mało aktywny, jednakże nie zaniknął nasz „oszczędny genotyp". Osoby z nadwagą częściej są narażone na wystąpienie chorób cywilizacyjnych – sercowo-naczyniowych, cukrzycy typu 2 oraz nowotworów. Czynniki środowiskowe skorelowane z występowaniem otyłości wśród dzieci i osób dorosłych są następujące: nieodpowiednie nawyki żywieniowe rodziców, brak wspólnego spożywanie posiłków, łatwy dostęp do żywności typu „fast food", słodyczy i napojów słodzonych, zbyt niskie spożycie warzyw i owoców oraz produktów pełnoziarnistych w połączeniu z niskim lub całkowitym brakiem aktywności fizycznej. Nadwaga i otyłość, w szczególności u dzieci, ma istotny wpływ na kondycję fizyczną i psychiczną.

Zasadniczym celem przeprowadzonych badań była analiza wpływu ekspozycji pulsacyjnego pola elektromagnetycznego (PEMF) o niskiej częstotliwości na żywotność i metabolizm komórek macierzystych pochodzących z tkanki tłuszczowej (ADSCs), izolowanych od szczurów utrzymywanych na diecie niskotłuszczowej i wysokotłuszczowej. W badaniach aktywności sekrecyjnej ADSCs eksponowanych na PEMF oznaczano produkowane przez komórki adipokiny takie jak rezystyna, leptyna, adiponektyna oraz mediatory reakcji zapalnej techniką ELISA.

Najbardziej znaczące efekty działania PEMF uzyskano w hodowli ADSCs izolowanych od dorosłych otyłych samic utrzymywanych na diecie wysokotłuszczowej (HF). W tym przypadku stymulacja indukowała najintensywniej apoptozę w porównaniu do innych hodowli ADSCs otrzymanych od zwierząt różniących się wiekiem, płcią i rodzajem diety. Oddziaływanie PEMF wykazało także korzystny wpływ na produkowaną adiponektynę (wzrost) oraz IL-6 (spadek) w hodowli ADSCs izolowanych od osesków płci żeńskiej utrzymywanych na diecie HF. Adiponektyna wykazuje działanie przeciwzapalne, przeciwmiażdżycowe i poprawia insulinowrażliwość komórek. W otyłości notuje się ujemną korelację tego hormonu. Natomiast IL-6 stymuluje produkcję białek ostrej fazy i nasila proces zapalny w otyłości. Szczególnie wrażliwe na działanie PEMF okazały się hodowle ADSCs pochodzące od samic utrzymywanych na diecie HF.

Uzyskane wyniki badań doświadczalnych prowadzonych na hodowlach ADSCs poddanych działaniu pola elektromagnetycznego mogą przyczynić się w przyszłości do praktycznego wykorzystania oddziaływania pulsacyjnego pola elektromagnetycznego o niskiej częstotliwości jako nieinwazyjnego czynnika fizycznego, który korzystnie wpływa na metabolizm komórkowy, proces zapalny i żywotność komórek

VIII. Streszczenie w języku angielskim

Childhood and adult obesity is currently the main health problem that poses a challenge to public health. The high prevalence of obesity in modern times might be partly explained by evolutionary reasons. Excessive consumption of high-fat and sugar-rich products combined with a lack of physical activity is incompatible with the “thrifty-gene” hypothesis (Neel J. 1962). This hypothesis assumes that our ancestors living in times of chronic starvation had genetic variants leading to the accumulation of adipose stores. These previously beneficial genotypes have survived to modern times when the body resides in the

environment with unlimited access to food, leading to the excessive accumulation of energy, especially in the form of visceral adipose tissue. The right body mass is the result of maintaining homeostasis achieved from a balanced supply and energy expenditure. The imbalance in favour of energy supply can be caused by genetic and environmental factors leading to the development of obesity. The contribution of genetic factors is emphasised by population studies showing that obese parents tend to have obese children more often than parents with normal weight. Industrialisation has resulted in a change of lifestyle to a more comfortable and less active, however, our "thrifty genotype" has not disappeared. Overweight people are prone to lifestyle diseases – cardiovascular diseases, diabetes type 2 and cancer. Environmental factors correlated with the occurrence of obesity among children and adults are as follows: inappropriate parenting habits, lack of shared meals, easy access to fast food, sweets and sugary drinks, inadequate intake of vegetables, fruits and whole grains in combination with low or no physical activity. Overweight and obesity, especially in children, has a significant impact on physical and mental condition.

The main aim of the research was to examine the influence of low-frequency pulsatile magnetic field (PEMF) exposure on the viability and metabolism of adipose derived stem cells (ADSCs) isolated from rats maintained on low and high-fat diets. Adipokines, such as resistin, leptin and adiponectin as well as inflammatory mediators were determined by ELISA method to assess the secretion activity of ADSCs exposed to PEMF.

The most significant effects of PEMF were obtained in ADSCs cultures isolated from adult obese female rats maintained on a high fat diet (HF). The PEMF stimulation in this case induced the most intense apoptosis in comparison to other ADSCs cultures isolated from animals differing in age, sex and diet type. The PEMF also showed a beneficial effect on the secretion of adiponectin (increase) and IL-6 (decrease) in ADSCs isolated from female nursing rats maintained on the HF diet. Adiponectin has anti-inflammatory, anti-atherosclerotic activity and improves cellular insulin sensitivity. Obesity is associated with a negative correlation of this hormone. IL-6, on the other hand, stimulates the production of acute phase proteins and intensifies the inflammatory process in obesity. The ADSCs from females kept on the HF diet proved to be particularly sensitive to the influence of PEMF.

The experiments conducted on ADSCs cultures exposed to the electromagnetic field may contribute in the future to the practical use of the pulsed low frequency electromagnetic field as a non-invasive physical factor that enhances cell metabolism, inflammation and cell viability.

IX. Piśmiennictwo

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X. Aneks

A. Pełne teksty publikacji

1. *Changes in viability of rat adipose-derived stem cells isolated from abdominal/perinuclear adipose tissue stimulated with pulsed electromagnetic field. Baranowska A, Skowron B, Nowak B, Ciesielczyk K, Guzdek P, Gil K, Kaszuba-Zwoińska J. J Physiol Pharmacol. 2017 Apr;68(2):253-264*

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3. *Obesity related adipokines release in rat adipose derived stem cell cultures influenced by pulsed electromagnetic field. Baranowska A, Skowron B, Gil K, Kaszuba-Zwoińska J. Folia Med Cracov. Vol. LVIII, 2, 2018: 131–145.*

4. *Effect of the pulsed electromagnetic field on the release of inflammatory mediators from adipose-derived stem cells (ADSCs) in rats. Agnieszka Baranowska, Beata Skowron, Krzysztof Gil, Jolanta Kaszuba-Zwoińska, Folia Med Cracov Vol. LVIII, 4, 2018: 21–34.*

B. Oświadczenia współautorów

JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2017, 68, 2, 253-264
www.jpp.krakow.pl

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CHANGES IN VIABILITY OF RAT ADIPOSE-DERIVED STEM CELLS ISOLATED FROM ABDOMINAL/PERINUCLEAR ADIPOSE TISSUE STIMULATED WITH PULSED ELECTROMAGNETIC FIELD

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Previous experiments demonstrated that low-frequency electromagnetic field (LF-EMF) may activate cellular death pathways in proliferating cells. Therefore, we hypothesized that LF-EMF may also influence viability of highly proliferating undifferentiated adipose-derived stem cells. Obesity is classified as a civilization disease; its etiopathogenesis is presumed to include both genetic predisposition and influence of modified environmental factors, such as unbalanced diet with excess calories and/or too low physical activity. Obesity may lead to a number of metabolic disorders, including type 2 diabetes mellitus, cardiovascular diseases (associated with atherosclerosis) related to primary hypertension and ischemic heart disease, myocardial infarction and other complications. The aim of this study was to verify if LF-EMF alters viability parameters of adipose-derived stem cells (ADSCs) isolated from rats, cultured *in vitro* and exposed to pulsed electromagnetic field (PEMF; 7 Hz, 30 mT). ADSCs were obtained from healthy rats and animals with experimentally-induced obesity, both males and females, pups and adults. The animals were fed with chow with either low (LF diet) or high fat content (HF diet) for 21 days. Then, ADSCs were isolated from extracted adipose tissue and used to establish cell cultures. ADSCs from the first passage were exposed to PEMF three times, 4 hours per exposure, at 24-h intervals (experimentally developed protocol of PEMF stimulation). 24 hours after the last exposure to PEMF, viability parameters of ADSCs were analyzed by flow cytometry (FCM). The study demonstrated that LF diet exerted a protective effect on PEMF-exposed ADSCs, especially in the case of male and female pups. In turn, the proportion of early apoptotic cells in PEMF-treated ADSC cultures from adult female rats maintained on HF diet turned out to be significantly higher than in other experimental groups.

Key words: *adipose-derived stem cells, pulsed electromagnetic field, obesity, adipose tissue, low fat diet, high fat diet*

INTRODUCTION

Low-frequency electromagnetic field (LF-EMF) was shown to modulate several cellular processes, including proliferation, differentiation and viability of many cell types in an intensity- and field type-dependent manner. Due to its documented beneficial biological effects, LF-EM is commonly used in medicine for magnetotherapy, and recently also as tumor treating field (TTF) therapy, a novel antimitotic electric field-based treatment for glioblastoma (1).

Biological effects of EMF documented in living organisms, tissues and cell lines are heterogeneous and seem to depend on cell type, electromagnetic field characteristics, such as range, intensity, waveform and time of exposure. Nevertheless, many experiments demonstrated that EMF may induce changes in various metabolic pathways (2, 3). Previous studies involving variable cell types showed that EMF may interfere with nearly all cellular processes, affecting proliferation, inducing changes in signal transduction pathways, causing DNA damage, modulating function and viability of immune cells, and exerting tumoricidal/anti-tumoricidal effects. The most extensively studied of these processes was cell proliferation; electromagnetic stimulation was shown to impair cell division *via* activation of cell death pathways (4-13).

Apoptosis, i.e. programmed controlled cell death, plays a key role in tissue and organ development, as well as during cell turnover within mature tissues. Apoptosis is distinct from necrosis which is an uncontrolled cell death resulting from acute injury, lysis, inflammation, tissue damage or carcinogenesis (14, 15).

Stem cells can be found virtually in whole body, including bones, cartilage, fat, tendons, muscles and bone marrow. They control tissue regeneration and healing (16, 17). Adipose tissue (AT) is a heterogeneous body compartment with endocrine properties, playing complex role in maintenance of homeostasis. Aside from being energy resource, AT interferes with various metabolic pathways, as well as with the endocrine and immune system (18). Mature adipocytes represent approximately 30% of body fat. Aside from adipocytes, adipose tissue contains also multipotent stem cells, nerve fibers, small blood vessels, fibroblasts and preadipocytes at various stages of differentiation (19).

Obesity is classified as a civilization disease; its etiopathogenesis is presumed to include both genetic predisposition and influence of modified environmental factors, such as unbalanced diet with excess calories and/or too low physical activity. Obesity may lead to a number of metabolic disorders, including type 2 diabetes mellitus, cardiovascular diseases (associated with atherosclerosis) related to primary hypertension and leading to ischemic heart disease, myocardial infarction and other complications (20).

In this study, we used animal model for obesity, namely Wistar rats of various age (both pups and adults), exposed to high- (HF) or low-fat (LF) diet. ADSCs were isolated from two different compartments, subcutaneous adipose tissue in females and perinuclear fat in males, to reflect sex-specific differences in body fat distribution. ADSCs were cultured *in vitro*; cells from the first passage were exposed to PEMF (7 Hz, 30 mT) for 4 hours at 24-h intervals, which corresponded to a total of three exposures during a 3-day period. 24 hours after the last exposure, PEMF-induced changes in ADSC viability were studied by means of flow cytometry (FC).

The aim of this study was to analyze PEMF-induced changes in viability parameters of *in vitro* cultured ADSCs from female and male rats of various age.

MATERIALS AND METHODS

Animals

The studies included Wistar rats (WistarKrf: (Wi) Wu) because of the physiological parameters analysis of the particular researched groups. The animals were obtained from the central animal house at the Faculty of Pharmacy, Jagiellonian University in Cracow (breeder registration no. 0056). Upon admission to the local animal house at the Department of Pathophysiology, the animals were allowed to adapt to new living conditions for seven days (as recommended by Szarek *et al.* adaptation period should last 5 – 15 days). During this period, female rats with pups were housed under specific hygienic conditions: in an air-conditioned room with air temperature maintained at 21 – 25°C, 50 – 60% humidity, with 12-h day-night cycle and unlimited access to water and food. Protocol of the study was approved by the Local Bioethics Committee at the Jagiellonian University in Cracow, Poland (decision no. 84/2014).

Study groups and experimental procedures

The study included the following eight groups of rats, identified on the basis of sex, age (pups kept with their mothers and adult animals) and diet (LF and HF diet):

Groups 1 and 3 - female and male pups kept with their mothers and receiving LF diet for 21 days of the experiment;

Groups 2 and 4 - female and male pups kept with their mothers and receiving HF diet for 21 days of the experiment;

Groups 5 and 7 - adult female and male rats receiving LF diet for 21 days of the experiment;

Groups 6 and 8 - adult female and male rats receiving HF diet for 21 days of the experiment.

Animals from all experimental groups underwent the following procedures:

Procedure 1. Animal weighing. Body weight was measured twice a week using electronic scales (Scale OHAUS NAWIGATOR 2100/0.1 g)

Procedure 2. Temperature measurements. Body temperature was measured twice a week. To minimize stress and pain associated with the procedure, the measurements were taken with an infrared thermometer (Anima Vivari). The data on body weight and body temperature obtained within the framework of this project have already been published elsewhere (21).

Procedure 3. Diet. Pups and adult rats maintained on HF diet received chow with higher contents of fat and protein (32% and 22%, respectively) and less carbohydrates (40%) (VERSELE-LAGA Opti Life Adult Active) than in the LF chow (protein 25%, fat 8%, carbohydrates 67%; Labofeed B, Pasze Kcynia).

Procedure 4. Euthanasia and tissue specimen collection. On the 21st day of the experiment, all animals were euthanized by an overdose of anesthetic (Pentobarbital, Morbital, Pulawy, 200 mg per kg body weight intraperitoneally), and adipose tissue specimens, subcutaneous tissue in females and periepididymal fat in males, were harvested. The specimens were collected in sterile conditions, under a laminar flow, using sterile surgical instruments (ASHE/A, mp type steam sterilizer, 15 min at 121°C).

Isolation of adipose-derived stem cells and cell culture

Adipose tissue specimens were collected aseptically as described previously in adult BalbC mice (22). The specimens were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, Germany) containing 1% penicillin/streptomycin solution (Sigma-Aldrich, Germany), homogenized and digested with me type collagenase (1 mg/mL; Gibco by Life Technologies, USA) at 37°C for 1 hour. Enzymatic activity was neutralized with a Dulbecco's modified eagle's medium (DMEM Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS; Gibco by Life Technologies, USA) and 1% penicillin/streptomycin solution. Subsequently, the reaction cocktail obtained from disintegrated tissues was filtered (filters with 100-µm pore diameter, Fisher Scientific, USA) and centrifuged at 300 × g for 10 min to obtain a high-density cell pellet. The pellet was suspended in DMEM supplemented with 10% FBS (Gibco by Life Technologies, USA) and 1% penicillin/streptomycin solution (Sigma-Aldrich, Germany), placed in T75 flasks (Corning, Sigma-Aldrich, Germany) and incubated overnight at 37°C in a 5% CO_2 incubator with 90% humidity. After the 24-h incubation, non-attached cells and non-adherent erythrocytes were washed with antibiotic-supplemented PBS. Adherent cells (ADCSs) were suspended in DMEM with 10% FBS and 1% penicillin/streptomycin solution. The medium was changed every 72 h until the cells became confluent. After reaching an 80 – 90% confluence, the cells were placed in trypsin-EDTA solution (0.25% weight/volume; Sigma-Aldrich, Germany), incubated at 37°C for 10 – 15 min, and dissociated by trituration. The trypsin reaction was blocked by addition of DMEM with 10% FBS and 1% penicillin/streptomycin solution, and the cell suspension was centrifuged at 416 × g for 10 min. ADSCs were suspended in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin solution, counted with a hemocytometer, seeded on a 96-well plate (Corning, Sigma-Aldrich, Germany) in triplicate at density of 0.25×10^6 cells/ml per sample, and cultured at 37°C at humidified atmosphere of 5% CO_2.

