Title Page

The melatonin receptor agonist piromelatine ameliorates impaired glucose metabolism in chronically stressed rats fed a high-fat diet

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Running Title Page

Running head: Piromelatine ameliorates glucose impairment in IR rats

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Abbreviations: 11βHSD1, 11β-hydroxysteroid dehydrogenase-1; CORT, corticosterone; CF, chronically stressed rats fed a HFD; CS, chronic stress; FFA, free fatty acid; GCs, glucocorticoids; GLUT-4, glucose transporter type-4; GR, glucocorticoid receptor; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat
diet; HOMA, homeostasis model assessment; HPA, hypothalamic-pituitary-adrenal; IL-6, interleukin-6; IPGTT, intraperitoneal glucose tolerance test; IR, insulin resistance; ITT, insulin tolerance test; LDL-C, low-density lipoprotein cholesterol; MLT, melatonin; MT1/MT2, melatonin receptor-1/melatonin receptor-2; PPAR-γ, peroxisome proliferator-activated receptor-γ; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor-α.

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ABSTRACT

Modern lifestyle factors (high-caloric food rich in fat) and daily chronic stress are important risk factors for metabolic disturbances. Increased hypothalamic-pituitary-adrenal (HPA) axis activity and the subsequent excess production of glucocorticoids (GCs) in response to chronic stress (CS) leads to increases in metabolic complications, such as type 2 diabetes and insulin resistance (IR). Melatonin (MLT), which protects several regulatory components of the HPA axis from GC-induced deterioration, might improve glucose homeostasis. Piromelatine is a melatonin receptor-1/melatonin receptor-2 (MT1/MT2) agonist with high affinity for MLT receptors and a longer duration of action than MLT. The objective of the present study was to explore the potential effects of piromelatine on glucose and lipid metabolism and insulin sensitivity in rats with IR induced by a high-fat diet (HFD) combined with CS (CF). The results showed that piromelatine prevented the suppression of body weight gain and energy intake induced by CF and normalized CF-induced hyperglycemia and homeostasis model assessment (HOMA)-IR index, which suggests that piromelatine prevented whole-body IR. Piromelatine also prevented CF-induced dysregulation of genes involved in glucose and lipid metabolism, including pro-inflammatory cytokines, in adipose tissue. In addition, piromelatine also attenuated CF-induced excess free corticosterone (CORT) release, increased glucocorticoid receptor (GR) expression and decreased 11βHSD1 expression, suggesting that piromelatine might ameliorate impaired glucose metabolism and prevent IR by normalizing HPA axis functions. In
conclusion, piromelatine might be a novel therapeutic agent for glucose intolerance and IR.

Introduction

The prevalence of type 2 diabetes mellitus (T2DM) has been increasing rapidly over the last two decades, and modern lifestyle factors, including a hypercaloric diet and social and work pressure (psychological stress), are thought to be responsible for this increase (Stumvoll et al., 2005; Tirabassi et al., 2016; Hansen et al., 2010). It is clear that chronic stress and endocrine stress responses are significantly associated with glucose intolerance, insulin resistance (IR) and diabetes mellitus (Siddiqui, et al., 2015). It has been reported that stressed people often reach for “comfort” food, which includes palatable food rich in carbohydrates and fat (Fachin et al., 2008). A high-fat diet (HFD) might lead to obesity and exacerbate metabolic disorders.

The body responds to stress-induced homeostatic challenges by activating the hypothalamic-pituitary-adrenal (HPA) axis (Tirabassi et al., 2016). Chronic stress (CS) might impair the feedback mechanisms of the HPA axis that return these hormonal systems to normal, resulting in chronically elevated levels of glucocorticoids (GCs), catecholamines, and inflammatory markers (Joshua and Sherita, 2016). As the final output of the HPA axis, the release of GCs from the adrenal glands serves to oppose insulin-mediated energy storage, in part by suppressing insulin secretion (Dinneen et al., 1993). By inducing a transient state of IR, excessive amounts of GCs restrain hepatic glucose production and regulate glucose delivery to peripheral tissues, thereby
resulting in hyperglycemia (Rizza et al., 1982; Bjorntorp and Rosmond, 1999, 2000, 2001; Patterson and Abizaid, 2013). Therefore, counteracting HPA axis hyperactivity is a challenge in stress-related metabolic disorders.

Melatonin (5-methoxy-N-acetyltryptamine) (MLT) is a circulating hormone primarily produced and secreted by the pineal gland that acts as a major synchronizer of endocrine rhythms (Detanico et al., 2009) and is a homeostatic regulator or pharmacological buffer of the effects of stress (Kopp et al., 1999). Several studies have found that MLT administration is associated with diminished overall corticosterone (CORT) secretion and increased sensitivity to GC feedback (Houdek et al., 2015), which suggests that MLT might serve as a physiological regulator of the HPA axis (Konakchieva et al., 1997). Studies have suggested that chronic MLT treatment might protect several regulatory components of the HPA axis from GC-induced deterioration and might improve glucose homeostasis not only in pinealectomized rats that display glucose intolerance but also in rats with IR (Konakchieva et al., 1998; Kitagawa et al., 2012; Sartori et al., 2009; Shieh et al., 2009).

