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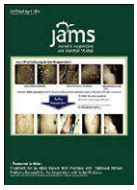

RESEARCH ARTICLE

Effect of Electroacupuncture on Inflammation in the Obese Zucker Fatty Rat Model of Metabolic Syndrome

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Abstract
Chronic inflammation is known to be associated with visceral obesity and insulin resistance and is characterized by altered levels of production of adipokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, leptin, and adiponectin. Metabolic syndrome (MetS) is a major and escalating public health and clinical challenge worldwide, and patients with MetS have an increased risk of developing cardiovascular disease and type 2 diabetes mellitus. Electroacupuncture (EA) was tested as a means of decreasing inflammation in genetically obese Zucker fatty rats, which serve as a model of MetS. Repeated application of EA at the Zhongwan/Guanyuan acupoints decreased serum TNF-α, but produced no significant alterations in serum leptin, adiponectin, or IL-10. EA had no significant effect on the levels of these four adipokines in white adipose tissue. These findings are consistent with the supposition that EA inhibits proliferation and/or infiltration of macrophages into the adipose tissue of obese rats and stimulates the release of IL-10 from the decreased numbers of macrophages present in adipose tissue. Compared with the control animals, no significant change in body weight occurred. The blood glucose (BG) level over a 30-minute interval in Week 2 was relatively the same as that in Week 1, suggesting that EA treatment does not increase the likelihood of developing hyperglycemia.

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1. Introduction

Obesity and diabetes mellitus have emerged as major health issues worldwide with increasing incidence and prevalence. According to the World Health Organization Global Health Estimates, in 2014, 39% of adults aged $\geq$ 18 years were overweight (body mass index $\geq$ 25 kg/m^2), 13% were obese (body mass index $\geq$ 30 kg/m^2) [1] and 9% had diabetes [2]. The International Diabetes Federation has defined metabolic syndrome (MetS) as a cluster of risk factors such as prediabetes, diabetes, abdominal obesity, hypercholesterolemia, and hypertension that collectively increase the risk of cardiovascular diseases. The International Diabetes Federation also estimates that a quarter of the global adult population has MetS and that this is likely to be an upward trend [3].

Obesity is described as a low-grade chronic inflammatory condition characterized by adipocyte hyperplasia and hypertrophy [4], increased levels of circulating leptin [5], decreased levels of circulating adiponectin [6], and increased numbers of classically activated macrophages in the white adipose tissue which produce many proinflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 [4]. Obesity often coexists with other serious morbidities such as hypertension, cardiovascular disease, diabetes, and cancer [7]. Treatments that have been used to reduce obesity include physical exercise, lifestyle changes including diet, psychosocial counselling, bariatric surgery, liposuction, and acupuncture. Medications have proven to be largely ineffective, often causing serious side effects including cardiovascular disease [8].

The development of obesity has been attributed to both genetic and environmental factors. Obese Zucker fatty rats have been widely investigated and used as genetic models of obesity and MetS. These obese rats possess a single gene (fa/fa) mutation characterized by a defect in leptin receptor [9—12] and can be visually distinguished from their lean littermates by 5 weeks of age due to excessive deposition of subcutaneous [13] and visceral adipose tissue. They are characterized by hyperphagia, insulin resistance, dyslipidemia, central adiposity, and hypertension [14], all risk factors involved in MetS [12]. These rats have also previously been used to evaluate the effects of antiobesity treatment like dehydroepiandrosterone [15], agonists of peroxisome proliferator-activated receptor alpha [16,17] and gamma [16] on serum lipids, body weight, and insulin sensitivity. Although effective in regulating certain parameters in obesity, many of them also cause serious side effects [8,18,19]. Habitual exercise was also shown to increase TNF-α in the adipose tissue of obese Zucker fatty rats [20].