Magnetic stimulation of adipose-derived stem cells cultures

PEMF stimulation was started after 24 hours of ADSC culture. A generator produced and kindly provided by the Institute of Electron Technology (Cracow, Poland) generated pulsed electromagnetic field with a frequency 7 Hz at a flux density of 30 mT inside the cell culture incubator. The reason behind choosing this particular PEMF frequency was its minimal heating effect and the fact that similar frequency EMF could be generated by household power devices. Furthermore, electromagnetic fields with similar parameters are used for medical purposes, e.g. magnetotherapy. A 96-well plate with ADSC culture was placed in the generator's pocket and exposed

to EMF for 4 hours daily at 24-h intervals, during three consecutive days; this corresponded to a total of three PEMF exposures over the 3-day period. Control samples were placed in the same incubator but at a 35-cm distance from the generator, additionally protected from PEMF exposure with an aluminum foil shield.

Cell viability assessment by means of flow cytometric analysis

Twenty-four hours after the last exposure to PEMF, ADSCs were detached from culture plates by trypsinization, washed three times with cold PBS (Sigma-Aldrich, Germany) and then stained exactly as described in FCM manufacturer's procedure. The proportion of apoptotic cells was determined with allophycocyanin (APC) conjugated annexin V (AnV-APC, BD, Pharmingen TM, USA). Propidium iodide (PI, BD, Pharmingen TM, USA) was used as a standard flow cytometric viability probe to distinguish necrotic cells from viable ones (23). AnV-APC-positive, PI-negative cells (AnV+) were classified as early apoptotic, AnV-APC- and PI-positive cells (AnV+PI+) as late apoptotic, and AnV-APC-negative, PI-positive cells (PI+) as necrotic. For staining, ADSCs were washed twice with cold PBS and resuspended in 1 × binding buffer (BD, Pharmingen TM, USA) at a concentration of 1 × 10^6 cells/ml. Then, the solution (100 µl) was transferred to a 5-ml culture tube, and AnV-APC and PI were added, 5 µl each. The cells were vortexed gently and incubated in darkness at room temperature for 15 min. After adding 1 × binding buffer (400 µl), the cells were analyzed on a FACS CALIBUR flow cytometer (Becton Dickinson, San Jose, CA) using Cell-Quest

Table 1. Viability of ADSCs originated from female and male pups maintained on low fat (LF) or high fat (HF) diet. Results for PEMF-exposed cells and cells from non-exposed control cultures (means ± S.D.). Differences significant at $P < 0.05$ marked with asterisks (*).

Comparison of viability parameters of rat ADSCs PEMF-exposed and controls (means ± S.D.)						
Experimental group	Percentage of early apoptotic cells. PEMF-exposed	Percentage of early apoptotic cells. Controls	Percentage of late apoptotic cells. PEMF-exposed	Percentage of late apoptotic cells. Controls	Percentage of necrotic cells. PEMF-exposed	Percentage of necrotic cells. Controls
Female pups on LF diet	3.85 ± 1.32	4.02 ± 1.49	11.84 ± 4.58	1.49 ± 1.84	1.13 ± 0.59*	6.97 ± 0.64
Male pups on LF diet	5.06 ± 3.08	4.47 ± 1.18	12.35 ± 4.66*	12.35 ± 4.66	0.58 ± 0.54	0.59 ± 0.45
Female pups on HF diet	8.74 ± 3.01*	10.92 ± 3.11	12.15 ± 2.44*	13.66 ± 1.23	0.56 ± 0.20*	0.74 ± 0.12
Male pups on HF diet	3.36 ± 1.48*	4.62 ± 1.93	8.42 ± 3.06	8.90 ± 2.99	1.20 ± 0.74	1.09 ± 0.47

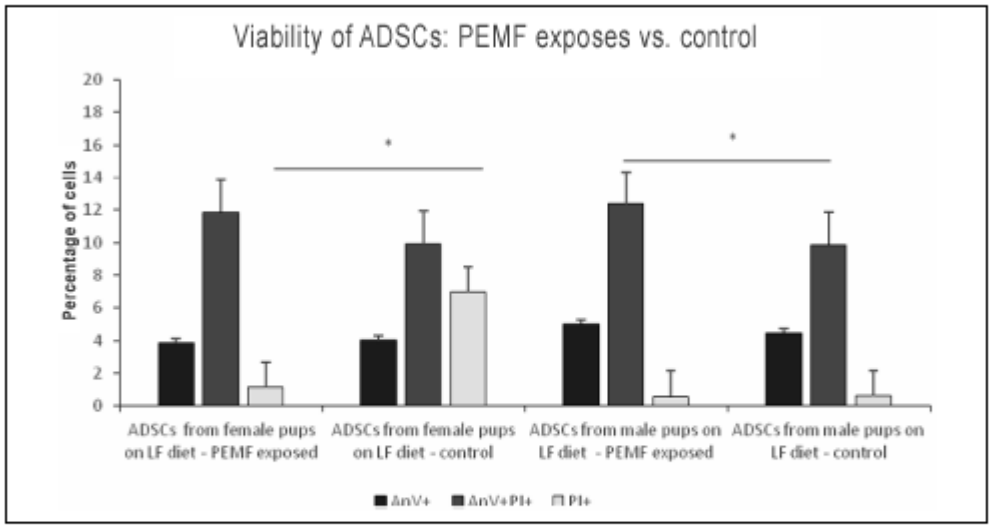

Fig. 1. Viability of ADSCs from female and male pups maintained on LF diet (groups 1 and 3). Results for PEMF-exposed cells and cells from non-exposed control cultures, presented as means (± S.D.). Statistical significance of intergroup differences verified with Student's t-test; differences significant at $P < 0.05$ marked with asterisks (*).

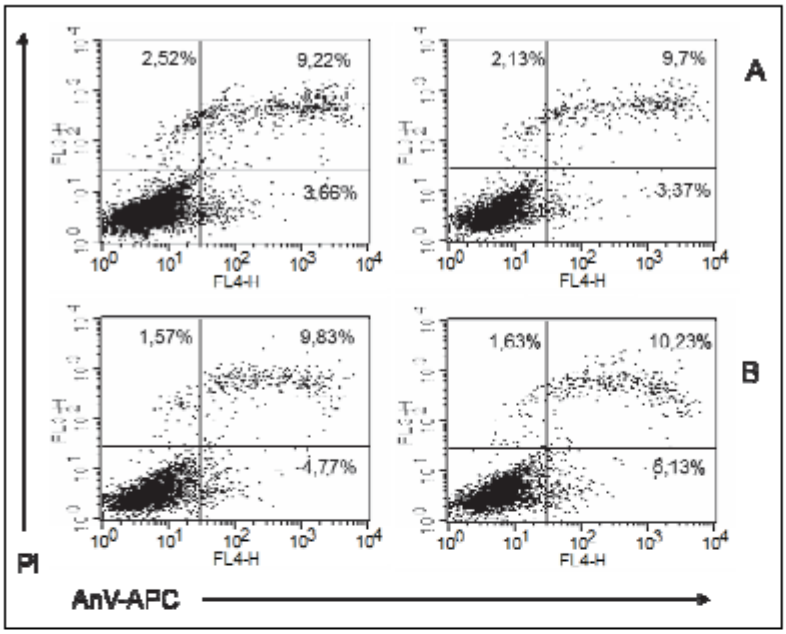

Fig. 2. Representatives log fluorescence dot plots of ADSCs originated from female pups grown on low fat diet in control (A) and PEMF exposed cells (B). Log propidium iodide (PI) fluorescence (FL3) versus log annexin V-APC (AnV-APC) fluorescence (FL4) dot plots of ADSCs.

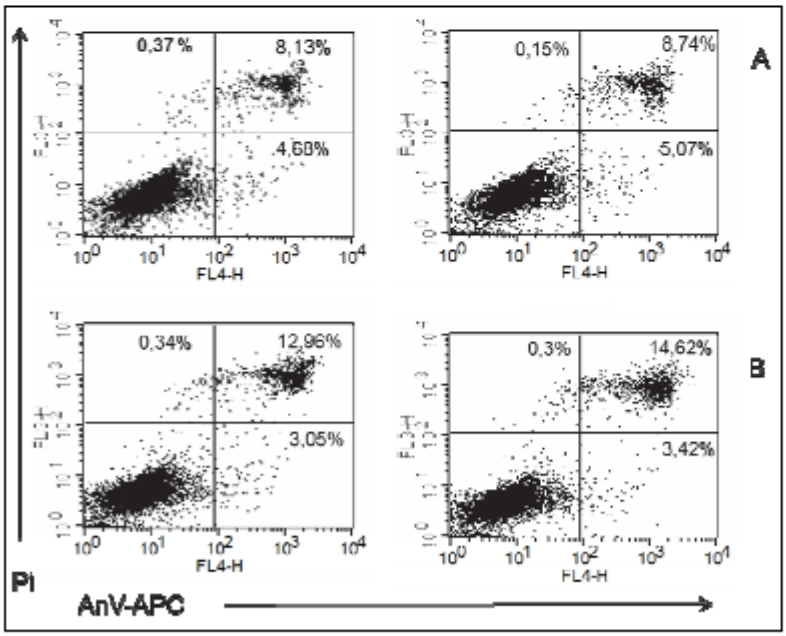

Fig. 3. Representatives log fluorescence dot plots of ADSCs originated from male pups grown on low fat diet in control (A) and PEMF exposed cells (B). Log propidium iodide (PI) fluorescence (FL3) versus log annexin V-APC (AnV-APC) fluorescence (FL4) dot plots of ADSCs.

software to calculate the proportion of ADSCs representing various types. Unstained cells, cells stained with AnV-APC alone (for FL-4 fluorescence) and cells stained with PI alone (detected in FL-3) were used as the controls to set up compensation and appropriate quadrants. At least 10,000 events were acquired for each sample (16).

Statistical analysis

The results were presented as means (±) their standard deviations (S.D.). Intergroup comparisons were conducted with Student's t-test. The differences were considered statistically significant at $P < 0.05$.

RESULTS

The study included eight groups of rats, differing in terms of age, sex and diet. Animals maintained on fat- and carbohydrate-rich diet, both pups and adults, presented with obesity and lipid disorders (21). ADSCs isolated from various anatomical regions were cultured *in vitro* and exposed to PEMF, and then their viability parameters were determined.

Pulsed electromagnetic field-induced changes in viability of adipose-derived stem cells from female and male pups maintained on low fat diet

While PEMF-exposed ADSC cultures from female pups contained a larger proportion of late apoptotic (AnV+PI+) cells and a significantly lower percentage of necrotic cells (PI+) than the non-exposed control cultures, virtually no intergroup differences were found in the percentages of early apoptotic cells (AnV+). In ADSC cultures from male pups, exposure to PEMF resulted in a significant increase in the proportion of late apoptotic cells (AnV+PI+), as well as in a mild, statistically insignificant, increase in the percentage of early apoptotic cells (AnV+); virtually no changes were observed in the case of necrotic cells (PI+) (*Figs. 1, 2, 3*); *Table 1*.

Pulsed electromagnetic field-induced changes in viability of adipose-derived stem cells from female and male pups maintained on high fat diet

PEMF-exposed ADSC cultures from female pups contained significantly smaller proportions of early apoptotic (AnV+), late apoptotic (AnV+PI+) and necrotic cells (PI+) than the non-exposed control cultures. Similar pattern of PEMF-induced viability changes was observed in the case of ADSC cultures from male pups, except from an increase in the percentage of necrotic cells. However, only the difference in the proportion of early apoptotic cells among ADSCs from male pups turned out to be statistically significant (*Figs. 4, 5, 6*); *Table 1*.

Pulsed electromagnetic field-induced changes in viability of adipose-derived stem cells from adult female and male rats maintained on low fat diet

PEMF exposure of ADSCs from adult female rats maintained on LF diet resulted in a statistically insignificant decrease in the proportions of early apoptotic (AnV+) and necrotic (PI+) cells. In the case of ADSC cultures from adult male rats maintained on LF diet, exposure to PEMF contributed to a significant decrease in the percentage of early and late apoptotic cells (AnV+) and (AnV+PI+), respectively), as well as to a significant increase in the proportion of necrotic cells (PI+) (*Figs. 7, 8, 9*); *Table 2*.

Pulsed electromagnetic field-induced changes in viability of adipose-derived stem cells from adult female and male rats maintained on high fat diet

In ADSC cultures from adult female rats maintained on HF diet, exposure to PEMF was reflected by a significant

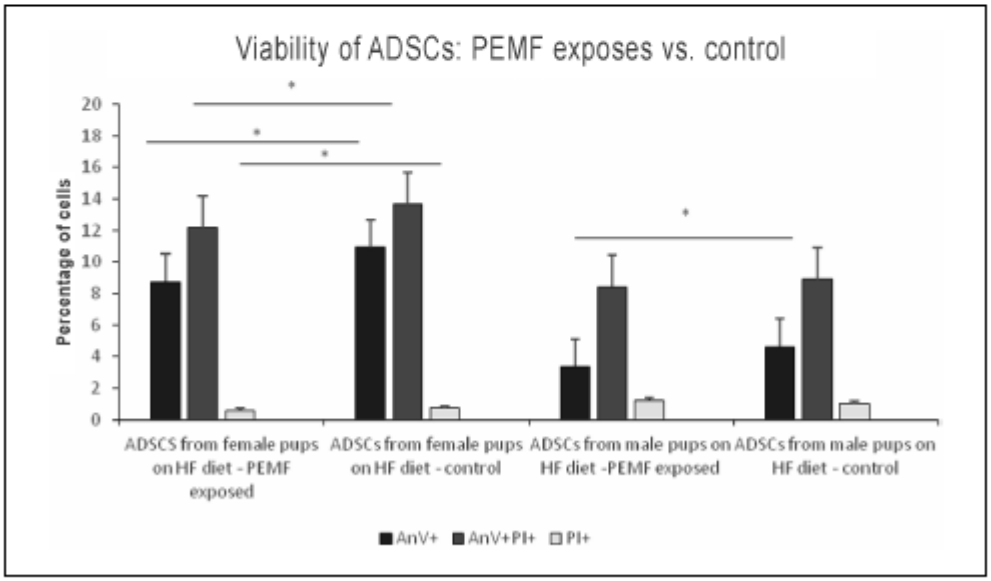

Fig. 4. Viability of ADSCs from female and male pups maintained on high fat diet (groups 2 and 4). Results for PEMF-exposed cells and cells from non-exposed control cultures, presented as means (± S.D.). Statistical significance of intergroup differences verified with Student's t-test; differences significant at $P < 0.05$ marked with asterisks (*).

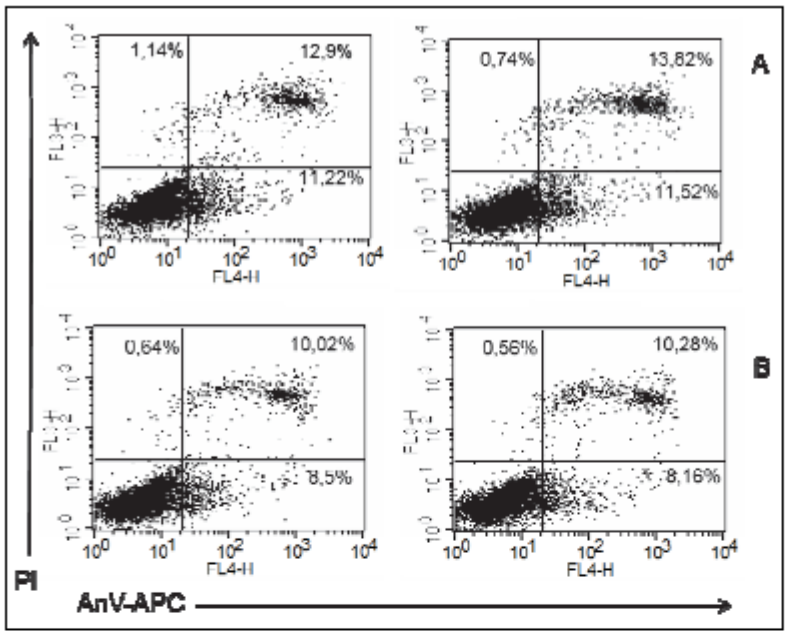

Fig. 5. Representatives log fluorescence dot plots of ADSCs originated from female pups grown on high fat diet in control (A) and PEMF exposed cells (B). Log propidium iodide (PI) fluorescence (FL3) versus log annexin V-APC (AnV-APC) fluorescence (FL4) dot plots of ADSCs.