As a melatonin receptor-1/melatonin receptor-2 (MT1/MT2) agonist, piromelatine [N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-4-oxo-4H-pyran-2-carboxamide] displays high affinity for MLT receptors and has a longer duration of action than MLT (Carocci et al., 2014; Yalkinoglu, et al., 2010), as shown in Table 1. The chemical structures of MLT and piromelatine are presented in Figure 1. Studies have shown that piromelatine functions similarly to MLT, and this molecule promotes sleep
(Laudon et al., 2008), alleviates lipid peroxidation, protects mitochondria from myocardial ischemia/reperfusion injury (Yu et al., 2014), exerts neuroprotective effects against brain ischemia (Buendia et al. 2015), exhibits antidepressant and anxiolytic activities (Tian et al., 2010), inhibits body weight gain, and increases sensitivity to insulin in obese rodent models (She et al., 2009). In our research, we explored the potential effects of piromelatine on glucose metabolism and insulin sensitivity in chronically stressed rats fed a HFD (CF).

**Materials and methods**

**Experimental Animals**

Adult male Wistar rats (200 ± 20 g) were provided by the Animal Facility Center of Lanzhou University (Approval No. 14-006), China. The animals were allowed to acclimatize for at least 6 days before the experiments and were housed under controlled illumination (12-h light/12-h dark cycle; lights on from 7:00 a.m. to 7:00 p.m.) and ambient temperature (20–24°C) and humidity (40%–60%) with food and water ad libitum. Animal welfare and experimental procedures complied with the guidelines of the Gansu Experimental Animal Center (Gansu, China) and were officially approved by the Ministry of Health, P. R. China, in accordance with NIH guidelines (NIH, 2002). The laboratory procedures were carried out under the permission and surveillance of the PLA Lanzhou General Hospital Ethics Committee.

**Reagents**

MLT and piromelatine were synthesized and provided by Neurim Pharmaceuticals
Ltd. (Tel-Aviv, Israel). The compounds were dissolved in ethanol and diluted in sterile saline to obtain a final ethanol concentration of 0.02%. All drugs were administered by oral gavage in a volume of no more than 10 mL/kg.

**Experimental designs**

**Experiment I-Effect of a HFD and stress on rat metabolism**

Adult male Wistar rats (200 ± 20 g) were randomly divided into four experimental groups: a Control group, which was fed commercial standard chow; a HFD group, which was fed high-fat chow instead of commercial standard chow; a CS group, which was fed the same diet as the Control group and exposed to chronic, unpredictable stress; and a CF group, which was fed the same diet as the HFD group and exposed to chronic stress.

The standard rat chow diet (13 kJ/g; 5% saccharides; 12.5% fat) contained 53.1% carbohydrates and 4% lipids. The HFD (24 kJ/g; 25% saccharides; 62% fat) consisted of 40% standard diet, 20% edible lard, 5.5% carbohydrates, 35% egg (w/w) and 0.9% NaCl. The chows were prepared by the Animal Experimental Center of Lanzhou General Hospital, PLA, China.

The stress methods were improved based on the report by Zardooz *et al.* (2006) and included the following stressors: (1) absorbent gauze wrap secured with tape, (2) restraint in a polyvinyl chloride tube (L = 25 cm, ID = 5 cm) closed at both ends, (3) immobilization on a board with tape, and (4) tail suspension. The stress protocol involved exposure to one of the first three restraint stressors for a 2-h period from 9:00 to 11:00 am or to the fourth stressor for 30 min from 9:00 to 9:30 am once daily.
The animals were exposed in turn to one of the above stressors once a day and then returned to the animal facility 15 min after the stress exposure to minimize disturbance to the control group.

Body weight and fasting blood glucose (6 h) were measured weekly. The insulin tolerance test (ITT, 0.5 IU/kg) was performed at day 21, and the intraperitoneal glucose tolerance test (IPGTT, 2.5 g/kg) was performed at day 28. At the end of the 33 days of treatment, animals were fasted for 12 h and then killed via decapitation. Their blood was sampled to perform lipid, free fatty acid (FFA), CORT, insulin, glucagon and leptin measurements, and their liver and muscle tissues were sampled for glycogen assays.

Experiment II-Induction of insulin resistance and experimental design

After an acclimation period, the rats were randomly divided into 5 groups: a normal control group (Control group, sterile saline 2 ml/kg), a chronically stressed and HFD-fed group (CF group, sterile saline 2 ml/kg), an MLT-treated CF group (MLT group, 20 mg/kg), a low-dose piromelatine-treated CF group (PMT-L group, 10 mg/kg), and a high-dose piromelatine-treated CF group (PMT-H group, 20 mg/kg). The treated rats were administered drugs by gavage from 8:00 to 9:00 am, whereas the Control group and CF group were administered an equal volume of sterile saline. After drug administration, the Control group was fed a standard diet and was not exposed to any stressors, whereas the other experimental groups were fed a HFD and simultaneously stimulated with chronic stress for 33 days.