Based on the antiinflammatory effects of electroacupuncture (EA) [21], it has been proposed as a means to control the low-grade inflammation in the obese Zucker fatty rat model of MetS. The present study was designed to test whether repeated application of EA in obese Zucker fatty rats would lead to a change in the imbalance of proinflammatory and antiinflammatory cytokines produced by white adipose tissue. The effect of EA on body weight and levels of glucose, insulin, leptin, adiponectin, TNF-α, and IL-10 were measured. A change in the relative concentrations of IL-10 and TNF-α in blood and adipose tissue would indicate a reversal in activation state of adipose tissue macrophages, while a change in the relative concentrations of adiponectin and leptin levels would indicate an alteration in insulin resistance. Antiobesity treatments or medications should ideally not increase blood glucose (BG) levels, as otherwise, there would be a tendency towards diabetes which often coexists with obesity.

2. Materials and methods

2.1. Animals

Male obese Zucker fatty rats, 12—14 weeks of age, were obtained from a breeding colony maintained at the Taieri Animal Station and delivered to the Hercus Taieri Research Unit, University of Otago, Dunedin, New Zealand. The rats were fed standard rat chow (Specialty Feeds irradiated rat and mouse cubes, 4.8% fat, 20% protein, amino acids, vitamins and minerals, 3.34 kcal/g; Specialty Feeds, Glen Forest, Western Australia, Australia) and acclimatized to the new environment for 1 week. They were housed in individual cages with food and water *ad libitum* in a room with a 12 hour/12 hour light/dark cycle at constant temperature. Animals were deprived of food at 3:00 PM on the day prior to the experiment to ensure an overnight fast of at least 17 hours. This study was approved by the University of Otago Animal Ethics Committee.

2.2. Treatment of animals

2.2.1. Anesthesia and EA

The obese Zucker fatty rats were divided into two groups. Rats in both groups were anesthetized with halothane (1%) in 3:1 mixture of nitrous oxide:oxygen 1.2 L/min. This involved placing the nose of each animal in the nose cone of the anesthetic apparatus. BG was measured with a handheld glucometer (Accu-Chek Advantage, Roche, Roche Diagnostics NZ Ltd., Mt Wellington, Auckland, New Zealand) after needle pricking the lateral saphenous vein of one of the hind limbs at 10 minutes and 20 minutes following anesthesia. This was to confirm that at 20 minutes, BG is relatively stable and can be used as a baseline.

The obese Zucker fatty rats in Group 1 [$n = 5$; mean age (standard error) 14.6 (0.40) weeks; mean weight 528 (6.6) g] were treated with EA applied at the Zhongwan (CV12) and Guanyuan (CV4) acupoints, while obese Zucker fatty rats in Group 2 [$n = 7$; mean age 14.1 (0.40) weeks; mean weight 540 (13.0) g] were not treated with EA and served as controls. EA treatments were given on alternate weekdays giving a total of six applications of EA over 2 weeks. The acupoints were located using the acupoint detector of the EA unit. The Zhongwan acupoint is 9/14 above the pubic crest of the distance measured between the top of the xiphoid process and the pubic crest, whereas the Guanyuan acupoint is 2/14 of this distance above the pubic crest [22]. The hair at the acupoint sites was removed with electric clippers. Sterile acupuncture needles (Seirin Corporation, Shizuoka, Japan, 0.25 mm, 15 mm) were inserted into the muscle layer at the chosen acupoints to a depth of 4 mm. EA

was carried out for 30 minutes at frequency 10 Hz, pulse width 200 μS, and intensity 15 mA using Hans E600 EA unit (Han's Healthronics, Likon, Taipei, Taiwan). The positive and negative charges were connected to the Zhongwan and Guanyuan acupoints, respectively. The frequency and intensity of the electrical stimulation were monitored in a previous study [23] with an oscilloscope located at both ends of a resistor (20 Ω) inserted into the circuit. On each day at the completion of treatment, BG was measured again (i.e., at 50 minutes) and the weights of the animals measured.

2.2.2. Collection of blood serum and white adipose tissue
At the completion of the study on Day 12, the rats were given sodium pentobarbitone (60 mg/mL in sterile saline, 0.4 mL i.p.). Blood was collected by cardiac puncture into tubes and white adipose tissue harvested from the pelvic region and frozen immediately in liquid nitrogen. The blood samples were centrifuged (1500*g*, 10 minutes) and the sera stored at −20°C and adipose tissue samples stored at −80°C until assays for adiponectin, leptin, TNF-α, IL-10, and insulin were performed.

