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Effects of Pioglitazone On the Lipid Profile, Serum Antioxidant Capacity, and *UCP1* Gene Expression in Mouse Brown Adipose Tissue

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Abstract

Background: Pioglitazone increases insulin sensitivity and improves glycemic control in type 2 diabetics. In this study, we evaluated the effects of pioglitazone on the uncoupling protein 1 (UCP1) expression in mouse brown adipose tissue (BAT), and on recovery from oxidative stress due to a high-fat diet.

Methods: 30 mice were divided into three groups: group 1 received a normal diet, group 2 received a high-fat diet, and group 3 received a high-fat diet plus 30 mg/kg pioglitazone. After treatment, the cholesterol, triglyceride, paraoxonase 1 (PON1), total serum antioxidant capacity (TAC), malondialdehyde (MDA), and specific activity of hepatic catalase were measured. BAT UCP1 expression was evaluated at both the mRNA and protein levels.

Results: The weights differed between the groups ($p<0.05$). Serum MDA was greater and TAC, liver catalase, and PON1 were less than in group 2 than in group 1 ($p<0.05$). In Serum MDA was less and catalase activity was greater in group 3 than in group 2 ($p<0.05$). UCP1 gene expression was less in group 2 than in group 1 ($p<0.05$) but greater than in group 3 ($p<0.05$).

Conclusions: Pioglitazone may have a protective role in high-fat-diet-induced oxidative stress by increasing the antioxidant capacity. Moreover, it can induce weight loss by increasing *UCP1* mRNA and protein expression.

Keywords: High-fat diet, MDA, pioglitazone, PON1, TAC, UCP1.

Introduction

Obesity is a chronic and multifactorial disease affected by various psychological, behavioral, cellular, molecular, and metabolic factors (1). Obesity is characterized by the accumulation of fat in body tissues (2), and on a global scale, is considered our biggest health problem. Studies show that the prevalence of obesity is increasing in developing countries, especially in women (3). Obesity is one of the most important risk factors for developing cardiovascular and chronic kidney diseases, as well as diabetes and cancer (4, 5). Cardiac fibrosis, causing metabolic disorders such as heart failure and arrhythmia, is also linked to obesity (6, 7). In obesity, white adipose cells bulge due to the accumulation of triglycerides. In this case, fatty acids are released into the bloodstream and insulin sensitivity decreases, leading to diabetes. Peroxisome proliferator-activated receptors (PPARs) act by stimulating adipogenesis, regulating fat metabolism in adipocytes, and increasing the differentiation of brown adipose tissue (BAT) cells. Pioglitazone, a derivative of thiazolidinedione, affects lipid metabolism via PPARα activation. Activation of PPARs types 1

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and 2 increases insulin sensitivity and controls blood sugar in type 2 diabetics. Because of these interactions, free fatty acids reduce types 1 and 4 glucose transporters and stimulate glucose consumption in peripheral tissues, leading to induction of insulin signaling pathways and a decrease in insulin resistance (8-9). Humans have both brown and white adipose tissues; the BAT contains polyhedral fat cells with abundant mitochondria; unoupling protein 1(UCP1) is highly expressed in the mitochondria and plays an important role in the thermogenic activity of fatty tissue (8). UCP1 decreases the proton gradient by transferring protons across the membrane into the mitochondrial matrix; in this state, the energy from aerobic oxidation produces heat instead of ATP. This process controls energy homeostasis and keeps living creatures alive at cold temperatures. The BAT, as the body's natural anti-obesity organ, burns fat, produces heat, and causes weight loss via UCP1 gene expression, without affecting the appetite (9, 10). For this reason, BAT is widely used in current diabetes and obesity-related research (9). Pioglitazone is routinely used as an anti-diabetic drug. Therefore, the aim of this study was to evaluate the effects of pioglitazone on the lipid profile, serum antioxidant capacity, and UCP1 gene expression in mouse BAT.