Food intake and water consumption were recorded daily. Body weight and fasting
Blood glucose (6 h) were measured weekly. The IPGTT (2.5 g/kg) was performed at day 28. At the end of the treatment, the animals were fasted for 12 h and then killed via decapitation. Whole blood and tissue samples were collected for subsequent analyses.

Measurement of body weight, food intake and water consumption

Body weights were monitored weekly in the morning. The rats were fed daily on a precise feeding schedule (at 8:00 a.m.) with equal amounts of food and water. Each rat was provided with approximately 30 g of chow and 180 g of water. Food intake and water consumption were measured by subtracting the remaining food and water before feeding. The relative food intake was calculated as grams of daily food intake per 100 grams body weight, and the relative water consumption was calculated as grams of daily water intake per 100 grams body weight.

Measurement of fasting blood glucose levels

The fasting blood glucose level (after 6 h of fasting) was measured weekly on d0, d7, d14, d21 and d28. Fasting blood glucose levels were estimated using commercially available glucose kits based on the glucose oxidase method according to the manufacturer’s instructions. The results are expressed as mmol/L plasma.

IPGTT

All animals were fasted for 6 h and then injected intraperitoneally with a single dose of 50% glucose at a concentration of 2.5 g/kg body weight. Blood samples were collected from each group just before glucose administration (0 min) and at 30, 60 and 120 min after glucose administration. Plasma glucose levels were determined by
the glucose oxidase method. The results are expressed as mmol/L.

**ITT**

All animals were fasted for 6 h and then injected intraperitoneally with a single dose of insulin at a concentration of 0.5 IU/kg body weight. Blood samples were collected from each group immediately before insulin administration (0 min) and at 30, 60 and 120 min after insulin administration. Plasma glucose levels were determined via the glucose oxidase method and are expressed as mmol/L plasma.

**Plasma lipid determination**

Plasma was collected after rat sacrifice as described above. The cholesterol oxidase-peroxidase-4-aminoantipyrine-phenol (CHOD-PAP) method was used to determine the plasma total cholesterol (TC) levels, and the glycerol phosphate oxidase-peroxidase-4-aminoantipyrine-phenol (GPO-PAP) method was used to determine the plasma triglyceride (TG) levels according to the instructions provided with the kit. The plasma high-density lipoprotein cholesterol (HDL-C) levels were determined by the phosphotungstic acid-magnesium$^{2+}$ (PTA-Mg$^{2+}$) precipitation method, whereas the plasma low-density lipoprotein cholesterol (LDL-C) levels were determined by the polyethylene-sulfuric acid-precipitation (PSAP) method according to instructions provided with the kit. Free fatty acids (FFAs) were measured via the biscyclohexanoneoxaiydihydraone coloration method using commercial assay kits according to the manufacturer’s directions. All values are expressed as mmol/L.

**Measurement of plasma glucagon, insulin and leptin levels and the HOMA-IR index**
The glucagon, insulin and leptin levels in frozen plasma samples were measured using Rat Enzyme Immunoassay Kits. The IR index estimated by the homeostasis model assessment (HOMA) was calculated using relationships between the blood glucose and insulin levels according to the following formula (Matthews et al., 1985):

\[ \text{HOMA-IR} = \left( \frac{\text{fasting serum insulin (mIU/L)} \times \text{fasting blood glucose (mmol/L)}}{22.5} \right) \]

**Measurement of glycogen levels**

The glycogen contents of liver and skeletal muscles were measured using the anthrone-sulfuric acid colorimetric assay method as previously established (Sadasivam and Manickam, 1996), according to the manufacturer’s instructions. The amounts of glycogen in the tissue samples are expressed as mg glucose/g tissue (wet weight).

**Measurement of plasma CORT and 24-h urinary CORT**

Using commercially available radio-immunoassay kits for rat CORT, the plasma and 24-h urinary levels of CORT were estimated according to instructions provided with the kit. The results are expressed as nmol/L.

**Total RNA isolation and quantitative real-time PCR**

For mRNA analysis, total RNA was extracted from tissues using a MiniBEST Universal RNA Extraction Kit (Takara Bio Inc., Shiga, Japan), and cDNA was synthesized using a PrimeScript RT Reagent Kit (cDNA Synthesis Premix; Takara Bio Inc.). The cDNA was amplified by real-time PCR with SYBR Premix Ex TaqII (qPCR Premix; Takara Bio Inc.). The thermocycling conditions were as follows: initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 3 s,
annealing at 72°C for 30 s and extension at 60°C for 30 s. The β-actin gene served as the internal control. The cycle threshold (Ct) value obtained for each target gene was normalized to that of the β-actin gene using the formula $2^{-\Delta\Delta Ct}$. The relative quantity of each gene was expressed as the fold change with respect to the control after normalization to β-actin. The gene-specific primers used in this study to amplify the glucose and lipid metabolism-related genes glucocorticoid receptor (GR), 11β-hydroxysteroid dehydrogenase (11β-HSD) 1, adiponectin, leptin, tumor necrosis factor (TNF)-α, interleukin (IL)-6, peroxisome proliferator-activated receptor (PPAR)-γ, and glucose transporter type (GLUT)-4 are shown in Table 1.

