Research Article

Adiponectin Has Anti-inflammatory Effects on Adipose Tissue in Mice Fed Methionine-Choline Deficient Diet

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Abstract

Background: Adiponectin is an adipokines secreted by adipose cells and found to be lower in obese subjects than in lean subjects.
Aim: To examine potential anti-inflammatory effects of adiponectin on adipose tissue of animals on methionine-choline deficient diet.
Materials&Methods: 25 adult male albino mice were used in the study. The animals were divided into three groups: Group M1 animals (10 mice) were fed methionine-choline deficient diet for three weeks. On the third week, they were treated with intraperitoneal adiponectin injections (1.5 mg/kg/day. Group M2 animals (10 mice) were fed the same diet for three weeks. On the third week, they were treated with intraperitoneal 0.9% saline placebo injections. Control animals (5 mice) were fed regular chow for 3 weeks. The epididymal fat pad was bluntly dissected and transferred to fixative. It was prepared for routine paraffin sectioning and stained with H&E. The number of cells per mm^2, adipocyte diameter and surface area were assessed using *Image J Adipocytes Tool Macros.*
Results: Adipocyte measurements were significantly reduced in both groups M1 and M2 in comparison to control animals. Fat sections from group M1 showed a marked reduction in fat cell size and tissue surface area with enlarged and congested blood vessels were surrounded by small rounded adipocytes and preadipocytes. Fat sections from group M2 animals showed an unusual picture of fatty microsyst formation, enlarged congested blood vessels surrounded by thick connective tissue cells mantle and in between the cysts, small fat cells and inflammatory infiltrate were observed. Some large cysts surrounded areas loaded with macrophages and preadipocytes at different stages of maturation.
Conclusion: The less amount of inflammation and absence of fatty microcysts in adiponectin treated animals suggests that adiponectin may have anti-inflammatory effects in rapidly remodeling adipose tissue.

Key words: Adiponectin, Methionine-Choline Defecient Diet, Adipose Tissue.

INTRODUCTION

Adiponectin is a 30 kDa protein consisting of 244 amino acids and structurally belongs to the soluble defense collagen superfamily [(1)]. Adiponectin was initially thought to be produced exclusively by adipocytes. However, several studies have shown it to be expressed, both at mRNA and protein levels, in other tissues such as human and murine osteoblasts [(2)], Hepatocytes [(3)], myocytes [(4)], epithelial cells [(5)] and placenta [(6)]. In contrast to most adipokines, plasma adiponectin levels are found to be lower in obese subjects than in lean subjects, and strong negative correlations between plasma adiponectin levels and body mass index (BMI)

have been shown both in humans and in animals [7]. Adiponectin has various biological functions resulting from a combination of endocrine and autocrine/paracrine effects. They include, but are not limited to, insulin-sensitizing, anti-atherogenic, anti-inflammatory and anti-tumor functions [8].

The current study was designed to investigate potential anti-inflammatory effects of adiponectin on adipose tissue of animals on methionine-choline deficient (MCD) diet, a diet that causes rapid adipose tissue loss and inflammation.

MATERIALS & METHODS

Twenty five adult male albino mice (Mus musculus) were used in the study. They were 7-8 weeks of age with an average weight of 27.1 grams. The animals were purchased from the Iraqi Center for Cancer and Medical Genetics Research, Baghdad – Iraq. They were transported and studied at the Postgraduate Lab of Al-Mustansiriyah University, College of Medicine - Department of Anatomy, Histology and Embryology. The study began in October 2013 and lasted 4 months.

All mice were housed in plastic tub cages with a stainless steel grid lid and wood shavings scattered on the floor, under controlled room temperature (22±2 °C) and humidity with 12:12 hour light / dark cycle. All the animals were provided with commercially available regular mouse chow and water *ad libitum*, prior to dietary manipulation. The animals were left to adapt to the lab conditions for one week before starting the study protocol.

The animals were divided into three groups:

- **Group M1** animals (10 mice) were fed MCD diet for three weeks. On the third week, they were treated with intraperitoneal adiponectin injections. They served to study the effects of adiponectin on adipose tissue.
- **Group M2** animals (10 mice) were fed MCD diet for three weeks. On the third week, they were treated with intraperitoneal 0.9% saline placebo injections. They served as model control for group A1.
- **Control** animals (5 mice) were fed regular chow for 3 weeks and were given intraperitoneal 0.9% saline placebo injections. They served as healthy controls.