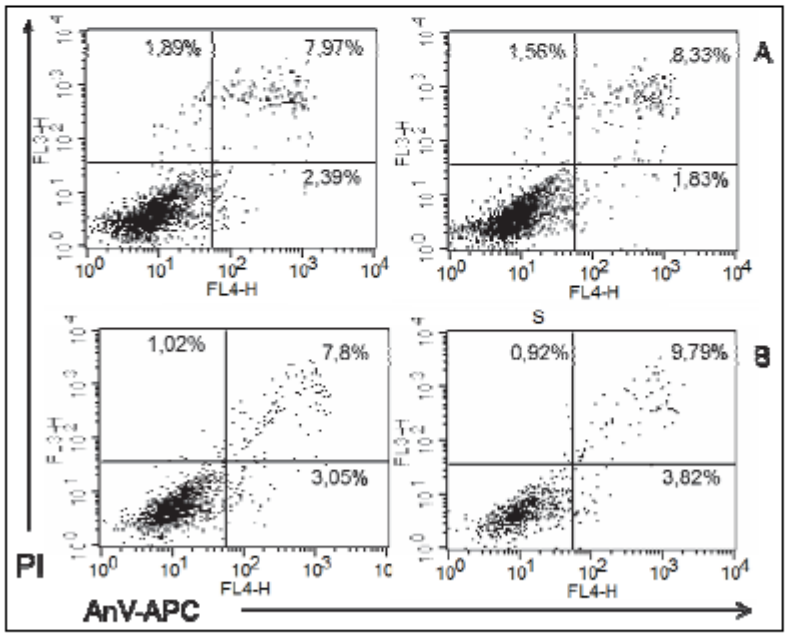

Fig. 6. Representatives log fluorescence dot plots of ADSCs originated from male pups grown on high fat diet in control (A) and PEMF exposed cells (B). Log propidium iodide (PI) fluorescence (FL3) versus log annexin V-APC (AnV-APC) fluorescence (FL4) dot plots of ADSCs.

increase in the proportion of early apoptotic cells (AnV+), as well as by a mild albeit still significant increase in the percentage of necrotic cells (PI+). No statistically significant PEMF-induced changes were observed in the viability parameters of ADSCs from adult male rats maintained on HF diet (*Figs. 10, 11, 12*); *Table 2*.

Table 2. Viability of ADSCs originated from adult female and male rats maintained on low fat (LF) and high fat (HF) diet. Results for PEMF-exposed cells and cells from non-exposed control cultures (means ± S.D.). Differences significant at $P < 0.05$ marked with asterisks (*).

Comparison of viability parameters of rat ADSCs PEMF-exposed and controls (mean ± S.D.)						
Experimental group	Percentage of early apoptotic cells. **PEMF-exposed**	Percentage of early apoptotic cells. **Controls**	Percentage of late apoptotic cells. **PEMF-exposed**	Percentage of late apoptotic cells. **Controls**	Percentage of necrotic cells. **PEMF-exposed**	Percentage of necrotic cells. **Controls**
Adult females on LF diet	9.11 ± 9.79	13.25 ± 10.84	9.40 ± 6.07	9.46 ± 4.05	1.19 ± 0.93	1.72 ± 3.49
Adult males on LF diet	1.91 ± 2.21*	5.41 ± 3.83	4.16 ± 5.54*	10.67 ± 6.71	29.72 ± 5.68*	7.68 ± 5.05
Adult females on HF diet	17.48 ± 10.52*	8.84 ± 5.30	5.92 ± 2.06	6.37 ± 1.83	0.82 ± 0.47*	0.50 ± 0.28
Adult males on HF diet	4.60 ± 3.53	5.20 ± 3.96	6.35 ± 3.11	6.39 ± 2.75	0.52 ± 0.52	0.38 ± 0.15

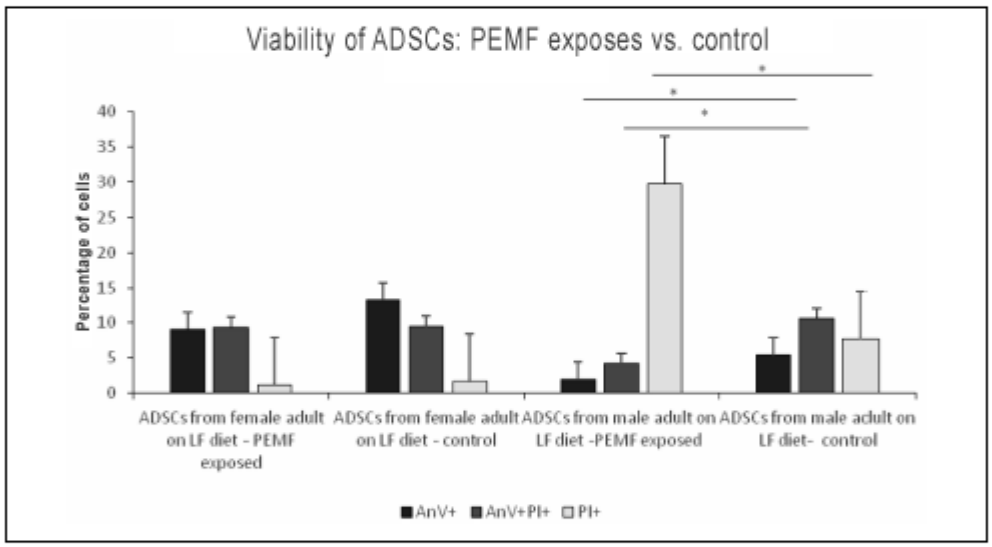

Fig. 7. Viability of ADSCs from adult female and male rats maintained on low fat diet (groups 5 and 7). Results for PEMF-exposed cells and cells from non-exposed control cultures, presented as means (± S.D.). Statistical significance of intergroup differences verified with Student's t-test; differences significant at $P < 0.05$ marked with asterisks (*).

DISCUSSION

Proportion of apoptotic and necrotic cells among adipose-derived stem cells from female and male pups maintained on low fat and high fat diet

To the best of our knowledge, none of the previously published experiments analyzed the issue of obesity starting from *in vitro* ADSC cultures and changes in their viability in response to an external stimulus, such as exposure to electromagnetic field. Typical frequencies of magnetic fields used for medical purposes range between 10 Hz and 20 Hz, and never exceed 100 Hz (24). In our present study, exposure to PEMF (frequency 7 Hz, magnetic induction 30 mT, 3 daily sessions, each lasting 4 hours) contributed to sex-specific changes in the viability of *in vitro* cultured ADSCs. Whereas exposure to PEMF seemed to improve the viability of ADSCs from female pups maintained on LF diet, as shown by a decrease in the percentage of necrotic cells, an opposite effect, namely an increase in the proportion of late apoptotic cells, was observed in cultured cells from male pups. Meng *et al.* (25) investigated effects of high-intensity pulsed electromagnetic field (HI-PEMF, 0.1 Hz, 0.5 – 10 T, five exposures) on growing neural stem cells from neonatal rats. The treatment resulted in an increase in

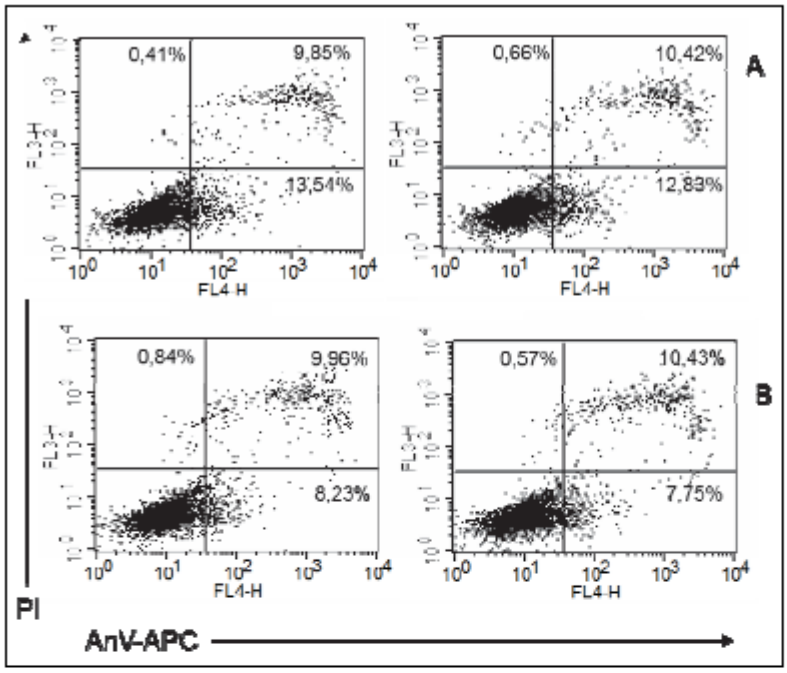

Fig. 8. Representatives log fluorescence dot plots of ADSCs originated from female adult grown on low fat diet in control (A) and PEMF exposed cells (B). Log propidium iodide (PI) fluorescence (FL3) vs. log annexin V-APC (Anv-APC) fluorescence (FL4) dot plots of ADSCs.

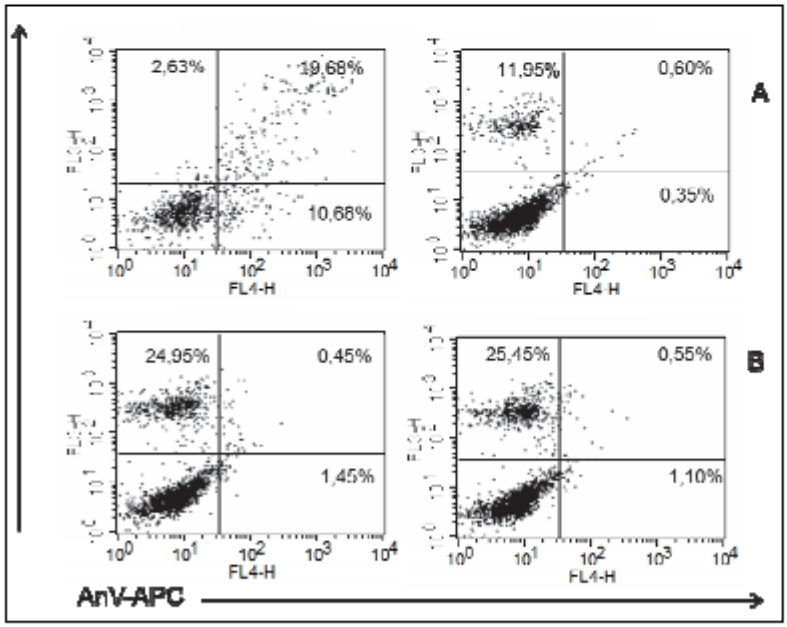

Fig. 9. Representatives log fluorescence dot plots of ADSCs originated from male adult grown on low fat diet in control (A) and PEMF exposed cells (B). Log propidium iodide (PI) fluorescence (FL3) versus log annexin V-APC (Anv-APC) fluorescence (FL4) dot plots of ADSCs.

proliferative activity, particularly evident after exposure to HI-PEMF with magnetic intensity of 4.0 T. However, no statistically significant differences were found in the viability of HI-PEMF-exposed and non-exposed neural stem cells (25). Perhaps, discrepancies between the abovementioned findings and our hereby presented results reflected different parameters of

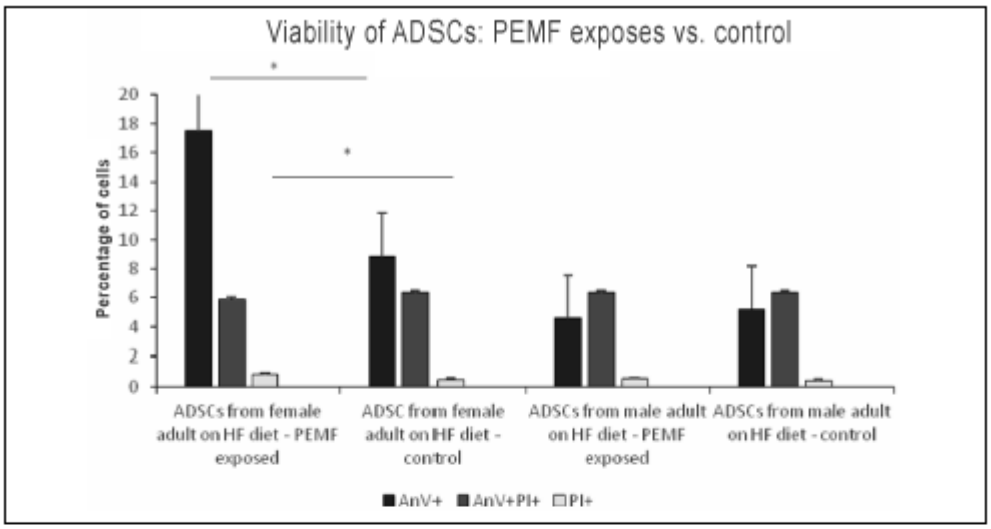

Fig. 10. Viability of ADSCs from adult female and male rats maintained on high fat diet. Results for PEMF-exposed cells and cells from non-exposed control cultures, presented as means (± S.D.). Statistical significance of intergroup differences verified with Student's t-test; differences significant at $P < 0.05$ marked with asterisks (*).

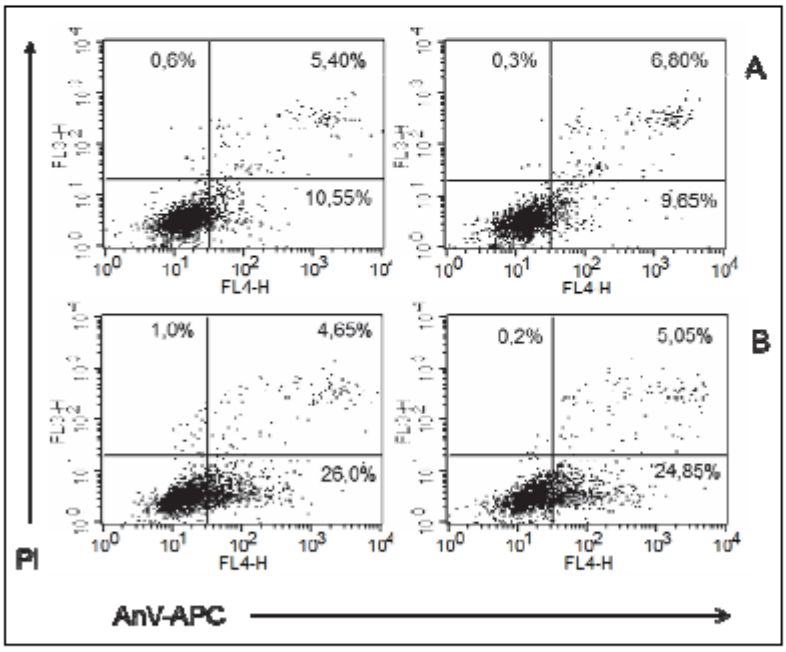

Fig. 11. Representatives log fluorescence dot plots of ADSCs originated from female adult grown on high fat diet in control (A) and PEMF exposed cells (B). Log propidium iodide (PI) fluorescence (FL3) versus log annexin V-APC (AnV-APC) fluorescence (FL4) dot plots of ADSCs.

electromagnetic fields. In another published study, the effects PEMF exposure on the activity and viability of neonatal rat osteoblast-like cells were similar to those documented in our present experiment. However, the authors of this study used PEMF with markedly lower magnetic induction values, 0.06 mT to 0.2 mT. An increase in cell number and proliferative activity was observed solely after exposure to PEMF with 0.2-mT value. The viability of cells exposed to 0.06-mT PEMF was lower than

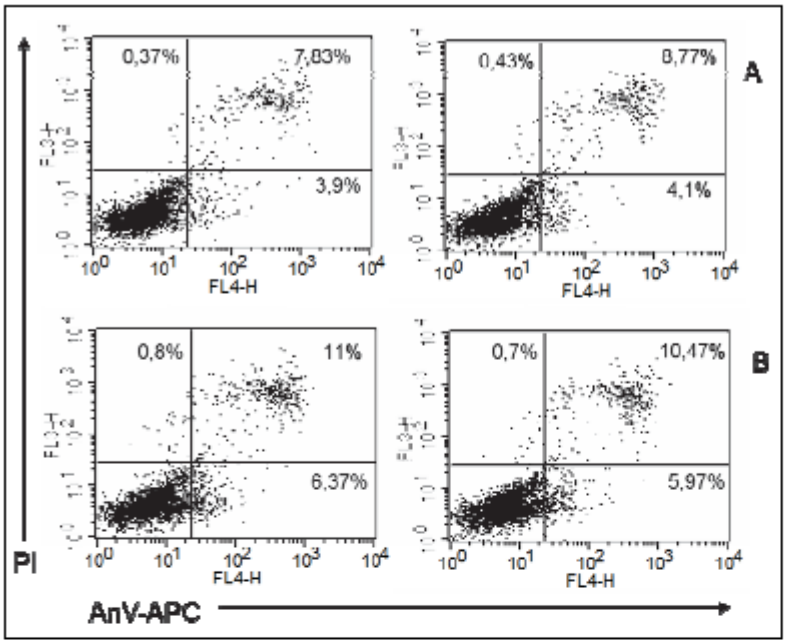

Fig. 12. Representatives log fluorescence dot plots of ADSCs originated from male adult grown on high fat diet in control (A) and PEMF exposed cells (B). Log propidium iodide (PI) fluorescence (FL3) versus log annexin V-APC (AnV-APC) fluorescence (FL4) dot plots of ADSCs.

in the case of control cells, and even more pronounced intergroup differences were observed following exposure to 0.2-mT PEMF (26).