**Western blot analysis**

The relative protein expression of GR and 11β-HSD1 in adipose tissue were determined according to methods described previously (Zhou et al., 2017). Tissues were washed with ice-cold phosphate-buffered saline. After homogenization and centrifugation, the proteins in the supernatant were quantified using a Pierce BCA protein assay kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Total proteins (20 mg) were separated by 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk for 1 h and then incubated with anti-GR or anti-11β-HSD1 polyclonal antibodies (diluted 1:1000; Abcam, USA) at 4°C overnight. The membranes were washed three times with Tris-buffered saline containing Tween 20 and then incubated for 60 min with the corresponding horseradish
peroxidase-conjugated goat anti-mouse antibody (diluted 1:5000; ZSGB-BIO, China). The proteins were then visualized with electro-chemiluminescence Prime Western blotting detection reagents (Solarbio, China) according to the manufacturer’s instructions. The protein bands were evaluated using a Tanon-4200SF Biomolecular Imager (Tanon Science & Technology Co., Shanghai, China) and analyzed with Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). All signals were normalized to that of the housekeeping protein β-actin.

**Statistical analyses**

Statistical analyses were conducted with GraphPad Prism 5.0 Software, and the results are expressed as the means ± S.D. Statistical significance was determined using one-way analysis of variance with Dunnett’s post hoc analyses or two-way analysis of variance with Bonferroni post hoc analyses according to the number of comparisons and variables. IPGTT and ITT data converted to area under the curve (AUC) values using one-way analysis of variance with Dunnett’s post hoc analyses. When an effect was statistically significant ($P < 0.05, P < 0.01$), mean comparisons were performed.

**RESULTS**

**Effects of CS and HFD on metabolic parameters in rats**

To assess the role of CS on the metabolic profile of rats exposed to a HFD, metabolic parameters were studied in the groups subjected to CS/HFD.

The body weights of the Control rats increased progressively throughout the 5-week experiment. The HFD showed a larger elevation in body weight compared
with itself in 0 week, but did not different with control rats significantly.

Compared with control group, CS challenge produced significant decreases in body weight gain from the 3rd week to the end of the experiment ($P < 0.05$), addition of the HFD to the CS challenge decreased the body weight gain from the 2nd week to the end of the experiment ($P < 0.05$) (Figure 2A).

Both the CS and CF groups exhibited higher glucose levels compared with their respective control ($P < 0.05$). From the 2nd week the end, CF rats increased the fasting glucose concentration compared with that in 0 week. There was no statistically significant difference between the control and HFD groups (Figure 2B).

Glucose tolerance was assessed via the IPGTT on day 28. After the 4-week experiment, compared with the normal group, the HFD or CS challenge alone significantly increased the glucose levels by 41.54% ($P < 0.05$) and 39.23% ($P < 0.05$), respectively, at 30 min, while increased the area under the curve (AUC) significantly ($P < 0.05$). Addition of the HFD to the CS challenge increased the blood glucose concentration by 71.54% ($P < 0.01$) and 48.35% ($P < 0.05$) at 30 and 60 min after glucose overload, respectively, and increased the AUC significantly ($P < 0.01$) (Figure 2C-D).

Insulin sensitivity was assessed via the ITT on day 21. Compared with the normal control group, the CS, HFD and CF groups displayed hyperglycemia, showing increases in glucose concentrations of 17.24% ($P < 0.05$), 17.24% ($P < 0.05$) and 31.03% ($P < 0.05$), respectively, at 30 min and of 35% ($P < 0.05$), 30% ($P < 0.05$), 40% ($P < 0.05$), respectively, at 60 min after insulin (0.5 IU/kg) injection. Only CF
increased AUC significantly ($P < 0.05$) (Figure 2E-F).

There was no statistically significant difference between the control and HFD groups in the liver and muscle glycogen contents. However, the CS and CF groups exhibited decreased glycogen storage in the liver and muscle tissues ($P < 0.05$) (Figures 2G-H).

The statistical analysis showed that significant effects of the CS and HFD treatment were observed on lipid metabolism. Both HFD-fed groups exhibited increased TG levels compared with the groups maintained on the standard diet ($P < 0.05$). Compared with the control animals, elevated TC concentrations were found in the blood of the CS group and both HFD-fed groups ($P < 0.05$) (Figure 2G). In addition, both stress groups showed increased FFA levels compared with the controls, but the FFA levels did not differ between the control and HFD group ($P < 0.05$) (Figure 2I).

The blood hormone data are shown in Figures 2J-N. There was a trend towards an increase in the CORT, insulin, glucagon and leptin concentrations in the groups that were subjected to CS/HFD; however, no statistically significant differences were observed.