Materials and methods

In this study, 30 six-week-old mice (C-57) with an average weight of 20–25 grams were used and kept in constant light (12 hours) and temperature (22±2 ºC) conditions. The study protocols were approved by the Shahrekord University of Medical Science Ethics Committee (IR.SKUMS.REC.1395.193).

Mice were randomly separated into three groups of 10 mice each. Mice in group 1 received a regular diet, mice in group 2 received a high-fat diet, and mice in group 3 received a high-fat diet plus 30 mg/kg body weight of pioglitazone by oral gavage. All the mice also received one milliliter of distilled water per day by oral gavage.

The study duration was 30 days. The pioglitazone was dissolved in one milliliter of distilled water, and all mice in group 3 received the pioglitazone by oral gavage in addition to high-fat diet daily. The high-fat diet consisted of 57 percent regular mouse food with 43 percent high-fat food, including 15 percent saturated animal fat, 5 percent vegetable oil, 20 percent sucrose, 2.5 percent cholesterol, and 0.5 percent cholic acid.

After 30 days, the mice were anesthetized with chloroform. Blood was collected by heart puncture and liver tissue was collected to measure hepatic catalase activity. The BAT was excised from between the shoulder blades at the back of the neck to evaluate UCP1 mRNA and protein expression. Tissue samples were washed with physiologic serum and stored at -70 °C until use.

Paraoxonase 1, malondialdehyde (MDA), and the lipid profile were measured. The serum lipid profile was measured using commercial kits (Pars Azmun Company) and an AutoAnalyzer (BT3000, Italia). The serum VLDL-C and LDL-C levels were calculated using the Friedewald formula. The serum antioxidant capacity was measured by evaluating its ability to reduce iron (III) to iron (II) using the FRAP method (11). The MDA concentration was determined by HPLC according to the Agarwal method (12). Paraoxonase 1 activity was measured on a spectrophotometer using phenylacetate as the substrate (13). Catalase activity was measured via the Abei method (14). To measure UCP1 gene expression, total RNA was extracted from the BAT using a commercial kit (Kit BIOFLUX Trizol Reagent). The RNA quality and concentration were determined on a Nanodrop (Thermo Scientific Nanodrop 2000 spectrophotometer, USA). cDNA was synthesized using the First Strand cDNA synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's guidelines. The primers were designed using Oligo 7 software. UCP1 gene expression was determined relative to β-actin expression. The β-Actin primers were: (Forward: 5'-CGGTCAGGTCATCACTATCGG-3', Reverse: 5'-TCTTTACGGATGTCAACTCACAC-3'); and the *UCP1* primers were: (Forward: 5'-TCAGGATTGGCCTCTACGACT-3', Reverse: 5'-GCATTCTGACCTTCACGACCT-3'). The *UCP1* and β-actin genes were amplified on a Rotor-gene 3000 (Corbett- Australia).The reaction mix was first incubated for 10 min at 95 °C, then the RT-PCR program consisted of 40

cycles of 15 sec at 95 °C, 20 sec at 62 °C, and 20 sec at 72 °C. UCP1 protein was isolated from the BAT using RIPA buffer and its concentration was determined using the Bradford method; afterward, 40 µg of each sample were electrophoresed by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with bovine serum albumin, and subsequently incubated with primary specific antibodies (anti-beta-actin [ab8227] or anti-UCP1[ab23841]). The membrane was washed and then incubated with the secondary antibody (anti-rabbit IgG). After further washes, one milliliter of enhanced chemiluminescence (ECL) solution was added and the membrane was scanned. Data were analyzed using SPSS version 20 software. The means of the groups were compared with one-way ANOVA and the groups were compared using the Mann-Whitney test. In this study, $p < 0.05$ was considered statistically significant.

Results

At the end of the study, the group 2 mice were significantly heavier than those in groups 1 and 3. Group 1 was also significantly heavier than group 3 ($p < 0.05$, Fig. 1). The weight differences began to occur between weeks 1 and 2.