Stem cell-based therapies can be used to treat many diseases, including several types of cancer, musculoskeletal, cardiovascular, metabolic, autoimmune diseases as well as in the treatment of wounds, ulcers, and burns. However, before clinical applications of stem cells, it is essential to isolate and multiply them *in vitro* to obtain a sufficient quantity.

Long term cell culture studies investigating changes in the biological and morphological properties of *in vitro* expanded human adipose tissue-derived stem cells (hADSCs) over 30 passages, showed that long-term expansion leads to significant changes in morphology and affects proliferation kinetics, but hADSCs maintained a prototypical immunophenotype, normal cell cycle, apoptosis regulator function, and possessed a low level of telomerase activity during later passages, what stays in agreement with findings of our studies (27)

Irrespective of sex, maintenance of rat pups on HF diet resulted in excess weight gain and development of lipid disorders (21). In the case of ADSCs from female pups maintained on HF diet, stimulation with low-frequency PEMF (7 Hz, 30 mT) contributed to a decrease in the proportion of early apoptotic, late apoptotic and necrotic cells; in contrast, only a decrease in the percentage of early apoptotic cells was observed in male pups. Quite opposite findings were reported by other authors, according to whom exposure to high-frequency electromagnetic field (900 Hz) enhanced apoptosis in differently localized cells obtained from rat pups. Specifically, Odaci *et al.* analyzed the effects of 60-min in utero exposure to high-frequency electromagnetic field (HF-EMF, 900 Hz) on brain granule cells. They showed that treatment with EMF contributed to a decrease in the proportion of viable cells, inducing apoptosis thereof (28). Similar effects were also reported by Hanci *et al.*, according to whom exposure to EMF enhanced apoptosis of rat testicular cells (29). The observation that exposure to electromagnetic field exerts sex-specific effects on viability of cells from rat adipose tissue seems to be the principal finding of our study. Furthermore, the hereby presented results imply that the effects of PEMF exposure on viability of ADSCs from adipose tissue of obese pups are different from those observed in normal-weight rats.

Proportion of apoptotic and necrotic cells among adipose-derived stem cells from adult female and male rats maintained on low fat and high fat diet

In this study, exposure to PEMF was reflected by a decrease in the proportion of early and late apoptotic ADSCs from male adult rats kept on LF diet, as well as by a concomitant increase in the percentage of necrotic cells. Our findings are consistent with the results published by Xie W. (30), according to whom 2-week PEMF therapy reduced apoptosis in rabbit chondrocytes. Recently, Lou F *et al.* reported the effects of PEMF on mesenchymal cells. According to these authors, PEMF induces differentiation of human mesenchymal stem cells (MSCs) (31), widely used in medicine and tissue engineering. However, in another study, exposure to low-frequency EMF (75 ± 2 Hz, 2 ± 0.2 mT, duration 3 – 40 min) exerted no effects on proliferation and differentiation of subcutaneous fat cells (ASCs) and bone marrow stem cells (BM-MSCs), except from an increase in the proliferation rate of the latter (32). Similar findings were also reported by Lu *et al.*, who analyzed BM-MSCs from adult male and female rats; exposure to PEMF (20 Hz, 2 mT, 6 hours per day for 12 days) promoted osteogenesis but not adipogenesis (33). These findings imply that PEMF exposure exerts an unfavorable effect on AT formation.

In our study, exposure to PEMF altered the proportion of apoptotic and necrotic cells among ADSCs from adult female rats maintained on HF diet. The unfavorable effects of PEMF on

viability of ADSCs from obese female rats were particularly evident, as shown by both a substantial increase in the proportion of early apoptotic cells and somehow less spectacular increase in the percentage of necrotic cells. Surprisingly, similar effects were not observed in ADSC cultures from male rats maintained on high fat diet.

Associations resulting from high fat diet during pregnancy in rats and adipokines level in obese humans

There are research data related to our findings in term of used diet in rats and its influence on hormones release, which show that, feeding a HF diet to dams during pregnancy and lactation disturbs plasma adiponectin levels and protein expression, both in female and male offspring; it is lowered adiponectin secretion and protein expression in the female whereas in male it is increased. Observed consequence is disruption of steroid secretion in offspring, towards testosterone and in females, and oestradiol in males (34).

Obesity in term of pathogenesis represents disease with participation of several adipokines such as leptin, adiponectin, resistin, tumor necrosis factor-α (TNF-α) support hepatic and peripheral carbohydrate homeostasis, insulin sensitivity, lipid metabolism, energy expenditure, hematopoiesis, wound healing, fertility and vascular hemodynamics in the pathomechanism.

As it regards obese patients with high BMI (BMI ≥ 40 kg/m^2) the study has shown that adiponectin has a positive effect on platelets activation through a possible reduction in sP-selectin and an elevated sE-selectin what revealed perspective about the endothelium stimulation, a higher risk of endothelial damage in morbidly obese patients.

The higher leptin level, leptin-to-adiponectin ratio and simultaneously lower concentration of leptin receptor were associated with leptin resistance, possible future risk of insulin resistance and development of diabetes type 2 in humans (35).

Our observation that PEMF induces apoptosis in cultured ADSCs from obese rats (especially in the cells from subcutaneous adipose tissue of females maintained on HF diet), implies that low-frequency electromagnetic field may be considered as an option of non-invasive anti-obesity treatment, free from adverse effects inherent to pharmacotherapy, e.g. drug interactions. However, this attractive hypothesis needs to be verified in future studies; specifically, future research should focus on the influence of PEMF on the differentiation of ADSCs to adipocytes.

Acknowledgements: The study was conducted at the Department of Pathophysiology, Jagiellonian University Medical College, and supported from the grant no. K/ZDS/006681 from the Jagiellonian University Medical College in Cracow, Poland.

Conflict of interest: None declared.

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Received: November 28, 2016
Accepted: April 24, 2017

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Research Article

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Journal of Animal Research and Nutrition
ISSN 2572-5459

2018
Vol.3 No.1:1

DOI: 10.21767/2572-5459.100039

Low Grade Inflammation in Visceral and Subcutaneous Adipose Tissue Originated from Adipose Derived Stem Cells in Experimental Model of Obesity in Rats under Influence of Pulsed Electromagnetic Field Interaction

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Rec date: January 01, 2018; **Acc date:** January 16, 2018; **Pub date:** January 19, 2018

Citation: Agnieszka B (2018) Low Grade Inflammation in Visceral and Subcutaneous Adipose Tissue Originated from Adipose Derived Stem Cells in Experimental Model Of Obesity In Rats Under Influence Of Pulsed Electromagnetic Field Interaction. J Anim Res Nutr Vol No 3: Iss no: 1: 1.

Abstract

Objective: Aim of our study was to examine if pulsed electromagnetic field (PEMF) treatment of the adipose derived stem cells (ADSCs) originating from differently localized adipose tissue, affects the pro-inflammatory cytokines and resistin release dependently on age, gender and used diet.

Methods: Cultures of ADSCs originated from pups and adult animals of both gender grown on low fat (LF) and high fat (HF) diet for 21 days of obesity inducing time, after which the adipose tissue and blood samples for serum were collected. ADSCs cultures were exposed to PEMF (7 Hz, 30 mT) thrice, 4 h per day. Proinflammatory cytokines and resistin levels were determined by ELISA.

Results: The serum level of TNFα and IL-6 cytokines in HF diet female and male adults was significantly higher as compared to LF diet serum level. HF diet also caused changes in resistin serum level. ADSCs of PEMF exposed LF diet female pups released more TNFα than unexposed ones. IL-6 level in ADSCs cultures of PEMF exposed female pups was higher than in controls. PEMF exposure of ADSCs of both genders of HF diet pups caused increased secretion of TNFα. On the other hand IL-6 release by ADSCs of female pups upon PEMF treatment was diminished. PEMF exposure of ADSCs of both genders of LF diet pups caused increased secretion of resistin. Different results were obtained in case of HF diet pups ADSCs. ADSCs cultures after PEMF exposure originating from male and female adults produced more resistin.

Conclusion: We found that PEMF exposure can modify metabolic activity of ADSCs.

Keywords: Low grade inflammation; Adipose tissue; Pulsed electromagnetic field; Obesity

Introduction

Nowadays low frequency electromagnetic fields are used in the form of magnetotherapy or magnetostimulation as a non-invasive and simple manner to treat a variety of conditions, like Alzheimer's, Parkinson's disease, multiple sclerosis or many diseases with inflammatory patomechanisms, both in medicine and dentistry [1]. The impact of pulsed magnetic field (PMF) is broad and includes anti-inflammatory, antiallodynic character and also and pro-regenerative effect in rodent models [2-6]. The current study revealed that PEMF exposure can modify metabolic activity of ADSCs. Several epidemiologic studies have implicated visceral fat (VAT) as a major risk factor for insulin resistance, type 2 diabetes mellitus, cardiovascular disease, stroke, metabolic syndrome and death [7]. Obesity is an inflammation of the body, showing signs of inflammation at a low level. In addition, it strongly influences the development of insulin resistance and type 2 diabetes [8]. It is well known that high fat containing diet induces obesity and metabolic disorders in rodent experimental studies [9]. Our experiment shows the ontogenic process in animals of both genders including a modified environmental issue - their diet. The research has shown changes in inflammatory cytokines both in serum and in vivo conditions. The consumption of food rich in fat during pregnancy and lactation predisposes to excessive weight gain in later life [10]. Maternal obesity is recognized to trigger stress factors including pro-inflammatory cytokines and oxidative stress molecules [11]. The accumulated knowledge allows for a statement that obesity plays an important role in the pathogenesis of low-grade inflammation related to metabolic disorders [12]. The adipose tissue (AT) is an endocrine organ which affects, among others, the vascular and immune systems and plays an important function in metabolism [13]. One of the AT roles is secretion of adipokines and cytokines which have a signaling character. The above factors play a significant role in maintaining health but from the other side they also participate in pathophysiology of obesity [14]. AT consists of a fraction of white adipose tissue (WAT) and brown adipose tissue (BAT).

Basic role of WAT is collection of triacylglycerols while BAT takes part in heat production [15]. WAT is responsible for production of bioactive molecules – adipokines, which include leptin, resistin, adiponectin, that affect the body homeostasis [16]. The expression of protein substances is related to the localization of two main fat depots: visceral (VAT) and subcutaneous (SAT) adipose tissues. Results related to the level of proinflammatory molecules showed higher levels in VAT, as compared to SAT, in diabetes mellitus type 2 [17]. Most of these adipokines have a proinflammatory character. Scientific reports showed that in fatty tissue: tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) are elevated [18,19]. Another pro-inflammatory adipokinin is resistin, produced by adipocytes and macrophages [20]. Resistin is also known as adipose tissue-specific secretory factor (ADSF) and was found to be released from adipose tissue and involved in other physiological systems, such as inflammation and energy homeostasis [21-23]. Resitin expression can be upregulated by some interleukins such as IL-6, IL-1 and TNFα as well as by some inflammatory stimuli like lipopolysaccharide (LPS) from Gram negative bacteria. Resistin is primarily related to insulin sensitivity and adipocyte differentiation [24]. In obesity and metabolic syndrome, higher levels of adipokines were observed in blood, like resistin, leptin, adiponectin and also C reactive protein (CRP) [25]. Research has shown that overweight reduces inflammation by decreasing the serum CRP, IL-6 leptin and resistin [26,27]. Shang et al. used ADSCs to supervise disease with inflammatory or autoimmune character, but the correlation between obesity and inflammation remains still unexplained [28]. Thus resistin is reputed to contribute to insulin resistance from one side, and from the other side some research results suggest that resistin may be a link in the well-known association between inflammation and insulin resistance [29]. WNT proteins are family of autocrine and paracrine growth factors that affect biological and developmental processes. They also mediate signal transduction pathways in fat tissue. WNT5A, a non-canonical WNT ligand, has been shown to promote AT inflammation and insulin resistance in animal studies. Elevated non-canonical WNT5A signaling in VAT contributes to the exacerbated IL-6 production in this depot and the low-grade systemic inflammation typically associated with visceral adiposity [30].

Materials and Methods

Animals and ethics statement

Animals were purchased from the central animal house of the Pharmacy Faculty at the Jagiellonian University Medical College (JUMC) in Cracow. All procedures related to laboratory Wistar rats were approved by the Animal Bioethical Committee of Jagiellonian University, Cracow, Poland (resolution no. 84/2014). Dams and adult rats were housed in air conditioned room with constant temperature of 20°C +/- 4 ° C with a 12 hour day/night cycle with humidity level of 55% ±-10%. To ensure that the welfare of the Wistar albino rats was at the highest level possible, we provided lux in an expedition room at 25-50 lux. The air exchange was at the recommended level of 15-20 exchanges per hour. The animals were randomized to an experimental group and received chow and water ad libitum. Female rats, during mating time, pregnancy and lactation (21 days) received the LF or HF diets, respectively. At birth, dams were sexed and randomized in groups with 8 litters. To implement one of the Principles of Humane Experimental Technique, the remaining pups were left to adulthood.

Diet

In our experimental model of obesity, we used the following dietary groups: (1,3) group on low fat (LF) diet, with female or male pups with mother; (2,4) group on high fat (HF) diet, with female or male pups with mother; (5,7) group on LF diet with adult male or female; (6,8) group on HF diet with adult male or female. The LF diet (Labofeed B, Pasze Kcynia, Poland) supplement contained 25% of protein, 8% of fat and 67% of carbohydrates. The obesity-inducing diet, DIO (VERSELE-LAGA Opti Life Adult Active, Belgium) contained: 32% of protein, 22% of fat and 40% of carbohydrates was shown in **Table 1**.

Table 1: Compositions of 1 gram of low fat diet (LF) versus high fat diet (HF).

Diet component	LF diet	HF diet
Carbohydrates, ashes and minerals [%]	67	46
Protein [%]	25	32
Fats [%]	8	22
Energy density [kcal/g]	2.75	4.7

Blood collection

After a 21-days period of feeding animals with different diets, all members of the experimental groups were terminally anaesthetized with pentobarbital (Morbital, Puławy, 200 mg per dose). We then collected blood samples from the cardiac vein using standard surgical techniques and centrifugation to obtain serum samples for further analytic procedures.

Isolation and cell culture of ADSCs

ADSCs in female rats were obtained from white adipose tissue from the subcutaneous area. From male rats, we isolated ADSCs from the visceral area according to a published method based on adult BalbC mice [31]. Isolated fat tissues were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, Germany) containing 1% penicillin/streptomycin solution (Sigma-Aldrich, Germany), minced and digested with collagenase type (1 mg/mL; Gibco by Life Technologies, USA) at 37°C for 1 h. Enzyme activity was neutralized with a Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich, Germany) containing 10% FBS (Gibco by Life Technologies, USA) and 1% penicillin/streptomycin solution. Afterwards, the reaction cocktail obtained from disintegrated tissues was filtered (100 µm pore diameter filters, Fisher Scientific, USA), centrifuged at 300 g for 10 min. to obtain a high-density cell pellet. The cell pellet was suspended in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco by Life Technologies, USA) and 1% penicillin/streptomycin solution (Sigma-Aldrich, Germany) and placed in T75 flasks

(Corning, Sigma-Aldrich, Germany), incubated overnight at 37°C in a 5% CO_2 incubator at 90% humidity. Unattached cells and non-adherent red blood cells were removed after 24 h by washing with antibiotic supplemented PBS. Adherent cells (ADCSs) were suspended in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. The medium was changed at 72 h intervals until the cells became confluent. When the cells reached 80-90% confluence, they were placed in trypsin - EDTA solution (0.25% wt/vol; Sigma-Aldrich, Germany) for 10-15 min. at 37°C, and dissociated by trituration. To arrest the trypsin reaction, DMEM containing 10% FBS and 1% penicillin/streptomycin solution was added the cell suspension and the cells were centrifuged at 416 g for 10 min. ADSCs were suspended in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin solution, counted with a hemocytometer and seeded on 96-well plate (Corning, Sigma-Aldrich, Germany) in triplicate at a density of 0.25 × 10^6 cells/ml per each sample and cultured at 37°C with 5% CO_2 and humidified atmosphere.

Magnetic stimulation of ADSCs cultures

PEMF stimulation was started after 24 h of ADSCs culture. A generator produced and kindly provided by the Institute of Electron Technology (Cracow, Poland) generated pulsed electromagnetic field with a frequency 7 Hz at a flux density of 30 mT inside the cell culture incubator. A 96-well plate with ADSC cultures was placed in the generator's pocket and exposed to EMF for 4 h daily at 24 h intervals, during three consecutive days; this corresponded to a total of three PEMF exposures over the 3-day period. Control samples were placed in the same incubator but at a 35 cm distance from the generator.