Effect of piromelatine on body weight, food intake, water consumption and fasting blood glucose in chronically stressed rats fed a HFD

To examine the effects of piromelatine on chronically stressed rats fed a HFD, we monitored the body weights of rats weekly in the Control, CF, MLT (CF+20
mg/kg MLT), PMT-L (CF+10 mg/kg piromelatine), and PMT-H (CF+20 mg/kg piromelatine) groups over a 4-week treatment period. Compared with the Control group, the weekly weight gains of the CF rats from the 2nd week to the end of the experiment were reduced by 5.48%, 8.65% ($P < 0.05$), and 13.51% ($P < 0.01$), and these reductions in weight gain were attenuated by piromelatine (Figure 3A).

The daily food intake and water consumption of the experimental rats were monitored to further examine the effects of piromelatine. CF animals exhibited significantly lower food intake levels ($P < 0.01$) and tended to have increased water consumption compared with control animals from the 2nd week to the end of the experiment. Piromelatine treatment significantly increased the food intake of CF rats ($P < 0.05$) on the 2nd week and resulted in higher energy consumption throughout the experiment (Figure 3B). MLT tended to reduce the increased water consumption caused by CF throughout the 4-week period, but piromelatine had no effect on water consumption (Figure 3C). These results suggest that piromelatine attenuates the lower food intake in CF rats.

To determine whether MLT and piromelatine can lower the increased blood glucose levels in CF rats, we measured the fasting blood glucose levels in each group of rats (Figure 3D). Compared with the control animals, CF rats exhibited higher fasting blood glucose levels from the 2nd week to the 4th week of the experiment ($P < 0.05$). However, in the 1st and 2nd weeks of treatment, MLT administration lowered the blood glucose levels by 15.74% and 13.81%, respectively ($P < 0.05$ and $P < 0.01$ vs. CF animals), whereas PMT-H treatment decreased the blood glucose levels by
20.98% and 11.23% ($P < 0.01$ and $P < 0.05$ vs. CF animals) in the 3rd and 4th weeks, respectively. These results indicate that piromelatine attenuates many of the basic physiological changes caused by chronic stress and a HFD in rats.

Effect of piromelatine on IPGTT in chronically stressed rats fed a HFD

To further examine the effects of piromelatine on glucose homeostasis, we performed an IPGTT to assess the tolerance of rats after a bolus injection of glucose (2.5 g/kg i.p.). After being challenged with an oral bolus of glucose, CF animals showed a higher glucose excursion than normal control animals, whereas treatment with PMT-H inhibited the increase in blood glucose level by 17.67% ($P < 0.01$), 11.07%, 7.37% and 10.36% ($P < 0.05$) at 0, 30, 60, and 120 min, respectively (Figure 3E), as indicated by approximately 13.8% drop in the AUC (Figure 3F), suggesting improved insulin sensitivity. The rats treated with MLT or a low dose of piromelatine did not exhibit significant differences in glucose tolerance compared with the CF group.

Effect of piromelatine on insulin level and HOMA-IR in chronically stressed rats fed a HFD

To determine whether piromelatine can improve IR in CF rats, we measured the fasting insulin levels and calculated the IR index in each group of rats (Figures 3G and 3H). The mean IR values calculated at the end of the study by the HOMA were higher in the CF group than in the control group. After 4 weeks of treatment, CF rats
that received a high dose of piromelatine had a significantly reduced IR index compared with the CF group \((P < 0.05)\). These results suggest that piromelatine attenuates IR and improves the insulin sensitivity caused by chronic stress and a HFD in rats.

**Effect of piromelatine on blood lipids in chronically stressed rats fed a HFD**

To determine whether MLT and piromelatine affect the blood lipid content in CF rats, we assessed the levels of different types of lipids. After 4 weeks, the levels of TC, TG and LDL-C were elevated by 21.13\% \((P < 0.01)\), 39.84\% \((P < 0.01)\) and 15.79\%, respectively, in the CF group compared with the control group. Treatment with MLT decreased the TC levels significantly by 16.37\% \((P < 0.05)\), and piromelatine tended to decrease the TC, TG, and LDL-C levels, although the differences did not reach statistical significance (Figure 4).

**Effect of piromelatine on CORT concentrations in chronically stressed rats fed a HFD**

GCs are a type of steroid hormone naturally produced in the adrenal cortex upon activation of the HPA axis, and their analog in rats is CORT. To assess the effects of piromelatine on CORT levels, we measured the total CORT concentrations (free and bound in circulation) in plasma and the CORT levels (average free in circulation) in urine. There were no significant differences in the plasma concentrations of CORT in CF rats compared with normal rats, whereas the administration of MLT and PMT-H
reduced the plasma CORT by 35.74% and 42.96%, respectively (both $P < 0.05$). In contrast, the urinary CORT in CF rats was elevated by 28.94% ($P < 0.05$) compared with that in control rats, and low-dose and high-dose piromelatine administration reduced urinary CORT by 13.38% ($P < 0.05$) and 16.55% ($P < 0.01$) (Figures 5A and 5B). MLT also decreased the urinary CORT by 10.50%, but the difference was not statistically significant. These results suggest that piromelatine decreases both the total and free CORT concentrations in rats subjected to stress and a high-fat intake.

**Effect of piromelatine on GR and 11βHSD-1 mRNA expression in adipose tissue in CF rats**

11βHSD1 is a prereceptor enzyme that intracellularly converts less active GCs to a more active form (Stimson, et al., 2013). The active GCs then mediate their effects through the GR, an intracellular hormone-activated transcriptional regulator (Kovačević et al., 2016).

