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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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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 TNF α 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 TNF α 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 TNF α . 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 pathomechanisms, 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 (TNF α), 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]. Resistin expression can be upregulated by some interleukins such as IL-6, IL-1 and TNF α 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% \pm 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 μ m 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% CO₂ 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 (ADSCs) 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% CO₂ 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 TNF α , 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 proinflammatory cytokines TNF α 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).

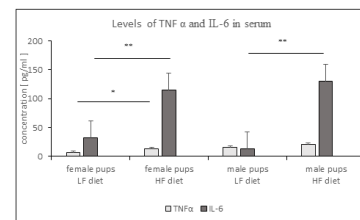


Figure 1: Concentration of proinflammatory cytokines TNF α and IL-1 in serum from female and male pups grown on low (LF) and high (HF) fat diet assessed by ELISA tests. The data are expressed as mean (+ SD), statistical significance was determined by Student t-test * $p < 0.05$, ** $p < 0.001$.

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).

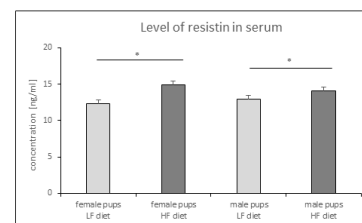


Figure 2: Concentration of resistin in serum from female and male pups grown on low (LF) and high (HF) fat diet assessed by ELISA tests. The data are expressed as mean (+ SD), statistical significance was determined by Student t-test * $p < 0.05$.

Serum level of proinflammatory cytokines and resistin in rat adults of both genders grown on LF and HF diet: The serum level of TNF α 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).

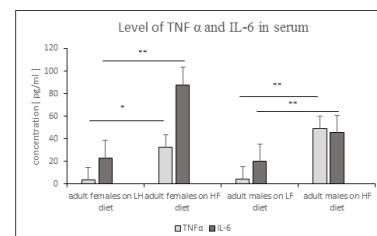


Figure 3: Concentration of proinflammatory cytokines TNF α and IL-6 in serum of female and male adults grown on low (LF) and high (HF) fat diet assessed by ELISA method. The data are expressed as mean (+ SD), statistical significance was determined by Student t-test * $p < 0.05$, ** $p < 0.001$.

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).

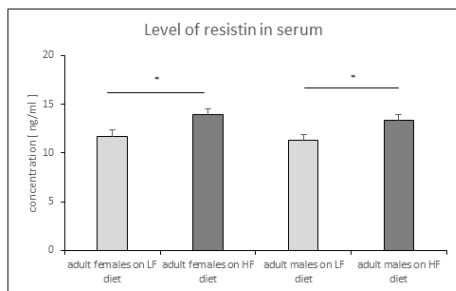


Figure 4: Concentration of resistin in serum samples obtained from female and male adults grown on low (LF) and high (HF) fat diet assessed by ELISA tests. The data are expressed as mean (+SD), statistical significance was determined by Student t-test * $p < 0.05$.

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 TNF α 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).

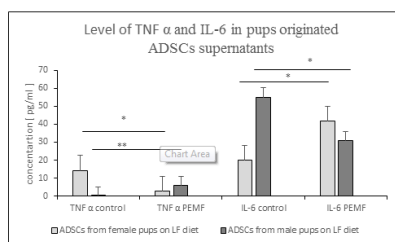


Figure 5: Concentration of pro-inflammatory cytokines TNF α and IL-1 in the supernatants from female and male pups originated from adipose derived stem cells (ADSCs) grown on low (LF) fat diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+SD), statistical 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 TNF α 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).