2.3. Homogenization of white adipose tissue

The pelvic region had the largest depot of white adipose tissue in the male obese Zucker fatty rats. Adipose tissue (0.200 g) was homogenized in radioimmunoprecipitation assay (RIPA) buffer (0.60 mL) using a Qiagen tissue lyser II (Venlo, Netherlands; Program P5, 20 Hz, 2 minutes). The RIPA buffer (0.625% Igepal CA-630, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid at pH 7.4) contained 10 μg/mL of protease inhibitor cocktail (Product P8340, Sigma Aldrich, St Louis, MO, USA). Homogenates were centrifuged at 4°C (12,000*g*, 5 minutes). An opening was made in the fat layer and the infranatants collected and stored at −20°C [24].

2.4. Assay of blood sera and white adipose tissue homogenates

The rat blood sera collected at the end of the study were assayed for glucose, insulin, adiponectin, leptin, TNF-α, and IL-10. The white adipose tissue homogenates were assayed for adiponectin, leptin, TNF-α, and IL-10. Glucose was measured spectrophotometrically by the hexokinase-glucose-6-phosphate dehydrogenase method (Modular analyzer, Roche Diagnostics). Insulin and the four mentioned adipokines were measured using rat enzyme-linked immunosorbent assay (ELISA) kits (Millipore, Abacus ALS, Auckland, New Zealand).

2.5. Statistical analysis

Mean values of BG for Group 1 and Group 2 on Day 1, Day 3, Day 5, Day 8, Day 10, and Day 12 were analyzed by analysis of variance (ANOVA) and when a significant p value was found, Duncan's *post hoc* test was performed. BG values were also compared over Week 1 (Day 1, Day 3, and Day 5 combined) and Week 2 (Day 8, Day 10, and Day 12 combined) by the unpaired t test to provide a better examination of possible changes in BG by EA. Body weights of rats in each group on Day 12 were compared with measurements on Day 1 by the paired t test. The levels of insulin, glucose, and adipokines for Group 1 and Group 2 were compared using the unpaired t test. Significance was taken to be at the level of $p < 0.05$.

3. Results

3.1. Effect of repeated EA on body weight

The mean body weight measurements on Day 1 and Day 12 for Group 1 and Group 2 are shown in Table 1. There was a significant decrease in mean body weight on Day 12 compared with Day 1 for both Group 1 and Group 2 ($p = 0.0161$, $p = 0.0295$, respectively). However, there was no significant difference in mean body weight between Group 1 and Group 2 on Day 1 or on Day 12 ($p > 0.05$ for both comparisons, unpaired t test).

3.2. Effect of repeated EA on BG

As in a previous study [23], the baseline BG level was taken to be at 20 minutes and the change in BG was measured over a 30 minute period from 20 to 50 minutes following anesthesia. Table 2 shows the mean BG levels at 20 minutes and 50 minutes and the change in BG on the various days of treatment for both groups. Combined BG measurements at 20 minutes and change in BG over 30 minutes for Week 1 and Week 2 for both groups are presented in Table 3.

By ANOVA, for Group 1, the mean BG at 20 minutes on Day 1 was significantly higher than on Day 3, Day 5, Day 8, and Day 12, but not on Day 10 ($p < 0.0005$). No significant changes were found in mean BG at 50 minutes ($p = 0.112$). For Group 2, the mean BG values at 20 minutes and 50 minutes on Day 1 were significantly higher than on all other days ($p = 0.002$, $p = 0.002$, respectively). In both Group 1 and Group 2, there were no significant changes in the mean change in BG over 30 minutes during the study period ($p = 0.486$, $p = 0.175$, respectively). For Group 1, there was a positive change in BG over 30 minutes on all the treatment days, whereas for Group 2 there was a negative change in BG over 30 minutes on all the treatment days except Day 10. Statistically significant differences were found between the mean change in BG for Group 1 compared with Group 2 on Day 5 (end of Week 1) and Day 12 (end of Week 2) (unpaired t test, $p = 0.0438$, $p = 0.0055$, respectively).