The methionine-choline deficient (MCD) diet was prepared according to Leclercq *et al.* [9] and consisted of the ingredients shown in table 1. The formula provided 4.2 kcal/g of energy derived from 65% carbohydrate (70:30 sucrose-starch), 17% protein (as defined amino acids) and 10% fat (as corn oil). The diet was prepared by mixing the ingredients with a small amount of water to form dough pellets that were then baked at 55 °C for 6 hours and dried. After baking, MCD pellets were stored in a refrigerator at 2-8°C.

Table 1: Composition of the methionine-choline deficient diet [9]

Ingredient	**Quantity (g/kg)**
Sucrose	455.3
Corn Starch	200
Corn Oil	100
Non-nutritive bulk	30
Amino acid mix (devoid of methionine)	174.4
Mineral Mix	35
Dicalcium phosphate	3
Vitamin Mix (devoid of Choline)	2.3

Adiponectin protein was purchased from abcam® (UK) in the form of histidine-tagged recombinant full-length mature mouse adiponectin expressed in E. coli and corresponding to 247 amino acids. It was provided as a liquid with a concentration of 1mg/ml. The protein was shipped at 4ºC. Upon arrival, it was aliquoted into 100µg portions that were stored at -20ºC until the time of injection, to avoid freeze-thaw cycles, as instructed by the manufacturer. A single daily intraperitoneal injection was given to treated animals at a dose of 1.5 mg/kg/day for a duration of 1 week [10].

Animals were euthanized by opening the abdominothoracic cavity and draining blood under anesthesia with chloroform. The epididymal fat pad was identified by applying gentle traction to the seminal vesicles to isolate the testis, epididymis and fat depot. The fat pads were bluntly dissected and weighed and transferred to fixative. The fat pad was prepared for routine paraffin sectioning and stained with H&E according to Bancroft and Stevens [11].

The number of cells per mm^2, adipocyte diameter and surface area were assessed using *Image J Adipocytes Tool Macros* with the *Watershed Algorithm*. The plugin macros renders the image through a series of processes that remove the background, exclude edging cells, segment cell spaces and calculate number, Ferret's diameter and surface area. The results for each image were reviewed carefully and overlapping or connected spaces were corrected manually.

Results were directly loaded into SPSS (v.20.0.0) statistics software for analysis. Values less than 100 μm^2 in surface area were assumed to represent artifacts from the image-conversion and thresholding processes and were excluded from analysis. By using analysis of variance (ANOVA), the results were evaluated statistically, and whenever there was a difference

between the correlated groups, student t-test was applied to estimate the degree of significance by comparing the mean of data and standard deviation of each group. Therefore, data are presented as measures of mean ± standard deviation, at 95% confidence interval.

RESULTS

The The fat tissue of control animals on regular chow showed normal histomorphology with a variety of medium-sized to large adipocytes, rounded and polygonal, with clear connective tissue septa and scattered blood vessels (figure 1).

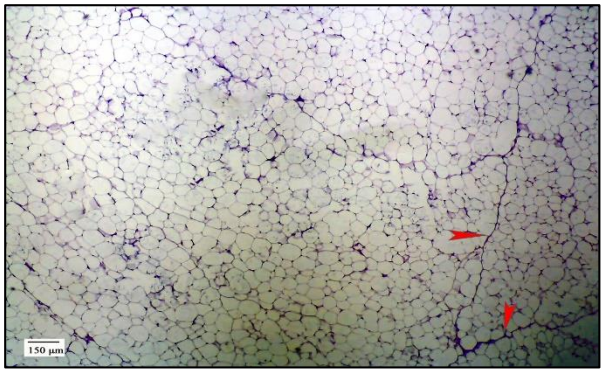

Figure 1: Epididymal fat tissue of control animals showing variable adipocyte sizes and clear connective tissue septa (red arrows). H&E X40

Epididymal fat sections from group M1 (adiponectin treated) showed a marked reduction in fat cell size and tissue surface area (figure 2, 3). The fat pad was surrounded by a thickened connective tissue capsule. Enlarged and congested blood vessels were surrounded by small rounded adipocytes. On high magnification, the smaller rounder fat cells had round eccentric nuclei. Preadipocytes were seen close to congested blood vessels (figure 4). They had clear pale nucleus with a dark staianinf nucleolus and a foamy cytoplasm. Inflammatory cells were scattered among fat cells and around thickened vascular walls.