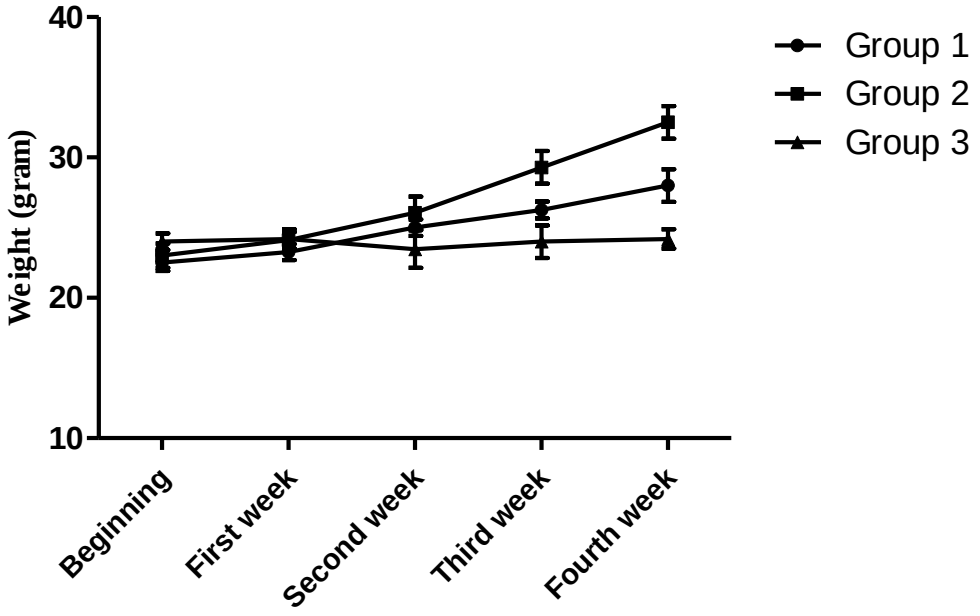

Fig. 1. Weekly weight chart. Ten mice were in each group. Group 1 received a normal diet, group 2 received a high-fat diet, and group 3 received a high fat diet plus 30 mg/kg pioglitazone. Mice were weighed weekly.

After 30 days the serum levels of TG, total cholesterol (TC), and LDL-C were greater (p=0.004) and HDL-C was less (p=0.03) in group 2 than in group 1. Total cholesterol and LDL-C were significantly less in group 3 than in group 2 (p=0.001). Serum VLDL was significantly greater in group 2 than in group 1 (p<0.05). Serum MDA was significantly greater in group 2 than in group 1 (p=0.001) and significantly less in group 3 than in group 2 (p=0.02); also, the serum TAC was significantly less in group 2 than in group 1 (p=0.003). Serum paraoxonase 1 and liver catalase activities were significantly less in group 2 than in group 1 (p=0.01). Hepatic catalase activity was significantly less in group 3 than in group 2 (p=0.003). Serum glucose in group 2 was significantly greater in group 2 than in groups 1 (p=0.02) and 3 (p=0.001) (Table 1).

Our data show that the level of the relative expression of *UCP-1* mRNA, compared to β-Actin was significantly decreased in group 2, compared to the group 1 (p=0.01); while it was significantly increased in the third group, compared to the second group (p=0.001) (Fig. 2A).

Also, UCP1 protein was significantly more abundant in groups 1 and 3 than in group 2, and most abundant in group 3 (Fig. 2B).