By the unpaired t test, for Group 1, there was no significant difference in baseline BG or change in BG for Week 2 compared with Week 1 ($p = 0.80$, $p = 0.43$,

Table 1 Body weight measurements (g).

	Obese Zucker fatty rats	
	Group 1	Group 2
Day 1	528 (6.6)	540 (13.0)
Day 12	512 (7.3)*	518 (9.2)*

Data presented as mean (standard error).
* Significantly different from Day 1 by paired t test ($p < 0.05$).

Table 2 Blood glucose measurements (mmol/L) on days of treatment.

Day of study	BG at 20 min	BG at 50 min	Change in BG
Group 1 (obese Zucker fatty rats, EA treated)			
Day 1	7.92 (0.53)[a]	8.54 (0.62)	0.62 (0.60)
Day 3	5.42 (0.41)[b]	5.90 (0.86)	0.48 (0.76)
Day 5	4.94 (0.21)[b]	5.96 (0.69)	1.02 (0.49)*
Day 8	6.08 (0.35)[b,c]	6.30 (0.50)	0.22 (0.18)
Day 10	7.04 (0.52)[a,c]	9.58 (2.21)	2.54 (1.79)
Day 12	5.54 (0.42)[b]	6.58 (0.54)	1.04 (0.16)*
Group 2 (obese Zucker fatty rats, control)			
Day 1	7.96 (0.64)[d]	7.64 (0.48)[f]	−0.32 (0.20)
Day 3	5.74 (0.61)[e]	5.14 (0.64)[g]	−0.60 (0.07)
Day 5	4.84 (0.55)[e]	4.66 (0.50)[g]	−0.18 (0.12)
Day 8	5.10 (0.38)[e]	5.06 (0.23)[g]	−0.04 (0.27)
Day 10	5.84 (0.37)[e]	6.06 (0.54)[g]	0.22 (0.30)
Day 12	5.40 (0.19)[e]	5.36 (0.20)[g]	−0.04 (0.24)

Data presented as mean (standard error). Data tested for each group by analysis of variance (ANOVA) and Duncan's test with significance taken to be at the level $p < 0.05$. For each group, values with different superscript letters within individual columns are significantly different.
* Significantly different from Group 2 by unpaired t test ($p < 0.05$).
BG = blood glucose; EA = electroacupuncture.

respectively). However, for Group 2, there was a significant rise in the value of change in BG in Week 2 compared with Week 1 ($p = 0.02$). For Group 1, the change in BG during Week 1 and baseline BG during Week 2 were significantly higher than for Group 2 ($p = 0.005$, $p = 0.03$, respectively).

3.3. Effect of repeated EA on adipokines, insulin, and glucose in blood sera

The mean values for adiponectin, leptin, IL-10, TNF-α, insulin, and glucose in the blood sera collected at the end of the study on Day 12 are presented in Table 4. There were no significant differences in the mean values of adiponectin, leptin, IL-10, insulin, and glucose between Group 1 and Group 2 ($p > 0.05$). The mean value of serum TNF-α was significantly lower in Group 1 than in Group 2 ($p = 0.0001$). The adiponectin:leptin ratio was not significantly different between the two groups ($p > 0.05$), but the IL-10:TNF-α ratio was significantly higher for Group 1 than Group 2 ($p = 0.05$).

Table 4 Measurements of adiponectin, leptin, interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), insulin, and glucose in blood sera.

	Obese Zucker fatty rats	
	Group 1	Group 2
Adiponectin (μg/mL)	15.28 (3.41)	21.72 (1.47)
Leptin (ng/mL)	66.16 (4.36)	63.22 (2.46)
Adiponectin:leptin ($\times 10^3$)	0.24 (0.07)	0.34 (0.02)
IL-10 (pg/mL)	12.91 (2.55)	20.15 (3.78)
TNF-α (pg/mL)	3.47 (0.89)*	18.89 (0.86)
IL-10:TNF-α	2.66 (0.54)*	1.00 (0.28)
Insulin (ng/mL)	13.63 (0.95)	12.42 (1.62)
Glucose (mmol/L)	12.23 (1.25)	9.18 (0.84)

Data presented as mean (standard error). The adiponectin:leptin ratios were determined from levels expressed in ng/mL.
* Significantly different from Group 2 by unpaired t test ($p < 0.05$).