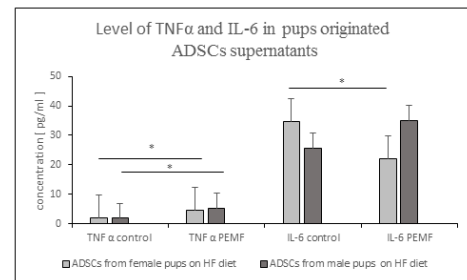


Figure 6: Concentration of pro-inflammatory cytokines TNF α and IL-1 in supernatants from female and male pups originated from adipose derived stem cells (ADSCs) grown on HF diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+SD), statistical significance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

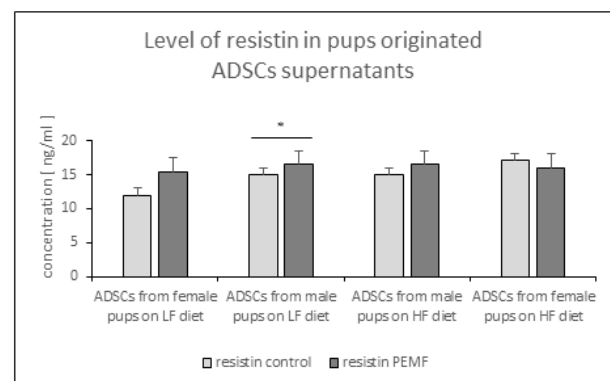


Figure 7: Concentration of resistin in supernatants from female and male pups in adipose derived stem cells (ADSCs) grown on low (LF) and high (HF) fat diet assessed by ELISA tests. The data are expressed as mean (+SD), statistical significance was determined by Student t-test * $p < 0.05$.

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**.

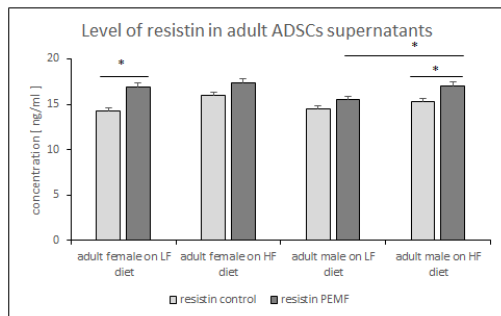


Figure 8: Concentration of resistin in supernatants from male and female adults grown on low (LF) and high (HF) fat diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+SD), statistical significance was determined by Student t-test * $p < 0.05$.

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 TNF α 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 TNF α and IL-6 (**Figure 9**).

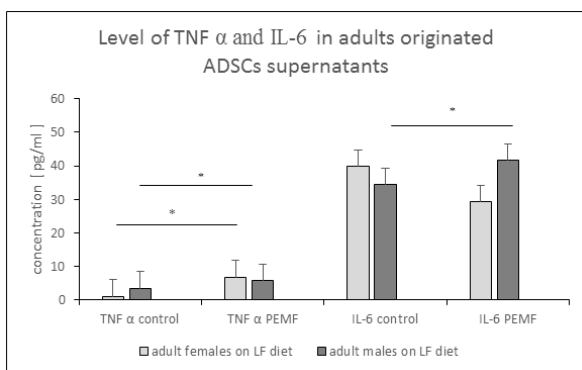


Figure 9: Concentration of pro-inflammatory cytokines and IL-6 in supernatants from female and male adults grown on low (LF) fat diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+ SD), statistical significance was determined by Student t-students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

Spectacular increase of the TNF α 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**).

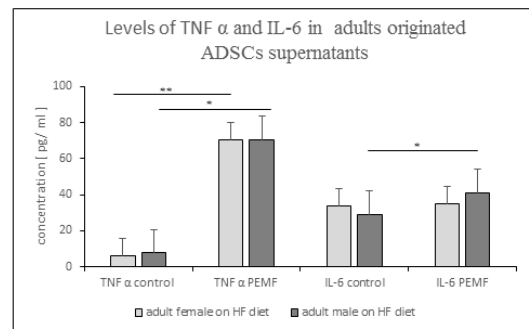


Figure 10: Concentration of pro-inflammatory cytokines TNF α and IL-6 in supernatants from male and female adults grown on high (HF) fat diet assessed by ELISA tests under pulsed electromagnetic field (PEMF). The data are expressed as mean (+ SD), statistical significance was determined by Student t-students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

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, TNF α was revealed in fetal plasma [33]. Also TNF α 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, TNF α 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 TNF α 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 TNF α , 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 TNF α 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 TNF α , 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 (TNF α , 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 TNF α 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 TNF α and IL-6 [52]. In our study, PEMF treatment of ADSCs from adult female rats on LF diet presented low IL-6 level, however TNF α 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) TNF α 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-1 β , TNF α) 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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