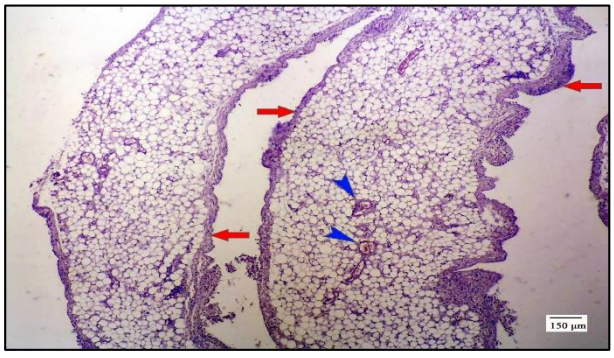

Figure 2: Epididymal fat tissue of group M1 animals showing thickened connective tissue capsule (red arrows) and engorged large blood vessels (blue arrowheads). H&E X40

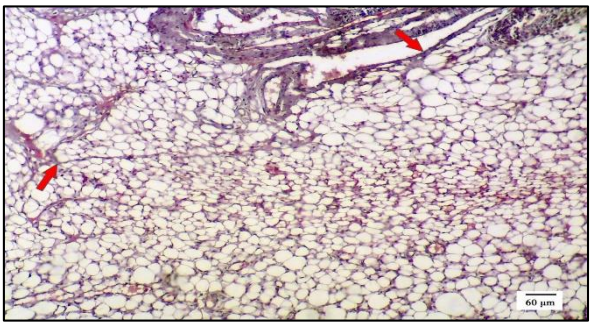

Figure 3: Epididymal fat tissue of group M1 animals showing thickened connective tissue capsule and septa (red arrows) and numerous small adipocytes H&E X100

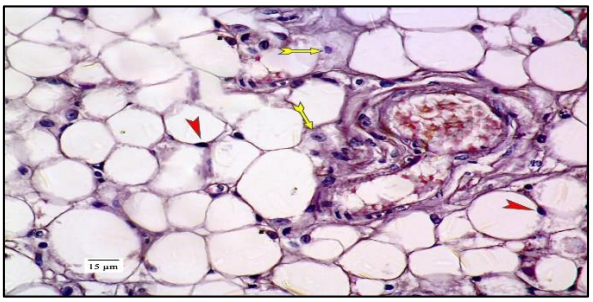

Figure 4: Epididymal fat tissue of group M1 animals showing numerous small adipocytes with eccentric round nuclei (red arrowheads), congested large blood vessels surrounded by maturating preadipocytes (yellow arrows). Inflammataory cells are scattered among adipocytes. H&E X400.

Fat sections from group M2 animals showed an unusual picture of fatty microsyst formation (figure 5, 6). The cysts were > 100 µm in diameter (range 110-223 µm), mostly rounded or oval and occupied by a large fat droplet. Enlarged congested blood vessels were seen surrounded by thick connective tissue cells mantle. In between the cysts, small fat cells and inflammatory infiltrate were observed. On high magnification (figure 7), some large cysts surrounded areas loaded with macrophages and preadipocytes at different stages of maturation.

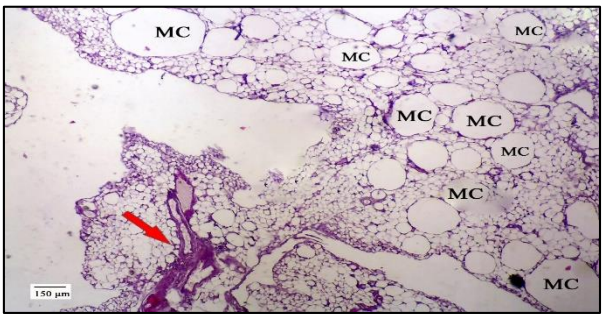

Figure 5: Epididymal fat tissue of group M2 animals showing fatty microsysts (MC) of variable sizes and congested large blood vessels (red arrow). H&E X40

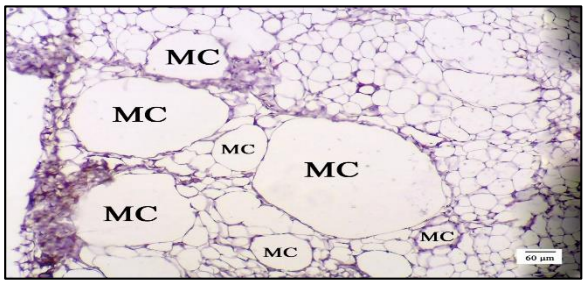

Figure 6: Epididymal fat tissue of group M2 animals showing fatty microsysts (MC) of variable sizes separated by small rounded adipocytes. H&E X100.

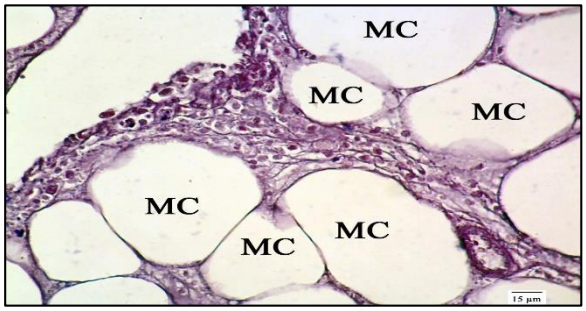

Figure 7: Epididymal fat tissue of group M2 animals showing large fatty microsysts (MC) surrounding an area crowded with preadipocytes and macrophages. H&E X400.