Cytokines production

Inflammatory cytokines and resistin levels were measured in blood serum samples obtained from animals of all experimental groups as well as in supernatants originated from ADSCs cultures 24 h after the last PEMF exposure and from unexposed control cultures, respectively. The cytokine concentrations TNFα, IL-6 (Diaclone SAS, France) and resistin (SunredBio, China) were estimated by enzyme-linked immunosorbent assay (ELISA) strictly according to the manufacturer's procedure.

Statistical analysis

The data were expressed as mean (+) standard deviation (S.D.) and compared using the Student t-test considering P<0.05 defined as significantly different.

Results

Serum level of proinflammatory cytokines and resistin in rat pups of both genders grown on LF and HF diet: In serum of female and male pups grown on HF diet the level of both pro-inflammatory cytokines TNFα and IL-6 was increased, but the really big difference was observed in IL-6 level in serum (p<0.02) comparing female and male pups fed with LF diet (**Figure 1**).

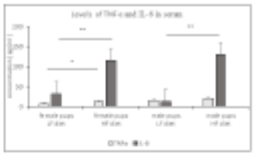

Figure 1: Concentration of proinflammatory cytokines TNFα and IL-1 in serum from female and male pups grown on low (LF) and high (HF) fat diet assessed by ELISA tests. The data are expressed as mean (+ SD), statistical significance was determined by Student t-test $^{*}p<0.05$, $^{**}p< 0.001$.

Resistin level in serum of female and male pups grown on HF diet was elevated in comparison to serum resistin level of rats of both genders fed the LF diet (**Figure 2**).

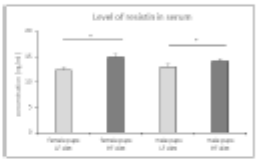

Figure 2: Concentration of resisting in serum from female and male pups grown on low (LF) and high (HF) fat diet assessed by ELISA tests. The data are expressed as mean (+ SD), statistical significance was determined by Student t-test $^{*}p<0.05$.

Serum level of proinflammatory cytokines and resistin in rat adults of both genders grown on LF and HF diet: The serum level of TNFα and IL-6 cytokines in the group of female and male adults grown on HF diet were significantly higher as compared to serum level of these cytokines in rats grown on LF diet (**Figure 3**).

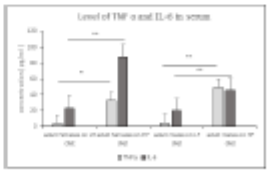

Figure 3: Concentration of proinflammatory cytokines TNF α and IL-6 in serum of female and male adults grown on low (LF) and high (HF) fat diet assessed by ELISA method. The data are expressed as mean (+ SD), statistical significance was determined by Student t-test $^{*}p<0.05$, $^{**}p< 0.001$.

HF diet caused changes in the serum level of resistin in adult rats of both genders when compared to LF diet; higher content

of resistin was measured in serum of adult animals induced by HF diet (Figure 4).

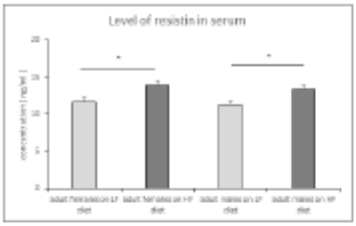

Figure 4: Concentration of resist in in serum samples obtained from female and male adults grown on low (LF) and high (HF) fat diet assessed by ELISA tests. The data are expressed as mean (+SD), statistical significance was determined by Student t-test *p<0.05.

PEMF exposure induced changes in proinflammatory cytokines level in ADSCs cell cultures originated from female and male rat pups grown on LF diet: In *in vitro* cell culture conditions, PEMF exposure of ADSCs isolated from female and male rat pups grown on LF diet caused changes in pro-inflammatory cytokines levels in ADSCs cell culture supernatants. PEMF exposed ADSCs cell cultures, originating from female pups, released more TNFα than unexposed ADSCs cell cultures, the opposite effect was obtained in case of male originated, PEMF exposed ADSCs cell cultures (Figure 5).

IL-6 level in PEMF exposed ADSCs cell cultures from female pups was higher than in controls not exposed to PEMF, the opposite effect was induced in ADSCs cell cultures from male pups. Upon PEMF treatment there was a significant increase of IL-6 cytokine produced to the milieu (Figure 5).

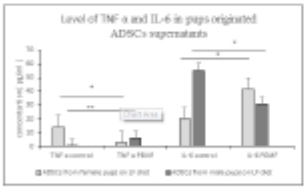

Figure 5: Concentration of pro-inflammatory cytokines TNFα and IL-1 in the supernatants from female and male pups originated from adipose derived stem cells (ADSCs) grown on low (LF) fat diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+SD), statistical significance was determined by Student t-test *p<0.05, **p< 0.001.

PEMF exposure induced changes in proinflammatory cytokines level in ADSCs cell cultures originated from female and male rat pups grown on HF diet: PEMF exposure of ADSCs cell cultures originating from pups of both genders grown on HF diet caused increased secretion of TNFα to cell culture medium by ADSCs, as opposed to ADSCs which were not exposed to PEMF. While the release of IL-6 by ADSCs originated from female pups upon PEMF treatment was diminished, ADSCs from male pups exposed to PEMF produced more IL-6 than ADSCs in controls (Figure 6).

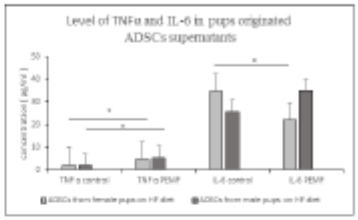

Figure 6: Concentration of pro-inflammatory cytokines TNFα and IL-1 in supernatants from female and male pups originated from adipose derived stem cells (ADSCs) grown on HF diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+SD), statistical significance was determined by Student t-test *p<0.05.

Resistin level changes induced by PEMF exposure in ADSCs cell cultures originated from rat pups and adults grown on HF or LF diet: PEMF treatment of ADSCs cell cultures from pups of both genders fed with LF diet caused increased secretion of resistin cell culture medium by ADSCs as compared to ADSCs cultures which were not treated with PEMF. Different results were obtained in case of ADSCs originated from HF diet pups. ADSCs cell cultures isolated from female pups grown on HF diet have shown slightly diminished resistin level upon PEMF influence (Figure 7).

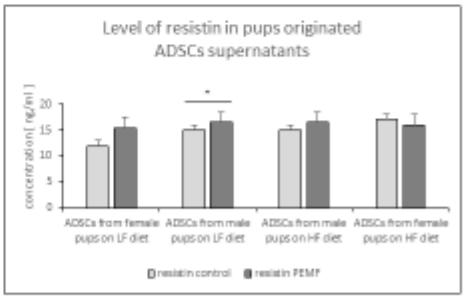

Figure 7: Concentration of resistin in supernatants from female and male pups in adipose derived stem cells (ADSCs) grown on low (LF) and high (HF) fat diet assessed by ELISA tests. The data are expressed as mean (+ SD), statistical significance was determined by Student t-test *p<0.05.

The ADSCs cell cultures from adults of both genders fed with LF and HF diet when treated with PEMF, a slight elevation of the

measured resistin content in cell culture supernatants was observed in Figure 8.

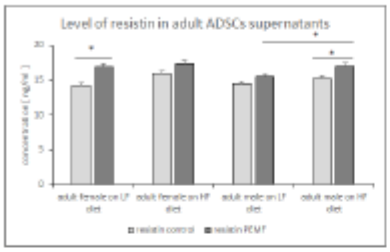

Figure 8: Concentration of resistin in supernatants from male and female adults grown on low (LF) and high (HF) fat diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+SD), statistical significance was determined by Student t-test *p<0.05.

PEMF exposure induced changes in proinflammatory cytokines level in ADSCs cell cultures originated from adult female and male rats grown on LF and HF diet: The level of IL-6 evaluated in ADSCs cell culture supernatants originating from adult females grown on LF diet was lowered under PEMF influence, an opposing effect was observed in ADSCs cell culture supernatants obtained from LF diet raised males, Figure 8.The TNFα level was increased in ADSCs cell cultures supernatants originating from both genders upon PEMF treatment (Figure 8). The ADSCs from male adult on HF diet under PEMF showed in inflammatory profile a significantly higher level of TNFα and IL-6 (Figure 9).

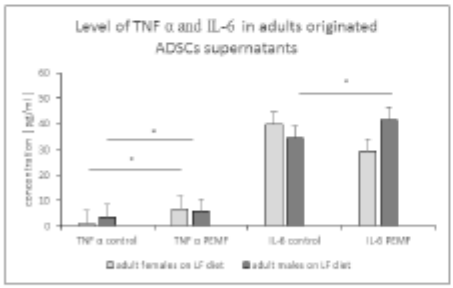

Figure 9: Concentration of pro-inflammatory cytokines and IL-6 in supernatants from female and male adults grown on low (LF) fat diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+ SD), statistical significance was determined by Student t-students *p<0.05.

Spectacular increase of the TNFα amount released to cell culture milieu occurred in ADSCs cell cultures isolated from both genders grown on HF diet, on the other hand when it comes to Il-6 measurements, there was nearly no effect in IL-6 level in ADSCs originated from HF grown females, and a slight increase in ADSCs supernatants obtained from HF diet males (Figure 10).

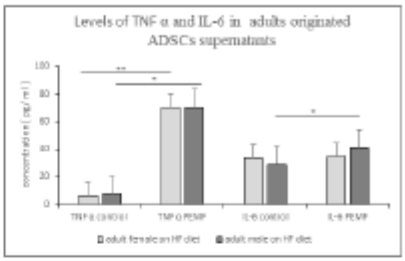

Figure 10: Concentration of pro-inflammatory cytokines TNFα and IL-6 in supernatants from male and female adults grown on high (HF) fat diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+ SD), statistical significance was determined by Student t-students *p<0.05, **p< 0.001.

Discussion

Maternal obesity caused by high fat diet is responsible for chronic low grade inflammation in progeny placenta, adipose tissue, liver, vascular system and brain [32]. During in utero time period, when the mother received obesity-inducing diet (Neeraj Desai et al.) a higher level of IL-6, TNFα was revealed in fetal plasma [33]. Also TNFα in maternal plasma of animals on HF diet was higher than in controls [34]. The obvious consequence of mother's diet with high fat levels is systematic inflammation in the offspring. Other studies also support our reports, that in offspring from mothers on HF diet from mating, gestation and lactation, TNFα may be evaluated in 3-weeks-old pups of both genders [35]. In contrast to the above statement, in pregnant rats on HF diet, the maternal plasma IL-6 and TNFα was reduced. These incompatibilities in inflammatory profiles are the results of short exposure to diet induced obesity (DIO) chow compositions and also timing of the harvested tissue [36]. Our findings support the resistin levels evaluated in male offspring from overweight mothers on HF diet in postnatal day (PND) [22,37].

Overweight contributes to collection fat in AT, liver and muscle to cause activation of macrophages by secretion a pro-inflammatory proteins like TNFα, CRP, MCP-1, leptin, IL-6, resistin [38]. Results of our study confirm earlier finds that exposure to HF diet is connected to higher level of TNFα and IL-6 in adult male rats [39]. Gil et al. state that long term HF diet in adult mice on the degree of pro-inflammatory cytokines (TNF-α, IL-6, and MCP-1) was higher than in control-animals on LF diet [40]. Introduction of HF diet to adult rodent male species resulted in higher concentration of TNFα, just like in our experiment, but also in growth of other pro-inflammatory biomarkers – CRP, IL-17 [41,42]. In obesity models, WAT accumulated excess fatty tissue and released resistin, with levels correlating to DIO and possibly gene–related issues [43,44].

Scores from Muthulakshmi et al. are consistent with our experiment pro-inflammatory markers panel (TNFα, IL-6, resistin) in male mice and rats on HF diet [45]. Our results are also compatible, with the statement that in rodent male model on HF diet, the level of resistin is higher [46,47]. Other studies which also support our work show that in both genders of adult rats, the level of resistin increased on HF diet [48]. Our data are in agreement with Christine et al. research who found that in adult female mice on high fat diet, there were higher levels of inflammatory molecules, including IL-6 [49]. Acute model of high fat diet was in many reports dedicated to males. Milles et al. proved that in the females on HF diet, the level of TNFα was lower as compared to animals on LF diet. The differences may have resulted from the short duration of the diet [50].

Conclusion

Our previous experiments showed PEMF impact on ADSCs obtained from rat pups of both genders on LF diet having protective effects on viability. Contrary, PEMF exerted influence on ADSCs cell culture originating from adult female rats grown on HF diet by inducing high percentage of early apoptotic cells [51]. Unfortunately, there are little data available in the literature concerning the influence of PEMF on pro-inflammatory markers in ADSCs from adult rats of both genders on LF and HF diets. However, the available publications provide information that PEMF impact on bone marrow cells from adult female rats on standard diet causes a decrease in TNFα and IL-6 [52]. In our study, PEMF treatment of ADSCs from adult female rats on LF diet presented low IL-6 level, however TNFα concentration increased. Incompatibilities in PEMF influence may have resulted from the time exposure of the cells. Other reports showed osteoclastogenesis in adult females under low frequency pulsed electromagnetic field (ELF-PEMF) exposure. In 6 and 8 day of ELF-PEMF stimulation (intensity of 7.5 Hz, tension of 12.2 mV/cm and time 2 h/ day) TNFα concentration grew as compared to controls [53]. Electromagnetic fields used in obese persons, both men and women, lead to high abdominal fat reduction [54]. In obese mice weight and fat loss was reported after activity of 0.5 T electromagnetic fields [55]. In addition, the use of extremely low frequency field (ELF-MF 7.5 Hz, 0.4 T) on human tissue induced an inhibitory effect on obesity. Research studies carried out on mesenchymal stem cells (MSCs) showed a restrictive influence of ELF–MF on adipogenic differentiation [56]. Mengyao et al. proved that PEMF influence promotes myoblast differentiation, obtained from mice in inflammatory and in vitro conditions [57]. Also in human fibroblast like-cells, the level of inflammatory cytokines (IL-1β, TNFα) was lower with PEMF treatment of 2.25 mT with a frequency of 50 Hz [58]. Our earlier experimental studies analyzing the lipid profile in offspring on DIO feed showed high levels of triglycerides (TG), total cholesterol (TC) and low density lipoproteins (LDL) [59]. ADSCs isolated from young female animals grown on HF diet were susceptible for PEMF treatment what decreased release proinflammatory cytokine like IL-6 and metabolic agent – resistin. Adipose derived mesenchymal stem cells isolated and characterized and then osteogenic differentiation of them was investigated after culturing on the surface of Poly (caprolactone) (PCL) scaffold under treatments of pulsed electromagnetic fields (PEMF) [3]. ADSCs-seeded PCL nanofibrous scaffold in combination with PEMF could be a great option for use in bone tissue engineering applications [60].

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FOLIA MEDICA CRACOVIENSIA
Vol. LVIII, 2, 2018: 131–145
PL ISSN 0015-5616
DOI: 10.24425/fmc.2018.124664

Obesity related adipokines release in rat adipose derived stem cell cultures influenced by pulsed electromagnetic field

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Abstract: Objective: The main goal of our studies was to investigate the effect exerted by pulsed electromagnetic filed (PEMF) on adipocytokines secretion in cell culture supernatants from rat adipose derived stem cells (ADSCs) grown on varied energy-rich diet. Offspring and adult animals were randomly selected for two types of experimental diets: low (LF) or high fat (HF) diet for 7 weeks. After the diet period, serum glucose level was measured, ADSCs were isolated from adipose tissues from different locations. ADSCs from all experimental groups were exposed to PEMF, supernatants collected and adipokines level was determined.
Results: HF diet feed in pups/adult animals elevated blood glucose level and increased the level of adiponectin (Apn) and leptin of both genders and age measured in serum.
ADSCs cell cultures originated from female pups on LF diet and exposed to PEMF released large amounts of Apn. PEMF effect exerted on Apn release was also observed in ADSCs isolated from male pups HF diet. ADSCs from female pups on LF diet exposed to PEMF released smaller amounts of leptin in comparison to cell cultures without PEMF treatment. PEMF exposure of ADSCs cell cultures originated from female adults on LF diet decreased release of Apn, contrary adult male on LF diet ADSCs under PEMF treatment produced more leptin. PEMF treated male HF diet-originated ADSCs cultures released significantly more leptin than controls.
Conclusion: Our results suggest that PEMF exposure is responsible for metabolic physiological balance effects obtained in ADSCs cultures originating from adult animals on HF diet.

Key words: high/low fat diet, adipose tissue derived stem cells, adipokines, glicemia, PEMF.