To further investigate the prereceptor GC metabolism of HPA axis regulators in chronically stressed rats fed a HFD, we assayed GR and 11β-HSD1 mRNA expression in adipose tissue by qPCR. The mRNA expression of 11β-HSD1, the key player in intracellular cortisol metabolism, was elevated in CF rats by 42.16% ($P < 0.01$) compared with control rats, which is consistent with active CORT release. CF also reduced GR mRNA expression by 9% ($P < 0.05$), indicating inhibition of HPA axis feedback (Figure 5C). 11β-HSD1 mRNA expression was decreased by both MLT and piromelatine treatment ($P < 0.01$ vs. CF), and GR mRNA expression was increased by
PMT-H treatment ($P < 0.01$ vs. CF), whereas MLT treatment had no significant effect on GR expression. These results indicate that piromelatine induces changes in gene expression in adipose tissue that might potentially ameliorate the effect of CF on the HPA axis via such actions as diminished active CORT secretion and increased sensitivity to GC feedback (Houdek et al., 2015).

Effect of piromelatine on adipose gene expression in CF rats

Adipose tissue is a complex endocrine and immune organ that responds to and, in turn, releases signals that represent or reflect metabolic risk factors (Kershaw et al., 2004). To further examine the changes in gene expression mediated by piromelatine treatment, we evaluated the expression of molecules involved in glucose and lipid metabolism in adipose tissue in CF rats. Upon IR induction, the relative mRNA expression of adiponectin, GLUT-4 and PPAR-γ was decreased by 57.00%, 44.00%, and 39.00%, respectively (all $P < 0.01$) (Figures 6A, 6F, and 6G), and the relative mRNA expression of leptin, TNF-α and IL-6 was increased by 17.00%, 64.00% ($P < 0.01$), and 14.00%, respectively (Figure 6B, D, E). The leptin/adiponectin ratio was increased by 64.00% ($P < 0.01$) (Figure 6C).

Thus, piromelatine caused dose-dependent increases in the relative expression of adiponectin and dose-dependent decreases in leptin expression, with both tested doses resulting in statistically significant changes. MLT treatment also mediated slight changes in the expression of adiponectin and leptin. Adiponectin exerts anti-inflammatory and anti-diabetic effects (Zhao et al., 2015), and leptin represents one of the key peripheral cues that signals the status of body energy reserves to the
brain (Burgos-Ramos et al., 2012). These results imply that several improvements in T2DM symptoms are potentially regulated at the transcript level by piromelatine.

Additionally, the expression of the pro-inflammatory cytokines TNF-α and IL-6 increased significantly in CF rats compared with normal rats. However, the administration of piromelatine potentially reversed the changes in inflammatory markers caused by chronic stress and a HFD. TNF-α and IL-6 induce IR, promote lipolysis, and reduce adipogenesis (Tahar et al., 2011). These results might further support the potentially beneficial effects of MLT and piromelatine on glucose homeostasis and insulin sensitivity. Piromelatine treatment also mediated slight changes in the expression of GLUT-4, but there were no significant differences.

Effect of piromelatine on adipose GR and 11βHSD1 protein expression in CF rats

To further examine the changes in HPA axis regulation, adipose GR and 11βHSD1 protein expression was analyzed by Western blotting. Compared with the control group, the protein level of GR was decreased in the CF group, whereas treatment with MLT and piromelatine attenuated this suppression ($P < 0.01$) (Figure 6). 11βHSD1 is a possible link between GC biology and the development of IR. After induction of IR for 4 weeks, 11βHSD1 expression in adipose tissue was markedly increased ($P < 0.01$). The higher protein levels were significantly reduced in CF rats by 44.24% ($P < 0.01$), 50.91% ($P < 0.01$) and 61.82% ($P < 0.01$) after MLT or low- or high-dose piromelatine treatment, respectively. These results verify the protective
effects of MLT and piromelatine on HPA axis function at the molecular level.

Discussion

Daily exposure to stress and consumption of high-caloric food rich in fat, which are features of a modern lifestyle, are correlated with an increased incidence of metabolic syndrome, T2DM and cardiovascular disease (Kovačević et al., 2016). A HFD has been shown to impair insulin sensitivity and lead to IR (Tanaka et al., 2007; Flanagan et al., 2008). Furthermore, chronic-stress-induced excessive GC production is known to result in whole-body IR and impaired glucose homeostasis, which have been suggested to trigger diabetes (Geer et al., 2014; Novak et al., 2013). In humans, prolonged chronic social stress is associated with higher cortisol levels, increased abdominal fat deposition, hyperglycemia and IR (Aschbacher et al., 2014; Epel et al., 2001). The results obtained in experiment I demonstrate that the combination of a HFD and chronic stress impairs glucose metabolism and induces IR (as demonstrated by the IPGTT, ITT and HOMA-IR index), with a loss of body weight gain and the gradual onset of symptoms of hyperglycemia (Zhang et al., 2014), which is in accordance with previous studies (Bruder-Nascimento et al., 2013; Kovačević et al., 2016; Zardooz et al., 2006; Zemdegs et al., 2016).