3.4. Effect of repeated EA on adipokines in white adipose tissue

The mean values for adiponectin, leptin, IL-10, and TNF-α in the white adipose tissue collected at the end of the study on Day 12 are given in Table 5. There were no significant differences in the mean adiponectin, leptin, IL-10, and TNF-α levels between Group 1 and Group 2 ($p > 0.05$). The adiponectin:leptin ratio and IL-10:TNF-α ratio were also not significantly different between the two groups ($p > 0.05$). Four of the five values for adiponectin in Group 1 were $>$ 9.00 μg/mL, whereas only one of the five values in Group 2 was $>$ 9.00 μg/mL (by 2 × 2 Chi-square test, $p \approx 0.05$).

4. Discussion

Obesity has become a major public health issue worldwide. It is a contributing factor in diabetes, cardiovascular disease, hypertension, stroke, cancer, osteoarthritis, asthma, and sleep apnea [25], and one of the components of MetS. MetS is a state of chronic low-grade inflammation as a consequence of complex interactions between genetic and environmental factors. It is characterized by the clustering

Table 3 Blood glucose measurements (mmol/L) over Week 1 and Week 2.

	Week 1		Week 2	
	Baseline BG (at 20 min)	Change in BG over 30 min	Baseline BG (at 20 min)	Change in BG over 30 min
Obese Zucker fatty rats				
Group 1	6.09 (0.41)	0.71 (0.34)*	6.22 (0.29)*	1.27 (0.61)
Group 2	6.18 (0.47)	−0.37 (0.09)	5.45 (0.19)	0.05 (0.15)[†]

Data presented as mean (standard error).
* Significantly different from Group 2 by unpaired t test ($p < 0.05$).
[†] Significantly different from Week 1 by unpaired t test ($p < 0.05$).

Table 5 Measurements of adiponectin, leptin, interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) in white adipose tissue.

	Obese Zucker fatty rats	
	Group 1	Group 2
Adiponectin (μg/mL)	13.35 (2.84)	7.35 (1.51)
Leptin (ng/mL)	48.16 (3.39)	43.06 (2.34)
Adiponectin:leptin ($\times 10^3$)	0.27 (0.05)	0.17 (0.03)
IL-10 (pg/mL)	469.4 (16.39)	486.7 (28.26)
TNF-α (pg/mL)	21.13 (1.63)	17.87 (1.70)
IL-10:TNF-α	22.70 (1.83)	27.97 (2.56)

Data presented as mean (standard error). The adiponectin:leptin ratios were determined from levels expressed in ng/mL. White adipose tissue (0.2 g) was homogenized in 0.6 mL radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor, centrifuged, and the infranatant assayed.

of multiple metabolic abnormalities, including obesity, hypertension, dyslipidemia, insulin resistance, and impaired glucose tolerance as its main components. Many animal models of MetS have contributed to an understanding of the pathophysiological basis and development of novel therapies. Although each animal model has limitations and strengths, used together in a complementary fashion, they are essential for research on the MetS and for understanding the etiology and pathogenesis towards a cure [26]. Insulin resistance and the compensatory hyperinsulinemia are considered to be key elements of MetS. Only a few naturally occurring diabetes-prone and genetically altered rodent strains present with insulin resistance and hyperinsulinemia spontaneously. The frequently used diabetes-prone rodent model, the Zucker fatty rat, has monogenic inheritance and development of insulin resistance and diabetes.

There is increasing evidence that obesity and insulin-resistant states are associated with a low-grade inflammation resulting from chronic activation of the innate immune system. Individuals with MetS produce a relative excess of proinflammatory cytokines such as TNF-α and IL-6, both of which are produced by adipose tissue, thereby leading to an imbalance of proinflammatory and antiinflammatory cytokines. Interventions aimed at causing weight loss in obese women had led to reductions in the levels of proinflammatory proteins including IL-6 [27].