Introduction

Obesity has become a worldwide society health problem associated with metabolic and chronic disorders. It concerns both male and female genders in most age groups [1, 2]. Adiposity is progressive, connected with adipocytokines such as adiponectin (Apn), by it decreased levels and the opposite effect is observed in leptin serum level. These metabolically active hormones in association with proinflammatory cytokines like TNFα and IL-6 are responsible for dysfunction of endothelium development and insulin resistance [3]. Apn plays an important role in metabolic balance through insulin-sensitizing, antidiabetic, antioxidant, anti-inflammatory, anti-atherogenic and vasoprotective effects [4, 5]. Leptin is engaged in obesity, insulin resistance, etiopathogenesis of cardiovascular diseases, inflammation, diabetes and plays an essential role in the long-term regulation/control of body weight [6, 7]. The western diet, rich in fat and sugar with combination of less activity results in excess weight and expression of genetic predispositions leading to type 2 diabetes mellitus (T2DM). This long-term developing disease with diagnosed hyperglycemia is caused by impaired insulin secretion, invalid insulin activity, defects of insulin receptors or post receptor insulin resistance [8]. Short term HF diet food highly increased plasma glucose and insulin level in adult male mice but didn't affect the body mass and inflammation [9, 10]. Rodent studies based on maternal HF diet during pregnancy and lactation resulted in later life obesity and metabolic syndrome in offspring [11]. Introduction of HF diet in utero during pregnancy caused disturbances in appetite and energy balance during formation of the hypothalamic nervous system. Increased blood glucose and leptin level was noted in the offspring of both genders [12].

Low frequency pulsed electromagnetic field (PEMF) exposure of different cell types is known to exert biological effects such as cell proliferation/differentiation, cell death induction/inhibition, changes in gene expression and release of cytokines, growth factors and hormones [13]. It has been proved that pulsed electromagnetic field (PEMF) in a non-invasive way affected the proliferation and osteogenic differentiation into stem cells [14, 15].

Although PEMF exposure induces a neuroprotective effect in patients with peripheral neuropathy in diabetes, usage of PEMF therapy in medicine remains controversial and needs further experiments to explain the cellular mechanism engaged in PEMF influenced activities [16].

Materials and Methods

Experimental animals

The use of animals in experimental research was approved by the First Local Ethics Commission of Jagiellonian University, Cracow, Poland (resolution no. 84/2014). Wistar rats [WistarKrf: (Wi) Wu] emanated from the Pharmacy Faculty at the UJCM in Krakow. Animals were acclimatized for 7 days before the research was started. Dams with offspring and adult animals were kept in an environmentally controlled room (20 ± 5°C, humidity 55 ± 10% and 12 h light-dark cycle with lights on from 07.00 h to 19.00 h). The intensity of light in experimental room was at 25 lux as a result of albinotic strain used. Animals were provided with enrichment in the cage in accordance with the guidelines of the 3R rule.

Diets

The rats were randomly divided into 8 groups and treated for 7 weeks: low fat diet group (LF), received a standard chow for rodents (Labofeed B Pasze Kcynia, Poland) and a high-fat group (HF) with higher content of fat (VERSELE-LAGA Opti Life Adult Active, Belgium). All experimental study groups based on different type of chow and also included age and gender characteristics. Standard diet contained the following: protein 25%, fat 8%, carbohydrates, ashes and minerals 67% (energy density was 2.75 kcal/g), HF diet with a higher fat content contained: protein 32%, fat 22%, carbohydrates, ashes and minerals 46%, (energy density 4.70 kcal/g).

Isolation and culture of ADSCs

Adipose tissue (AT) was obtained from animals from all 8 study groups under sterile conditions and placed in a sterilized culture dish under a laminar hood. AT was carefully collected according to a previously described methodology [17]. The specimens were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, Germany) containing 1% penicillin/streptomycin solution (Sigma-Aldrich, Germany), homogenized and digested with me type collagenase (1 mg/mL; Gibco by Life Technologies, USA) at 37°C for 1 h. Enzymatic activity was neutralized with Dulbecco's modified eagle's medium (DMEM Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS; Gibco by Life Technologies, USA) and 1% penicillin/streptomycin solution. Subsequently, the reaction cocktail obtained from disintegrated tissues was filtered (filters with 100-µm pore diameter, Fisher Scientific, USA) and centrifuged at 300 × g for 10 min to obtain a high-density cell pellet. The pellet was suspended in DMEM supplemented with 10% FBS (Gibco by Life Technologies,

USA) and 1% penicillin/streptomycin solution (Sigma-Aldrich, Germany), placed in T75 flasks (Corning, Sigma-Aldrich, Germany) and incubated overnight at 37°C in a 5% CO_2 incubator with 90% humidity. After the 24-h incubation, non-attached cells and non-adherent erythrocytes were washed with antibiotic-supplemented PBS. Adherent cells (ADCSs) were suspended in DMEM with 10% FBS and 1% penicillin/streptomycin solution. The medium was changed every 72 h until the cells became confluent. After reaching 80–90% confluence, the cells were placed in trypsin-EDTA solution (0.25% weight/volume; Sigma-Aldrich, Germany), incubated at 37°C for 10–15 min, and dissociated by trituration. The trypsin reaction was blocked by addition of DMEM with 10% FBS and 1% penicillin/streptomycin solution, and the cell suspension was centrifuged at 416 × g for 10 min. ADSCs were suspended in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin solution, counted with a hemocytometer, seeded on a 96-well plate (Corning, Sigma-Aldrich, Germany) in triplicate at a density of 0.25×10^6 cells/ml per sample, and cultured at 37°C at humidified atmosphere of 5% CO_2.

PEMF exposure of ADSCs cell cultures

PEMF stimulation was started after 24 h of ADSCs cell culture duration. A generator was designed and manufactured by the Institute of Electron Technology (Cracow, Poland) with a frequency of 7 Hz at a flux density of 30 mT inside the cell culture incubator. A 96-well plate with ADSC culture was placed in the generator's pocket and exposed to PEMF for 4 h a day at 24-h intervals, during three consecutive days.

Euthanized animals

At the end of the experiment (21st day), animals from all study groups were sacrificed with an anaesthetic (Pentobarbital, Morbital, Pulawy) at a dose of 200 mg per kg body weight to collected blood sample and AT.

Adipokines level measurement

Adiponectin was measured in serum and ADSCs cell culture supernatants with ELISA (Mediagnost /Germany) in duplicates. Leptin in rat plasma and supernatants from ADSCs cell cultures were determined by ELISA kit (BioVendor/Czech Republic) in duplicates.

Blood glucose level measurement

Rat blood was collected on heparin with anti-glycololytic fluoride for determination of blood glucose level. Glucose concentration was evaluated with an enzymatic method using the Cobas 80 000 analyzer.

Statistical analysis

The results were presented as means (±) and their standard deviations (SD). Intergroup comparisons were conducted with Student's t-test. The differences were considered statistically significant at $P < 0.05$.

Results

Adipokines serum level in animals of both genders grown on LF or HF diet

Serum adiponectin level in female pups and in male pups was elevated in animals grown up on HF diet in comparison to animals grown on LF diet. LF diet fed male pups showed to release more Apn into serum than female pups grown on the same type of chow (Fig. 1). Opposite effect was found in serum of adult animals grown on LF diet (both females and males) — adiponectin level was higher than in adult animals fed with HF diet (Fig. 1). Serum leptin level measured in pups of both genders was higher in animals fed with HF diet. Exactly the same relationship occurred in adult rats, which grown on HF diet produced more leptin

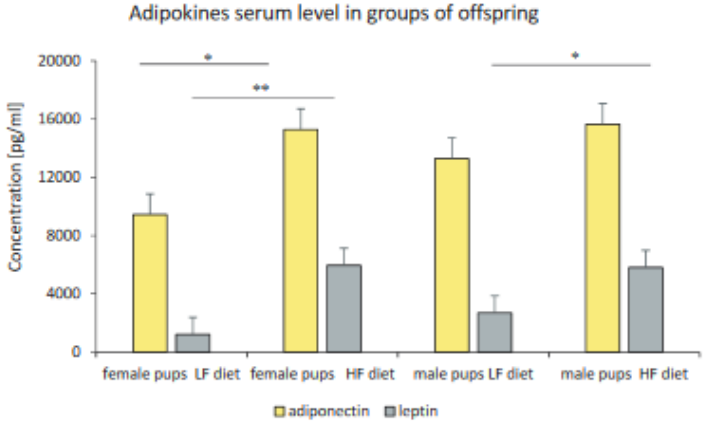

Fig. 1. The influence of LF/HF diet on sera adiponectin and leptin levels in offspring. Statistical significance of intergroup differences verified with Student's t-test; differences significant at $P < 0.05$ marked with asterisks (*).

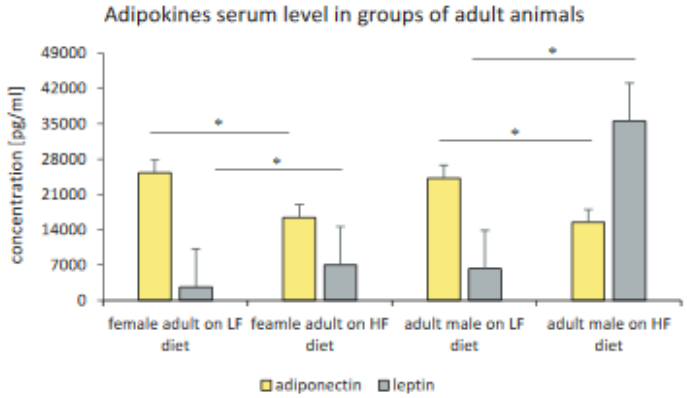

Fig. 2. The influence of LF/HF diet on sera adiponectin and leptin levels in adult animals. Statistical significance of intergroup differences verified with Student's t-test; differences significant at P <0.05 marked with asterisks (*).

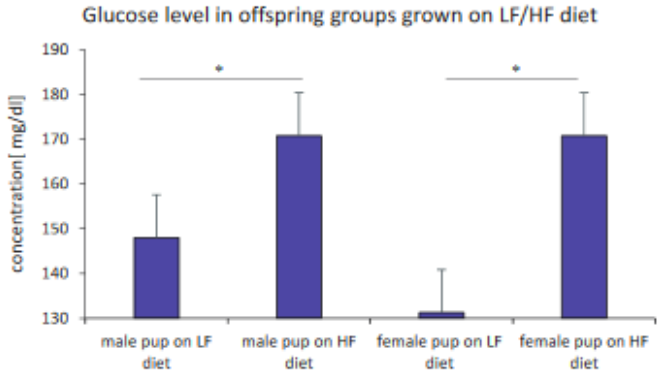

Fig. 3. The blood glycemia in pups. Statistical significance of intergroup differences verified with Student's t-test; differences significant at P <0.05 marked with asterisks (*).

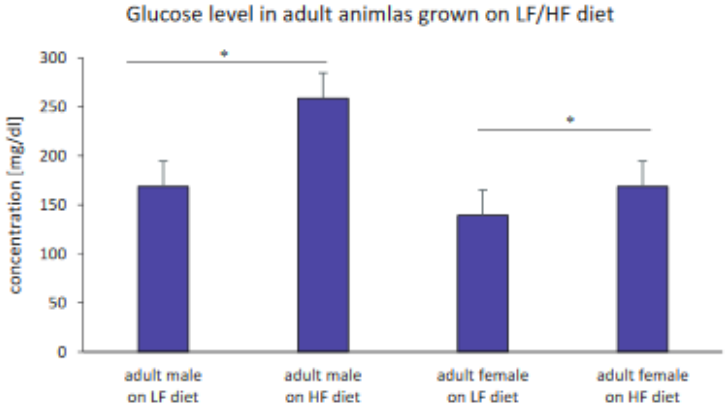

Fig. 4. The blood glycemia in adult animals. Statistical significance of intergroup differences verified with Student's t-test; differences significant at P <0.05 marked with asterisks (*).

than LF fed animals, respectively (Fig. 1). The highest amount of leptin was obtained in adult males fed with HF diet, nearly four times more than adult females on the same diet (Fig. 2).

Blood glucose level in animals of both genders grown on LF or HF diet

Glucose level in rat pups of both genders fed with HF diet was much higher than glucose level measured in pups grown on LF diet (Fig. 3). Blood glucose level in adult animals fed with HF diet exceeded 200 mg/ml in males — characteristic for diabetes mellitus diagnostic criterion, and was slightly elevated in females fed with HF diet in comparison to LF diet glucose level, however not exceeding the value of 200 mg/ml (Fig. 4).

Adipokines level in supernatants of ADSCs cell cultures isolated from pups exposed to PEMF

ADSCs cell cultures originating from female pups grown on LF diet when treated with PEMF exposure released extremal amounts of Apn in comparison to ADSCs cell cultures without PEMF treatment. The PEMF exposure effect exerted on Apn release was also observed in ADSCs cell cultures isolated from HF diet fed animals, but it was not as spectacular as in case ADSCs isolated from LF diet grown animals (Fig. 5). ADSCs cell cultures originating from female pups grown on LF diet exposed to PEMF released smaller amounts of leptin in comparison to ADSCs cell cultures without PEMF treatment, whereas PEMF has not caused any effect on leptin level in supernatants from male ADSCs cell cultures (Fig. 5).

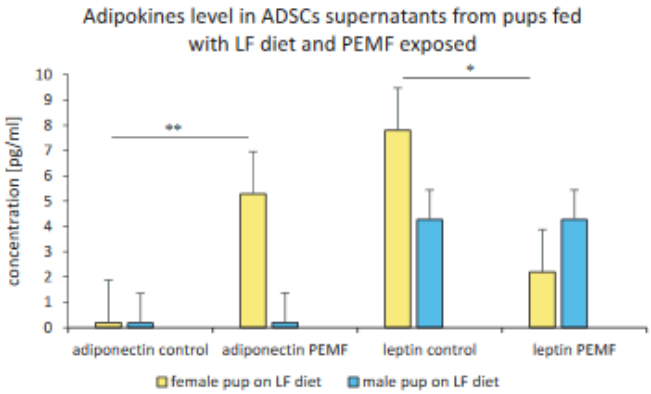

Fig. 5. Adiponectin and leptin levels in supernatants of ADSCs cell cultures isolated from pups on LF diet exposed to PEMF. Statistical significance of intergroup differences verified with Student's t-test; differences significant at $P < 0.05$ marked with asterisks (*).

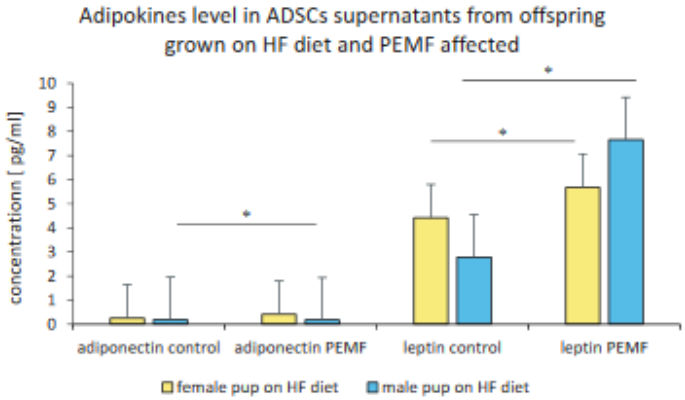

Fig. 6. Adiponectin and leptin levels in supernatants of ADSCs cell cultures isolated from pups grown on HF diet exposed to PEMF. Statistical significance of intergroup differences verified with Student's t-test; differences significant at P <0.05 marked with asterisks (*).

ADSCs cell cultures treated with PEMF originating from HF diet males and females produced less leptin to cell culture milieu than control ones (Fig. 6).

Adipokines level in supernatants from ADSCs cell cultures exposed to PEMF originating from adults

PEMF-exposed ADSCs cell cultures originating from female adults grown on LF diet released lower amounts of adiponectin in comparison to ADSCs cell cultures without PEMF treatment. The PEMF exposure effect exerted on adiponectin production was not observed in ADSCs cell cultures originating from female and male adults fed HF diet (Fig. 7). PEMF-exposed ADSCs cell cultures originating from adult males grown

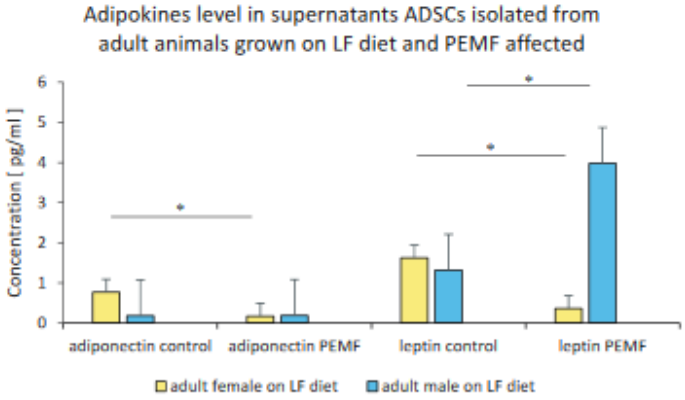

Fig. 7. Adiponectin and leptin levels in ADSCs cell culture supernatants originating from adult animals exposed to PEMF. Statistical significance of intergroup differences verified with Student's t-test; differences significant at P <0.05 marked with asterisks (*).

on LF diet produced more leptin than ADSCs cell cultures without PEMF treatment. PEMF-exposed ADSCs cell cultures originating from adult females grown on LF diet, produced less leptin than control ADSCs cell cultures (Fig. 7).