In experiment II, there was a slight increase in appetite in the 1st week and a significant reduction in food intake after 1 week in the CF group, and a suppression of body weight gain was also observed in the CF rats ($P < 0.05$ vs. the Control group), indicating a time-dependent interplay between the HFD and stress. Short-term stress
might increase the intake of highly palatable, energy-dense foods (Finger et al., 2012), whereas long-term stress exposure could inhibit an increase in weight gain and caloric intake (Marti et al., 1994), suggesting that chronic exposure to stress prevents the metabolic effects of a HFD on energy intake (Finger et al., 2012).

In clinical practice, psychological stress might cause some people to eat less but encourage others to eat more (Arthur et al., 1994), and chronic stress has been shown to cause both weight gain and weight loss (Nyberg et al., 2012). The associations between stress and eating and between stress and weight remain challenging due to the complexity of eating and body weight regulation. Eating and body weight are regulated by a number of factors, including gender, education, body mass index, the energetic state of the organism, gastrointestinal hormonal and neuronal signals, nutrients, free time during the day, food availability and food quality choice. In addition to these factors, disease state, genetics and ethnic differences also cause inter-individual differences in the responses of humans to stress (Iwasaki et al., 2017).

The animal model established in our present study represents a mechanistic model that incorporates most of the factors observed in humans and was used to analyze treatments for impaired glucose metabolism induced by chronic stress and a HFD.

There are clear gender differences in the responses to stressful daily problems, including eating (Arthur et al., 1994), body weight (Darling et al., 2017), hormonal regulation and other physiological and pathological outcomes (Deak et al., 2015; Fachin et al., 2008). In humans, the relationship between chronic life stress and
weight gain is stronger in males than in females (Torres and Nowson, 2007). In terms of HPA axis regulation, female rats exhibit higher GC levels and slower GC elimination after HPA axis activation (Paris et al., 2010; Babb et al., 2013). Another study demonstrated that estrogen impairs GC-dependent HPA axis negative feedback via estrogen receptor alpha within the hypothalamus (Weiser et al., 2009). A large number of studies use male rats to model stress (Detanico et al., 2009; Bruder-Nascimento et al., 2013; Kovačević et al., 2016; Zardooz et al., 2006; Zemdegs et al., 2016; Zhang et al., 2014); therefore, males have been investigated more often in studies of stress.

We demonstrated that piromelatine and MLT improved the dysfunction of glucose and lipid metabolism and insulin sensitivity in rats with IR induced by a HFD combined with chronic stress. Furthermore, mechanistic experiments showed that piromelatine administration effectively alleviated HPA axis hyperfunctioning and potentially restored the expression of several key genes that were impaired by a HFD and chronic stress exposure. Our results thus suggest the potential therapeutic effects of MLT and piromelatine on IR.

The HPA axis is regulated by GR and is responsible for the stimulation of adrenal corticosteroids required to maintain homeostasis in response to stress (Gupta et al., 2007). The plasma CORT level measured in this study, which represents the concentration of total CORT (free and bound) in circulation (Priyadarshini et al., 2016), remained unchanged in CF animals compared with control animals, although the CF rats did exhibit significantly elevated levels of urinary CORT, which reflects
the average amount of free and biologically active CORT in the body. In addition, we observed increased levels of 11βHSD1, which is a possible link between GC biology and the development of IR (Stimson et al., 2013). Both animal and human studies have indicated that metabolic disturbances are accompanied by increased intracellular 11βHSD1-driven regeneration of CORT (Kovačević et al., 2016). Decreased GR mRNA expression has been investigated in CF rats because it plays an important role in modulating GC feedback on the HPA axis (Moisan et al. 1990) in CF rats. CORT binds to GRs in the PVN, pituitary, and other central nervous system sites in the forebrain to mediate the negative feedback inhibition of CORT (Keller-Wood et al., 2015). Chronic psychosocial stress leads to increased HPA axis activity and impaired GR function, which is associated with hypercortisolemia and reduced GR-mediated negative feedback on the HPA axis (De Kloet et al., 1998). High concentrations of GRs enhance CORT negative feedback on the hypothalamus, thereby forcing the HPA axis into an alternative, low CORT state (Gupta et al., 2007).

Thus, decreased GR biosynthesis and elevated free CORT production are indicative of impaired functioning of the HPA axis feedback mechanisms (Konakchieva et al., 1998; Blanchard et al., 1995). Our results therefore suggest an impairment of HPA regulation, which is consistent with previous reports (Kovačević et al. 2016; Bruder-Nascimento et al., 2013).