The present study has used the Zucker fatty rat and was designed to test whether repeated EA application would reduce body weight and alter the imbalance of proinflammatory and antiinflammatory cytokines produced by white adipose tissue. The blood serum TNF-α level was significantly decreased on EA treatment, but there was no alteration in the serum levels of adiponectin, leptin, insulin, or glucose. The decrease in serum TNF-α level by EA is consistent with other reported studies [28,29]. TNF-α is principally produced by macrophages (classically activated, M1 activation state), and its decrease would indicate that EA treatment was effective in reducing the overall inflammatory state of the Zucker fatty rats and in reducing insulin resistance, even though no change was found in serum adiponectin and leptin. TNF-α induces insulin resistance by inhibition of the insulin receptor substrate 1 signaling pathway [30]. Previous studies showed that obesity in rodents resulted in an increase in macrophages in adipose tissue [20,31] due to an increased infiltration and/or proliferation of these cells [32], and infiltration of macrophages into adipose tissue of diet-induced obese rats was inhibited by EA [29]. Inhibition of macrophage infiltration would explain the decreased serum TNF-α level on EA treatment. The lack of a decrease in serum IL-10 by a reduced number of macrophages in the adipose tissue would suggest that EA has altered the activation state (to alternatively activated, M2 activation state). A question remains as to why the level of TNF-α in the adipose tissue was not decreased in EA-treated animals. A suggestion is that there is an increase in the number of other cell type(s) present in the adipose tissue that contain TNF-α, e.g., vascular endothelial cells, and which would be increased if EA led to an increase in vascularity.

An alternative explanation is that EA treatment does not inhibit infiltration of macrophages into the adipose tissue but rather suppresses the release of TNF-α from the macrophages present and does not alter the activation state of the macrophages. This would explain the decrease in serum TNF-α and the lack of change in serum IL-10 level. To determine which of these is the more likely explanation would require immunocytochemical staining of specific cell markers for M1 and M2 macrophages and also for vascular endothelial cells. Macrophages could also be isolated from adipose tissue of EA-treated and control animals and then characterized and quantitated by flow cytometry analysis [33].

EA treatment did not increase baseline BG or the change in BG over 30 minutes for Week 2 compared with Week 1, whereas for the controls, the change in BG over 30 minutes was significantly increased in Week 2 compared with Week 1. Thus EA treatment of Zucker fatty rats does not stimulate the development of hyperglycemia.

The body weights of the Zucker fatty rats decreased during the study period and EA had no apparent effect. Inhaled anesthetic gases, particularly nitrous oxide, have been reported to suppress appetite [34]. It seems likely that exposure to the anesthetic gas for long periods (50 minutes) is responsible for the decrease in body weight of EA-treated and control animals during the 2-week study period. In future studies to test the effect of repeated EA on body weight of Zucker fatty rats, it may be necessary to use conscious animals that have been trained to be restrained inside a plastic cylinder [35].

In a previous study, EA treatment of obese Zucker diabetic fatty rats 22—24 weeks of age did not significantly alter levels of leptin and adiponectin in blood serum and adipose tissue, although there was an indication that adiponectin levels were increased [36]. This is similar to the findings of the present study for obese Zucker fatty rats 15 weeks of age. It may be that with advanced metabolic derangement, EA given as six treatments over 2 weeks does not affect the adipose tissue to the same extent as when the metabolic disturbance is at an earlier, lesser stage.

In conclusion, this study has shown that EA in Zucker fatty rats at Zhongwan and Guanyuan acupoints decreased serum TNF-α, with no significant alteration in serum leptin, adiponectin, or IL-10. EA had no significant effect on the

measured adipokine levels in the adipose tissue, although there was an indication that adiponectin was increased. Further studies are warranted with a larger number of animals to obtain additional data on adipokine levels in serum and adipose tissue, and to characterize and quantitate macrophages and vascular endothelial cells in the adipose tissue.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material of this manuscript.

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