PEMF exposure of female HF diet grown ADSCs cell cultures caused slight decrease of leptin level in cell culture supernatants and significant leptin release in PEMF treated male HF diet grown ADSCs cell culture supernatants (Fig. 8).

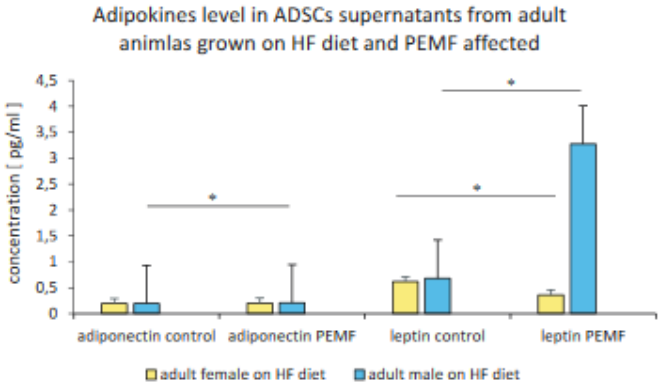

Fig. 8. Adiponectin and leptin levels in ADSCs cell culture supernatants originating from adult animals fed with HF diet, exposed to PEMF. Statistical significance of intergroup differences verified with Student's t-test; differences significant at $P < 0.05$ marked with asterisks (*).

Conclusion

ADSCs cell cultures isolated from adult males fed with LF or HF diet when PEMF exposed, released more leptin than those not exposed to PEMF. PEMF treated ADSCs cell cultures from male pups caused significant increase of Apn. The metabolic effects exerted by PEMF treatment of ADSCs cell cultures could be used as a possible antidiabetic, metabolic balance- influencing therapy.

Discussion

Adipokines and obesity

Obesity is nowadays considered as one of the leading health problems resulting in increased rates of cancers, heart disease, stroke and diabetes. The suggested solution for this problem was to restrict calories, make wiser dietary choices and encourage physical activity [18]. Research derived from murine projects based on HF diet has shown that females have lower body weight (BW), body mass index (BMI) and also smaller body waist circumference (WC) than male animals. Similarly, significantly higher values of fasting glycaemia were noticed in males on the same diet in

comparison to female ones [19, 20]. Hyperglycemia as a metabolic disorder was observed in offspring of both genders from dams fed with HF diet during pregnancy and lactation period, which is consistent with our experimental results [21]. Some signal transduction hormones like adipokinins are released from adipocytes and directly related with pathogenesis of obesity. Low level of Apn is negatively correlated with inflammation markers in overweight animals when BMI increases over 30 [22]. The leptin target activity is localized in the hypothalamus which regulates food intake and impact on BW [23]. It has been proved that increased leptin level is associated with the growth of AT. It defines pathogenesis of obesity and resulting complications [24]. Our data revealed that HF diet led to higher leptin and glucose levels in adult animals of both genders while adiponectin amount was decreased in adults. Similarly to our research studies, in adult male rats and other animal models (rabbits) grown on HF diet, blood glycaemia was out of normal range [25, 26]. Experiments carried out on adult obese females have shown high leptin concentration in their serum results, that stay in agreement with results obtained by our group [27]. Masuyama *et al.* stated that in offspring mice of both genders fed with HF diet, blood glucose level and leptin release were elevated contrary to adiponectin value [28]. Other results indicated that in adult male rats plasma adipocytokines — Apn and leptin amount increased in animals grown on high fat rich diet. Differences in our results in terms of adiponectin level might result from later introduction of HF diet (24 post natal day) [29]. Some research studies presented correlation between HF diet effect and increase of glucose and leptin level in young male rats as well as in adults [30]. Our data are in agreement with Hou *et al.* who proved that animals of age 3 and 8 weeks after HF diet had impaired glycaemia. Rodent studies showed fasting glycaemia level in young male species on HF diet and decreased adiponectin level [31, 32].

EMF influence

Electromagnetic field (EMF) interaction has been evidenced to be therapeutically effective and useful in a wide range of its parameters as well as medical implications. There are many research studies which confirm the hypoglycaemic activity of magnetic field exposure but still frequency and exposure time vary in different projects [33, 34]. Studies carried by Sieroń *et al.* which marked glucose by 3H uptake in organs and tissues of rats exposed to an extremely low electromagnetic field (ELEMF, 10 Hz, 1.8–3.8 mT), showed significantly higher glucose uptake in the liver, kidney, heart muscle, cartilage, connective tissue, skin and tendon. They hypothesised that ELEMF can facilitate glucose transport through the cell membrane [35]. Diabetic male mice when exposed to EMF (25 Hz, 250 μT) for 45 minutes for 2 weeks revealed lower blood glucose comparative to metformin and insulin application [33]. Research of Sakurai *et al.* run on hamster-derived insulin secreting cells (TIH-T15) with ELEMF

(60 Hz, 5 mT) has found increased concentration of intracellular insulin in the cell lines [36]. Öcal *et al.* investigated changes in blood glucose level in healthy and diabetic rats caused by alternating magnetic field (5 mT and 8 mT). They found out that alternative magnetic field exposure resulted in a decrease of blood glucose level in healthy and diabetic rats [37].

Endocrine system is sensitive to PEMF influence and possibly responds with changes of hormone production in the course of experimental animal research [38, 39]. Analysis of Holstein cow's milk productivity in the interaction of the vertical field and the horizontal magnetic field showed rise in insulin-like growth factor 1 (IGF-1) and milk production [40]. Influence of extremely low electromagnetic field on gender hormones is partially reversible [41]. Leoci *et al.* observed that application of EMF in the course Benign Prostatic Hyperplasia (BPH) in canine model led to a reduction a gland volume. Furthermore it did not negatively affect testosterone level and quality of the semen [42]. High rate of EMF (2450 MHz) in the prenatal period in female rats showed postnatal growth restriction and delayed maturation. In addition, in the brain and ovarian tissue EMF resulted in high oxidative stress index [43]. Currently, magnetotherapy is an easy and direct method used to treat a variety of illness and pathologies [44]. Studies with a low frequency electromagnetic field showed to affect different physiological processes in vitro and in vivo experiments, thus we chose the frequency of 7 Hz with a flux density of 30 mT [45]. Apn is a hormone from the fat tissue with anti-inflammatory effect in obesity, in contrast to the majority of adipocytokines [46, 47]. Apn lower values are associated with abundance of abdominal fat [48]. Unfortunately, there is little evidence when analyzing PEMF effect on metabolic hormones in rat ADSCs in experimental models of obesity. Therefore, we could not compare our findings with the results by other authors. In ADSCs cultures obtained from female pups grown on LF diet we proved strong PEMF effect with higher Apn level contrary to values of leptin. In turn, ADSCs cultures originating from HF diet grown animals showed the PEMF influence on higher serum leptin level in supernatants from ADSCs female pups. ADSCs from male offspring on the same diet presented elevated supernatant leptin level but adiponectin value was lower under PEMF exposure. Application of ELEMF from the 6th day of pregnancy across 21 days of lactation in male offspring did not affect the spermatogenesis and fertility [49].

Our PEMF treatment of ADSCs from adult females on LF diet presented lower Apn and leptin values but in males leptin scores were higher as compared to controls. Our PEMF application showed that ADSCs originating from obese adult male rats improved Apn on higher level, in turn ADSCs from females showed low leptin level after exposure. Research of Trent *et al.* presented activity growth and weight loss in obese adult knockout male mice in electromagnetic field of 0.5 Tesla (T) conditions. Other research studies dealing with human embryonic stem cells which

were underwent static magnetic fields exposure showed upregulation of genes encoding insulin factors [18]. Cell line HIT-T15 under ELEMF demonstrated limited insulin secretion in response to increased glucose level [50]. Contrary, in the same conditions, HIT-T15 cells treated with electromagnetic field at a frequency of 60 Hz, 5 mT without glucose therapy, responded with an increase in intracellular insulin concentration [51].

Acknowledgements

The work was supported by the grant no. K/ZDS/006681 from the Jagiellonian University Medical College in Cracow, Poland.

Conflict of interest

None declared.

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FOLIA MEDICA CRACOVIENSIA
Vol. LVIII, 4, 2018: 21–34
PL ISSN 0015-5616
DOI: 10.24425/fmc.2018.125701

Effect of the pulsed electromagnetic field on the release of inflammatory mediators from adipose-derived stem cells (ADSCs) in rats

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Abstract: Objective: The aim of this study was to verify if the exposure to the pulsed electromagnetic field (PEMF) influenced the release of proinflammatory cytokines from adipose-derived stem cells (ADSCs) of normal and overweight rats of various age and sex. Moreover, we compared body temperatures of normal-weight and overweight rats.
Methods: ADSCs of Wistar rats were isolated from the subcutaneous area in females and paratesticular region in males, cultured and exposed to PEMF (7 Hz, 30 mT). Concentrations of proinflammatory cytokines were determined in rat sera and supernatant from ADSCs cultures exposed and non-exposed to PEMF. Body temperature (BT) was measured twice a week, using an infrared and rectal thermometer.
Results: Irrespective of age and sex, animals maintained on low-fat (LF) diet had higher BT than those grown on high-fat (HF) diet. Exposure to PEMF reduced the release of TNF-α and enhanced the production of IL-6 in ADSCs cultures from female pups maintained on LF diet. In contrast, a decrease in IL-6 level was observed in PEMF-exposed ADSCs cultures from female pups grown on HF diet. A similar phenomenon, i.e. a post-exposure increase in IL-6 level was also observed in male pups fed with the LF diet. In the case of ADSCs cultures from adult rats maintained on an HF diet, either males or females, PEMF exposure contributed to a dramatic increase in TNF-α production.
Conclusion: Our findings suggest that PEMF exposure may affect the production of proinflammatory cytokines in ADSCs cultures. The intergroup differences in BT may result from the presence of an underlying inflammation in obese rats.

Key words: obesity, PEMF, ADSCs, proinflammatory cytokine, temperature.

Introduction

The effect of electromagnetic field (EMF) as a modulator of immune response has been recently a subject of many studies [1]. Previous research demonstrated that exposure to the pulsed electromagnetic field (PEMF) might affect proliferation, differentiation and viability of various cell types, as well as their metabolic and signal transduction pathways [2–6]. Extremely low-frequency electromagnetic fields (ELF-EMF) were shown to modulate the release of inflammatory mediators and keratinocyte proliferation [7]. According to Vincenzi *et al.*, the treatment of N9 microglial cell cultures with lipopolysaccharides and exposure to PEMF contributed to a decrease in concentrations of proinflammatory cytokines, such as tumor necrosis alpha factor (TNF-α), interleukin 6 (IL-6) and interleukin-1β (IL-1β), in cell culture medium [8]. Also, a study of adipose-derived stem cells (ADSCs) isolated from adipose tissue (AT) of male and female Wistar rats showed that exposure to PEMF modulated the synthesis of proinflammatory cytokines and adipokines by these cellular population [9].

Almost a half billion people worldwide are obese, and according to one hypothesis, the predisposition to overweight may correlate with metabolic activity and energy balance in homeothermy [10]. According to literature, normal body temperature (BT) of laboratory rats approximates 37.5–38.5/39.0°C. A decrease below those values may be a marker of immune response associated with an inflammatory process [11–13]. A drop off in BT below normal values was inter alia observed in adult female rats with experimentally-induced cystitis caused by *E.coli* strains [14].

The function of AT later in life is modulated by maternal nutritional status during fetal and immediate postnatal period; this phenomenon is referred to as metabolic programming [15]. Fat depots forming adipose tissue differ in terms of their structure and function [16].

Brown adipose tissue (BAT) is involved in thermogenesis acting via catecholamine signaling pathways. Homeostatic hormones, such as leptin and insulin, affect a release of uncoupling protein 1 (UCP-1) and the generation of thermal energy in brown adipocytes [17]. In turn, the primary function of adipocytes in white adipose tissue (WAT) is an accumulation of lipids and endocrine activity. Thus, an excess of WAT leads to obesity [18, 19]. In one study, rats maintained on high-fat (HF) diet showed an increase in UCP-1 level, but this effect was observed only in males. However, the authors of this study did not analyze changes in BT [20]. In contrast, Almeida *et al.* demonstrated that maternal HF diet contributed to an increase in UCP-1 and tyrosine hydroxylase (TH) contents in BAT from female, but not male pups [21]. Obesity was shown to cause disorders of BT in nonpregnant rats. Consumption of cafeteria diet contributed to a decrease (by up to 0.29°C) in BT of overweight female rats during the estrous cycle and pregnancy [22].

Excessive proliferation of AT can cause adipocyte dysfunction and stimulate secretion of proinflammatory cytokines [23]. Inflammatory mediators, such as TNF-α and IL-6, are the main proinflammatory cytokines associated with the development of endothelial dysfunction in obesity and type 2 diabetes mellitus (T2DM) [24]. TNF-α plays a crucial role as a chemotactic and activating agent attracting neutrophils and monocytes to the site of inflammation [25]. Overweight is known to be associated with excessive secretion of TNF-α in adipose tissue. Rat's male offspring from mothers maintained on HF diet during pregnancy and lactation presented with elevated serum levels of TNF-α [26]. An increase in serum TNF-α was also observed in HF diet-fed rats with experimentally induced kidney damage [27]. One study demonstrated that the development of low-grade inflammation in mice with diet-induced obesity was associated with upregulation of IL-6 [28]. In another experiment, male mice maintained on two types of diet, HF and cafeteria feeding, presented with elevated serum levels of IL-6 [29].

The aim of this study was to verify if the exposure to PEMF influenced the release of proinflammatory cytokines from ADSCs of normal and overweight rats of various age and sex. Before harvesting the AT, we measured BT of the study animals.

Material and Methods

Animal care and preparation

Wistar rats were obtained from the Animal House of the Faculty of Pharmacy, Jagiellonian University Medical College. Following a 5-day quarantine, 64 animals of various sex and age were randomized to eight groups, maintained on low- (LF) and high-fat (HF) diet. The rats were kept in an experimental room with controlled air temperature (20 ± 5°C) and humidity (55 ± 10%), under a 12-hour light cycle (light on from 7:00 AM to 7:00 PM), with unlimited access to water and chow. Every effort was made to provide animal welfare in line with the principles of the 3Rs.

Dietary treatment

The study animals were maintained on two types of diet: regular low-fat diet (LF, Labofeed B, Pasze Kcynia) containing 25% protein, 8% fat and 67% carbohydrates, and obesity-inducing high-fat diet (HF, DIO, VERSELE-LAGA Opti Life Adult Active) with 32% protein, 22% fat and 40% carbohydrates.

Cell culture and PEMF exposure

Adipose-derived stem cells (ADSCs) were isolated using the method described by Safford *et al.* [30]. AT were obtained from both control and obese animals. The tissues were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, Germany) containing

1% penicillin/streptomycin solution (Sigma-Aldrich, Germany), homogenized and digested with type 1 collagenase (1 mg/mL; Gibco by Life Technologies, USA) at 37°C for 1 hour. Enzymatic activity of the samples was neutralized with Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS, Gibco by Life Technologies, USA) and 1% penicillin/streptomycin solution. Then, the ADSCs were filtered (filters with a 100-µm pore diameter, Fisher Scientific, USA) and centrifuged at 300 g for 10 min. The cell pellets were suspended in DMEM supplemented with 10% FBS (Gibco by Life Technologies, USA) and 1% penicillin/streptomycin solution (Sigma-Aldrich, Germany), and left overnight in T75 flasks (Corning, Sigma-Aldrich, Germany) in a 5% CO_2 incubator set at 37°C and 90% humidity. After one day of culture, non-adherent cells were washed out with PBS containing 1% penicillin/streptomycin solution and resuspended in a fresh cell culture medium. Adherent cells were cultured until a 90% confluence was achieved, with cell culture medium changed every 72 hours. When the cells became confluent, they were treated with trypsin-EDTA solution (Gibco by Life Technologies, USA), followed by enzymatic neutralization. Then, the cells were centrifuged at 300 g for 10 min. Isolated ADSCs were counted with a hemocytometer and then cultured in triplicates onto 96-well plates, at the density of 0.25×10^6 cells/ ml. After a 24-hour incubation, the cells were exposed to PEMF (7 Hz, 30 mT, three exposures, each lasting 4 hours, with 24-hour intervals in between.

Euthanasia and tissue harvestings

On the 21st day of the experiment, animals from all groups were sacrificed by anesthetic overdose (Pentobarbital, Morbital, Puławy), to harvest adipose tissue specimens.

Temperature measurements

BT of rats from all the study groups was measured twice a week. To minimize stress and pain, BT of rat pups was measured with an infrared thermometer (Anima, Vivari), whereas the measurements in adults were taken with a rectal thermometer (Anima, Vivari).