Table 1. Data of the variables examined after the 30-day diet

Variables	Group 1	Group 2	Group 3
Triglyceride(mg/dl)	146.8 ± 6.3	185.4 ± 5.1 [a]	183.7 ± 2.8
Cholesterol (mg/dl)	107.7 ± 2.3	330.4 ± 22.7 [a]	212.8 ± 13.2 [b]
HDL-C (mg/dl)	71.7 ± 1.1	61.6 ± 1.6 [a]	62.25 ± 1.1
LDL-C (mg/dl)	6.7 ± 1.8	208.7 ± 21.4 [a]	104.8 ± 14.4 [b]
VLDL-C (mg/dl)	29.4 ± 1.3	37.0 ± 1.0 [a]	36.75 ± 0.7
MDA (µM)	25.1 ± 1.4	70.7 ± 2.4 [a]	38.6 ± 1.2 [b]
TAC (µM)	556.0 ± 27.1	360.9 ± 29.2 [a]	381.2 ± 15.9
Liver Catalase (unit/mg protein)	35.72 ± 2.7	21.1 ± 1.2 [a]	37.15 ± 1.21 [b]
Paraoxonase1 (Unit/ml)	46.3 ± 2.7	23.13 ± 0.8 [a]	28.9 ± 1.6
Glucose (mg/dl)	107.7 ± 2.5	153 ± 6.8 [a]	62.2 ± 3.7 [b]

Values are reported as means ± SEs. each group contained 10 mice. Mice in group 1 received normal diets, those in group 2 received high-fat diets, and those in group 3 received high-fat diets plus 30mg/kg pioglitazone. UCP1: Un-coupling protein 1, MDA: malondialdehyde, TAC: total antioxidant capacity, HDL-C: high-density lipoprotein, LDL-C: low-density lipoprotein, $P<0.05$ [a] is considered significantly different from group 1. $P<0.05$ [b] is considered significantly different from group 2.

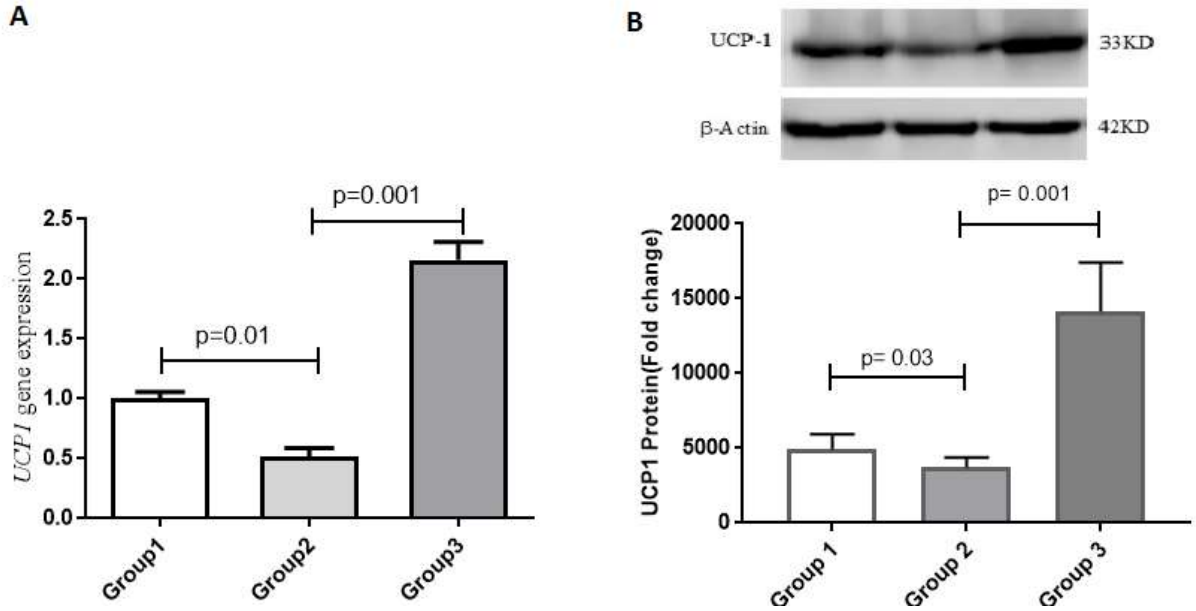

Fig. 2. A) Relative expression of *UCP-1* mRNA. Each group contained 10 mice. Mice in group 1 received normal diets, those in group 2 received high-fat diets, and those in group 3 received high-fat diets plus 30 mg/kg pioglitazone. Mice were fed for 30 days. B) Protein level of UCP1/ β-actin. Proteins were extracted from brown adipose tissue, electrophoresed by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-UCP or anti-β-actin antibody (upper panel). The differences in blot intensities are reported as fold-change (lower panel).