Adipocyte measurements were significantly reduced in both groups M1 and M2 in comparison to control animals, as shown in table 2.

Table 2: Histomorphometric measurements of epididymal adipose tissue in experiment 2 animals. (data expressed as Mean ± Standard Deviation, **= highly significant statistical difference (P<0.01))

Adipocyte Measurements	Groups		
	M1	M2	Control
Diameter (µm)	38.2±4.3	37.7±6.8	72.2±4.1**
Surface area (µm^2)	1189.4±50.3	1098.9±29	4113.6±62.4**
Number (cells/mm^2)	816.6±43.3	860.9±32.6	261±25.5**

DISCUSSION

Adipocyte size reduction seen in MCD fed animals represent the effects of increased secretion and circulation levels of adiponectin due to weight loss. Interestingly, inflammatory features and fatty microcysts were also found, features not consistent with reduction in fat mass. To our knowledge, no previous study on visceral fat in animals fed MCD diet reported such changes. The less amount of inflammation and absence of fatty microcysts in adiponectin treated animals suggests that adiponectin may have acted on three levels.

Firstly, adiponectin reduces adipose tissue inflammation via its anti-inflammatory actions. Early research on adiponectin identified a domain that is homologous to complement protein 1 and a domain with structural similarity to TNFα. Such homology and similarity encouraged the investigation of adiponectin as an inflammatory protein. However, clinical observations identified an inverse correlation between adiponectin and mild inflammation, suggesting that adiponectin may repress inflammation [(12)]. Adiponectin is also a known inhibitor of NFκB and ROS and promotes the production of the anti-inflammatory cytokine IL10. It has been found to accumulate at the stromal vascular fraction of inflamed adipose tissue of obese animals [(13)].

Secondly**,** adiponectin decreases adipose tissue inflammation through the reduction of macrophage infiltration. It reduces the adipose tissue macrophage population through multiple mechanisms including inhibition of monocyte adhesion to epithelial membranes, reduction of pro-macrophage cytokines, and retardation of macrophage growth [(14).]

Lastly, adiponectin may affect adipose tissue by preventing lipotoxicity and insulin resistance. Fu and colleagues [(15)] demonstrated that adiponectin has a new role as an autocrine factor in adipose tissues: promoting cell proliferation and differentiation from preadipocytes into adipocytes, augmenting programmed gene expression responsible for adipogenesis, and increasing lipid content and insulin responsiveness of the glucose transport system in adipocytes. Kim *et al.* [(16)] published a report supporting these findings that suggests adiponectin promotes adipose tissue expansion. This study examined the effects of transgenic overexpression of a mutated form of adiponectin in the obesity prone ob/ob mouse model. The combination of the *ob/ob* obesity model and high concentrations of adiponectin produced the fattest obese mouse model to date. Intriguingly, these massively obese mice presented serum metabolic markers within the normal range. The authors concluded that massive expansion of adipose tissue allowed for excessive lipid storage, thereby preventing the adverse consequences related to lipotoxicity. They further concluded that the increase in subcutaneous adipose tissue mass was a result of adipogenesis, as based on histological procedures. Evidence from an *in vitro* 3 cell culture model indicates proliferation and differentiation of preadipocytes is increased with the hyperexpression of adiponectin.

The current work supports these findings, as adiponectin treatment in MCD-inflamed adipose tissue reduced inflammatory features and promoted adipogenesis evident by increased pictures of preadipocytes. The presence of fatty microcysts in placebo treated MCD fed animals represent abnormal adipose tissue remodeling changes similar to lipodystrophy characterized by unhealthy adipose tissue shrinkage with immune cell infiltration, defective angiogenesis and extracellular matrix (ECM) overproduction [17]. With increased inflammation, dipocyte death ensues and adjacent adipocytes may fuse into microcystic structures. Adipocyte death acts as a driving stimulus for macrophage infiltration. Macrophages aggregate around the dead or dying adipocytes and may fuse to phagocytize the residual lipid droplet [18]. The presence of numerous small adipocytes may be related to increased lipolysis caused by insulin resistance in the absence of adiponectin.

Conclusion

MCD diet feeding results in rapid visceral fat pad shrinkage with inflammatory changes that are ameliorated by concomitant adiponectin treatment.

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