ELISA tests

Concentrations of cytokines, TNF-α and IL-6, in serum and ADSCs cultures were measured using ELISA with commercially available kits purchased from Diaclone (SAS, France), strictly following the manufacturer's instructions.

Statistical analysis

All results are presented as arithmetic means ± their standard deviations (SD). Intergroup comparisons were carried out with Student t-test, with the threshold of statistical significance set at $p<0.05$. Statistically significant differences were designated with asterisks.

Results

Female pups maintained on LF diet had significantly higher BT than female pups grown on HF diet. The same phenomenon was also observed in the case of male pups. BT of rat pups turned out to be lower than in adult rats, but this difference might be associated with the fact that the measurements in these two age groups were taken with different types of thermometer, infrared and rectal one, respectively (Fig. 1 and 2).

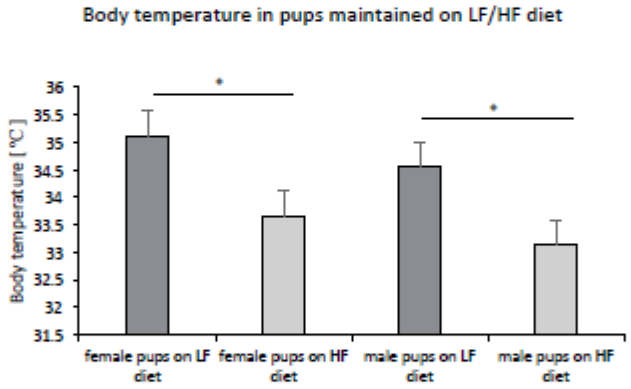

Fig. 1. Effect of HF/LF diet on body temperature in female and male rat pups. The results are presented as mean (+SD), the statistical significance of intergroup differences verified with Student t-test, * $p<0.05$.

BT of female adult rats maintained on LF diet was significantly higher than the temperature of adult females kept on HF diet. Also, male adult rats maintained on LF diet presented with significantly higher BT than the males receiving HF diet (Fig. 2).

Serum concentrations of cytokines, TNF-α and IL-6, in female pups and adult females maintained on HF diet were significantly higher than in their counterparts grown on LF diet (Fig. 3).

Both female and male pups received the same type of diet (HF or LF) as was given to their mothers during pregnancy.

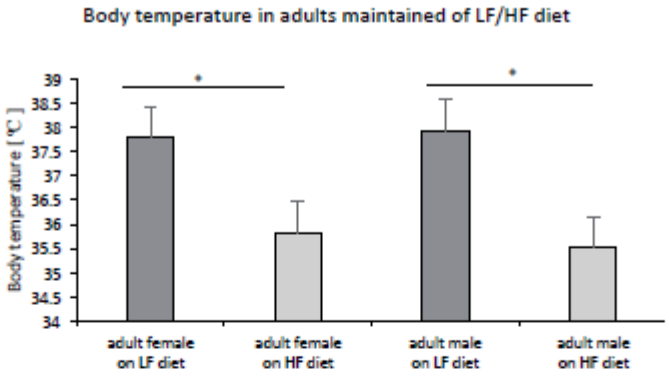

Fig. 2. Effect of HF/LF diet on body temperature in female and male adult rats. The results are presented as mean (+SD), the statistical significance of intergroup differences verified by Student t-test, * $p < 0.05$.

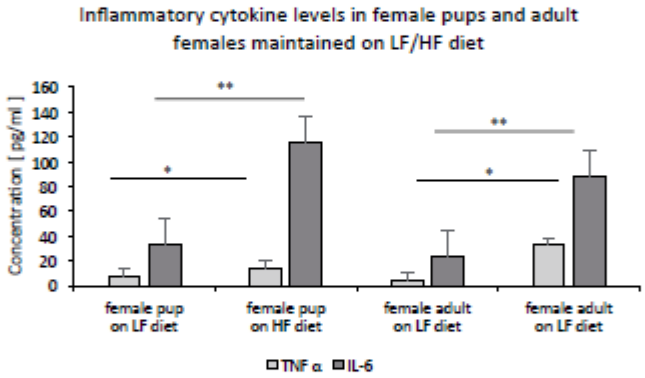

Fig. 3. Serum concentrations of TNF-α and IL-6 in female pups and adult females maintained on LF and HF diet, as determined by ELISA. The results are presented as mean (+SD), the statistical significance of intergroup differences verified by Student t-test, * $p < 0.05$, ** $p < 0.001$.

Serum concentrations of TNF-α and IL-6 in male pups and adult males grown on HF diet were significantly higher than in respective groups of male rats maintained on LF diet (Fig. 4).

While the exposure to PEMF contributed to a significant decrease in the release of TNF-α from ADSCs obtained from female pups grown up on LF diet, the amount of TNF-α synthesized by PEMF-exposed ADSCs from female pups maintained on HF diet was significantly higher than in non-exposed ADSCs from the same group of animals. Conversely, the exposure to PEFM resulted in a significant increase in the amount of IL-6 secreted by ADSCs from female pups maintained on LF diet, but PEMF-treated ADSCs from female pups grown on HF

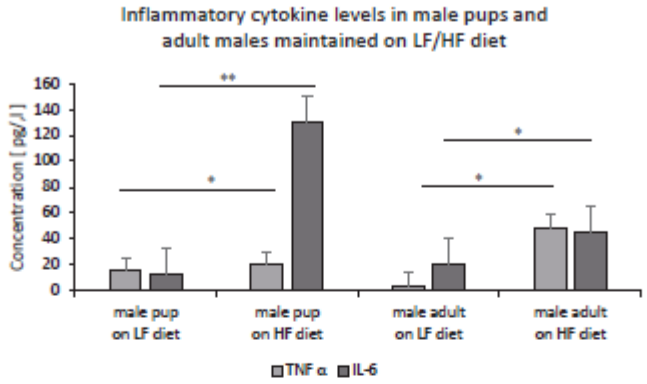

Fig. 4. Serum concentrations of TNF-α and IL-6 in male pups and adult males maintained on LF and HF diet, as determined by ELISA. The results are presented as mean (+SD), the statistical significance of intergroup differences verified by Student t-test, * $p < 0.05$, ** $p < 0.001$.

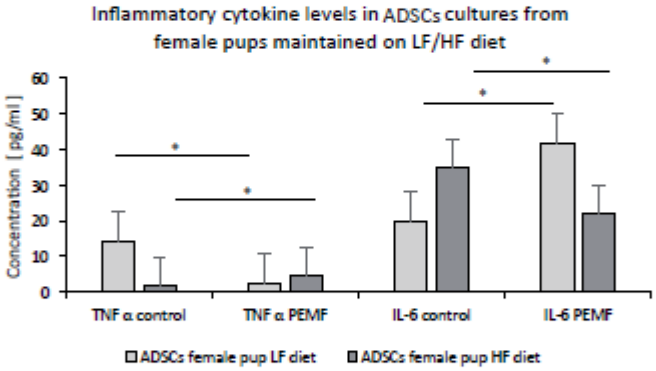

Fig. 5. Concentrations of TNF-α and IL-6 in supernatants from adipose-derived stem cell (ADSCs) cultures from female pups maintained on LF and HF diet, as determined by ELISA. The results for control cultures and cultures treated with the pulsed electromagnetic field (PEMF) are expressed as mean (+SD), statistical significance of intergroup differences verified by Student t-test, * $p < 0.05$.

diet produced significantly lesser amounts of this cytokine than the non-treated cells (Fig. 5).

PEMF-exposed ADSCs from adult females grown on either LF or HF diet produced significantly larger amounts of TNF-α than respective non-exposed ADSCs cultures. While the exposure to PEMF contributed to a significant decrease in the amount of IL-6 synthesized by ADSCs from adult females maintained on LF diet, no significant differences were found in the concentrations of this cytokine in PEMF-treated and non-treated ADSCs cultures from females grown on HF diet (Fig. 6).

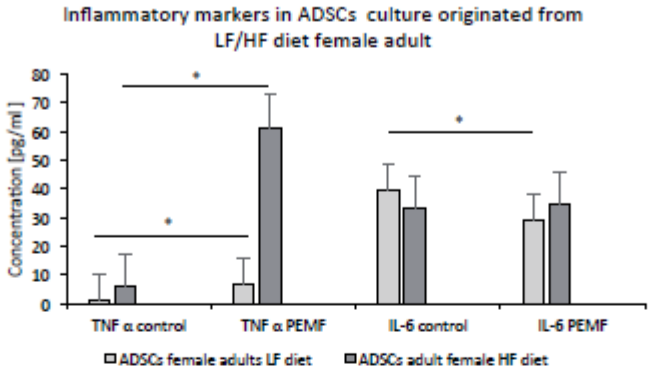

Fig. 6. Concentrations of TNF-α and IL-6 in supernatants from adipose-derived stem cell (ADSCs) cultures from adult females maintained on LF and HF diet, as determined by ELISA. The results for control cultures and cultures treated with the pulsed electromagnetic field (PEMF) are expressed as mean (+SD), statistical significance of intergroup differences verified by Student t-test, * $p < 0.05$.

Irrespective of the diet type, exposure to PEMF contributed to a significant increase in the concentration of TNF-α in the supernatants of ADSCs cultures from male pups. While IL-6 concentration in PEMF-exposed ADSCs cultures from male pups maintained on LF diet was significantly lower than in non-exposed cells, an opposite effect, i.e. post-exposure increase in IL-6 level was observed in ADSCs from male pups fed with HF diet (Fig. 7).

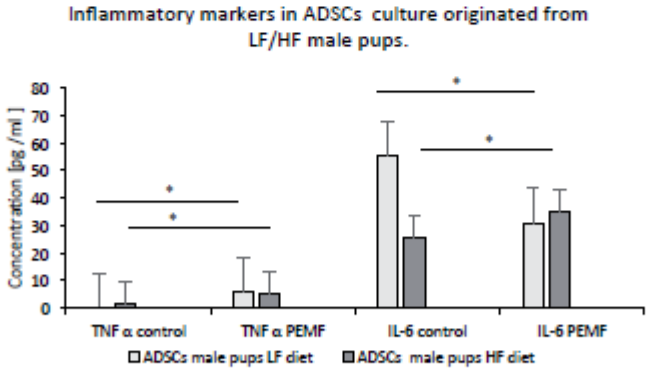

Fig. 7. Concentrations of TNF-α and IL-6 in supernatants from adipose-derived stem cell (ADSCs) cultures from male pups maintained on LF and HF diet, as determined by ELISA. The results for control cultures and cultures treated with the pulsed electromagnetic field (PEMF) are expressed as mean (+SD), statistical significance of intergroup differences verified by Student t-test, * $p < 0.05$.

Following the exposure to PEMF, ADSCs from adult male rats maintained on HF diet, but not from animals kept on LF diet, produced significantly more TNF-α than non-treated cells. Irrespective of the diet type, the exposure to PEMF contributed to a significant increase in the production of IL-6 by ADSCs (Fig. 8).

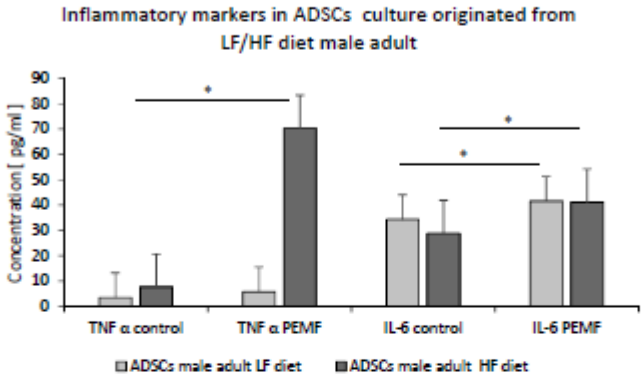

Fig. 8. Concentrations of TNF-α and IL-6 in supernatants from adipose-derived stem cell (ADSCs) cultures from adult males maintained on LF and HF diet, as determined by ELISA. The results for control cultures and cultures treated with the pulsed electromagnetic field (PEMF) are expressed as mean (+SD), statistical significance of intergroup differences verified by Student t-test, * $p < 0.05$.

Discussion

Non-shivering thermogenesis in mammals is associated with the activity of BAT, and is responsible for the maintenance of BT, especially during inflammatory processes [31]. BAT differs morphologically and functionally from WAT as it contains small intracellular lipid droplets, a greater number of mitochondria, synthesizes UCP-1 and shows enhanced metabolic activity [32]. Some published evidence suggests that the activity of BAT may be limited in obese humans [33]. In our present study, male and female rat pups grown on a standard LF diet had similar BT, 35.5°C ± 0.1 and 35.6°C ± 0.9, respectively [34]. Our findings are consistent with the results published by Tsushima *et al.* who demonstrated that HF diet had an effect on BT in adult male rats, which was lower than in the controls [35]. Also in another study, in which BT was measured twice a day, male adult rats fed with HF diet presented with lower body temperatures than the controls [36]. We observed that male and female rats maintained on an HF diet, either pups or adults, had increased piloerection (not shown) which may indicate disturbances at the BAT function level.

The results of our study are consistent with the observations made by De Almeida *et al.* according to whom male offspring from mothers grown on HF

diet during lactation period (with light and dark cycle kept) had lower BT then the controls maintained on LF diet [37]. However, another study conducted in mice produced contradictory findings, since 4-week-old animals that were grown on HF diet presented with higher BT than their counterparts maintained on LF diet; this effect was observed during the day and was followed by a decrease in early-night thermogenesis [38]. Finally, some authors did not find significant diet-related differences in rectal temperatures of adult male rats [39].

Maternal HF diet is known to promote the signs of early obesity, excessive proliferation of white adipocytes and enhanced accumulation of BAT in the offspring [40]. Male and female offspring from dams fed with HF diet during mating, gestation and lactation were overweight and showed greater body adiposity. However, some sex-specific differences were observed in the offspring's response to HF diet, as only males presented with hyperleptinemia and had higher energy expenditures [41]. Maternal HF diet was also shown to contribute to elevated plasma levels of TNF-α and IL-β in the offspring [42]. Our findings are consistent with the results of an *in vitro* study conducted by Tinkow *et al.*, in which the levels of IL-6 in adult female rats maintained on HF diet were significantly higher than in animals grown on a standard LF diet. Also, serum concentrations of IL-6 in male adult mice and rats grown on HF diet were shown to be higher than in control groups fed with LF diet [43, 44]. Likewise in our study, Díaz-Rúa *et al.* demonstrated that HF diet had an effect on serum TNF-α level in adult male rats [45]. Another study, conducted in overweight rodents, showed that elevated concentration of TNF-α was a marker of underlying inflammation [46]. An increase in serum TNF-α level was also previously observed in young male mice grown on HF diet [47]. Elevated levels of TNF-α and IL-6 were also found in visceral adipose tissue (VAT) harvested from male offspring grown on HF diet. Interestingly, however, the level of TNF-α in subcutaneous adipose tissue (SAT) from female pups maintained on the HF diet was similar as in the controls [48].

PEMF treatment is a non-invasive method to deliver electric and magnetic fields to tissues especially those affected by various pathological processes. Published evidence from clinical studies in humans and animal experiments suggests that PEMF treatment may produce beneficial effects in bone and wound healing, inflammation, treatment of post-operative pain and edema [49]. *In vitro* studies demonstrated that PEMF exerts an anti-inflammatory effect in cell culture models [50, 51].

Following the exposure to PEMF, the cells of the nucleus pulposus from adult male rats released less IL-1β and TNF-α to cell culture medium [52]. In another study, low-frequency PEMF treatment (2.5 ± 0.3 mT, 75 Hz, 1.3 ms pulse duration) maintained at low levels the production of proinflammatory cytokines (IL-1β, TNF-α and IL-6) in mononuclear cells obtained from adult male rats [53]. To the best of our knowledge, none of the previous studies except those conducted by our group [9, 54], have analyzed the effect of PEMF on ADSCs in an animal model for obesity. Our

findings suggest that the exposure to PEMF may alter the profile of biomarkers synthesized *in vitro* by undifferentiated ADSCs and that this effect may depend on animal age, sex and the type of diet.

Conflict of interest

None declared.

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Kraków, dnia.13/02/2019

Mgr inż. Agnieszka Baranowska
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

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Baranowska Agnieszka

(podpis współautora)

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Bartłomiej Noszczyk

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Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr inż. ...Agnieszki Baranowskiej .... przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Kaszuba-Zwoińska

(podpis współautora)

Kraków, dnia 12/02/2019

Dr hab. Krzysztof Gil, prof. UJ
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Effect of the pulsed electromagnetic field on the release of inflammatory mediators from adipose-derived stem cells (ADSCs) in rats"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

- wsparcie finansowe projektu
- rewizja tekstu manuskryptu
- akceptacja wersji finalnej manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez Panią mgr inż. Agnieszkę Baranowską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr inż. Agnieszki Baranowskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

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p.o. Kierownik

.....
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