Discussion

Obesity causes an increase in free radical production, leading to an increase in oxidative stress. In obesity, a reduction in total antioxidant capacity and the activity of enzymes such as catalase, contribute to other obesity-related problems (1,4,5). Pioglitazone activates PPARγ types 1 and 2 and improves insulin sensitivity and glycemic control in type 2 diabetics (8-9). In a study by Bahramikia and colleagues.

LDL-C, TC, and TG were greater, and HDL-C less, in rats fed high-fat diets than in controls (15). In our study, LDL-C, TC, and TG were significantly greater and HDL-C was significantly less in group 2 than in group 1. Asdaq *et al.*

evaluated the effects of saffron on rats fed high-fat diets and reported that the specific activity of catalase and the serum total antioxidant capacity was less in those rats than in controls ($p<0.05$) (16). Yekta *et al.* also found that pioglitazone activates and increases liver catalase activity (17). In our study, catalase activity was greater in group 3 than in group 2 ($p<0.05$). Noeman *et al.* reported that MDA was greater and PON1 less in rats fed high-fat diets than in controls (18). In our study serum PON1 was also less in group 2 than in group 1. These changes suggest that in the high-fat group, lipids and fatty acids have increased and the liver tends to produce more triglyceride, resulting in a reduction in HDL synthesis. Further, HDL is necessary for PON1 to migrate from the liver to the bloodstream, whereas a decrease in HDL secretion is concomitant with a decrease in serum PON1. Serum MDA was greater in group 2 than in group 1 likely due to the increase in fatty acids and decrease in serum antioxidant capacity. Singh *et al.* reported that 30 mg/kg pioglitazone reduced serum MDA levels (19). In our study, serum MDA in group 3 was less than in group 1. Hussian *et al*. reported that serum glucose, LDL, and cholesterol were less in non-diabetic hyperlipidemia rats than in controls (20). We found that serum glucose, LDL, and serum cholesterol levels were significantly less in group 3 than in group 2. The UCP1 protein expression differences seen in our study may be due to the high fat consumption in these groups.

Weight gain and decrease in TAC, catalase activity, and serum antioxidant capacity in group 2 may be due to the high calorie regimen and the free radicals produced by the high-fat diet.

The BAT has an important role in weight and metabolism regulation and converts the stored energy of TG to heat, via UCP1. Lee *et al.* reported that UCP1 gene expression increased in rats fed high-fat diets (21). Shen *et al.* reported that in mice, cinnamon intake decreased blood glucose and displayed anti-diabetic effects by increasing UCP1 gene expression in BAT and by increasing GLUT-4 in muscle and BATs (22). Rossato *et al.* reported that UCP1 gene expression in white adipose tissue was induced by the activation of cold receptors, subsequently increasing heat production (23). Lisa *et al.* reported that pioglitazone increased UCP1 mRNA and protein expression in the BAT of obese diabetic rats (24). Camirand *et al.* reported that thiazolidinediones increase UCP-2 gene expression and energy expenditure via PPARγ production (25). We found that UCP1 gene and protein expression was significantly less in group 2 than in group 1 ($p<0.05$), which is in contrast with the study of Lee *et al*.; however, UCP1 protein production was significantly greater in group 3 than in group 2 ($p<0.05$), consistent with previous studies. The lack of weight gain seen in group 3 is likely due to the increased UCP1 gene expression in this group. This study suggests that pioglitazone reduces the high-fat-diet-induced oxidative stress by increasing lipid and glucose catabolism in the body.

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