Recently, MLT has received attention as a potential chronotherapeutic drug for metabolic disease. Evidence suggests that MLT, a neurohormone that is secreted from the pineal gland, might normalize CORT secretion or the activity of the HPA axis
Increasing evidence suggests that MLT plays an important role in glucose metabolism in both animals and humans (Peschke and Mühlbauer, 2010; Agil et al., 2012). In the present study, MLT and piromelatine enhanced GR expression and inhibited 11βHSD1 expression, reflecting an enhanced negative feedback on the HPA axis and decreased active CORT secretion, which would result in a reduction of the active free GCs in circulation. This finding suggests that piromelatine exerts a strong normalizing effect on HPA axis hyperfunctioning, thus ameliorating IR, lowering fasting plasma glucose levels and improving other metabolic dysfunction symptoms induced by chronic stress and a HFD (Kovačević et al., 2016). Our data showed that MLT and piromelatine treatment improved glucose tolerance in CF rats, as demonstrated by decreased blood glucose levels after glucose load in an IPGTT and a decreased HOMA-IR index. Notably, these results are similar to those obtained in another study, in which the administration of piromelatine improved insulin sensitivity in sleep-deprived rats (She et al., 2009). This result further supports the beneficial effects of piromelatine on insulin sensitivity.

GCs (hyperglycemic effect) and insulin (hypoglycemic effect) play important roles in energetic balance, and this balance can be impaired by exposure to chronic stress (Bruder-Nascimento et al., 2013). GCs exert diabetogenic effects that interfere with insulin action at different levels, causing, for example, impaired regulation of energy homoeostasis through leptin and adiponectin (Lee and Shao., 2014; Marangon et al., 2014; Isorna et al., 2016), disruptions in glucose transport to intracellular spaces through GLUT4 (Coderre et al., 1996), and inhibition of the potent insulin
sensitizer PPARγ (Rosen and Spiegelman, 2001). We observed several changes in the expression of genes that possibly regulate glucose and lipid metabolism after MLT and piromelatine administration, although a direct assessment of blood lipid levels did not show significant differences.

Adiponectin is an adipokine with anti-inflammatory and anti-diabetic properties. Hypoadiponectinemia is closely associated with endothelial dysfunction and IR in obesity and diabetes (Zhao et al., 2015). The adipose tissue-derived hormone leptin has a well-characterized role in energy homoeostasis and modulates appetite, food intake, body weight, adipose stores and immune functions (Burgos-Ramos et al., 2012). Both adiponectin and leptin are regulated by GCs and in turn might impair insulin sensitivity (Castan-Laurell et al., 2011). As a potent insulin sensitizer, adipocyte PPARγ increases the expression of genes that promote triacylglycerol storage and induce lipolysis, thus decreasing ectopic lipid deposition (Rosen and Spiegelman, 2001; Berthiaume et al., 2004). GLUT-4 in adipose tissue transports glucose into fat cells, which is a rate-determining step for glucose consumption in adipocytes (Coderre et al., 1996).

Our data demonstrated that CF decreases PPARγ, GLUT-4 and adiponectin expression and increases leptin expression at the transcriptional level, which is partly consistent with previous findings (Kovačević et al., 2016; Bruder-Nascimento et al., 2013). Our results also suggest that piromelatine relieves the suppression of adiponectin expression and the overexpression of leptin but has no effect on PPARγ or GLUT-4 mRNA expression, demonstrating that piromelatine might potentially
promote energy homoeostasis and improve whole-body IR but does not affect adipogenesis or lipid disposal, which is supported by the observation that the blood lipid levels did not change. Thus, the effects of piromelatine on the expression of several genes could possibly contribute to their ability to regulate glucose homeostasis.

Low-grade inflammation has been linked to risk of T2DM and atherosclerotic vascular diseases (Uusitupa et al., 2013). In response to excess adiposity and macrophage infiltration in adipose tissue, numerous pro-inflammatory cytokines, including TNF-α and IL-6, are concomitantly increased in adipose tissue, and their levels correlate with the degree of IR (Galic et al., 2010; Kern et al., 2001). The increased levels of TNF-α and IL-6 gene expression observed in the CF group might indicate the presence of low-grade inflammation in chronically stressed rats fed a HFD. MLT has been reported to reduce inflammatory injury by downregulating pro-inflammatory molecules, e.g., TNF-α and IL-6, in an NF-κB-dependent manner in young male Zucker diabetic fatty (ZDF) rats or in rats fed a HFD (Cutando et al., 2015; Agil et al., 2013; Cano et al., 2014). In our study, piromelatine administration appeared to reverse the increases in pro-inflammatory cytokines in the adipose tissues of rats subjected to CF, indicating the potential alleviation of inflammation and IR.

In recent years, the beneficial effects of MLT in experimental or even clinical conditions of obesity and metabolic syndrome have been demonstrated, but its therapeutic use has been limited by its short half-life (0.5 h) (Paulis et al., 2012), and the administration of higher doses of MLT might desensitize MLT receptors (Gerdin
et al., 2004). Piromelatine was developed to extend the half-life of MLT to up to 1.5-3.0 h, leading to a prolonged duration of action and greater efficiency than other MLT supplements.

MLT appears to have an approximately 4.7-fold higher affinity for the MT1 receptor [dissociation constancy for binding affinity \( (K_i) = 0.081 \text{ nM} \)] than for the MT2 receptor \( (K_i = 0.383 \text{ nM} \) ). Piromelatine shows a similar binding affinity to both types of MLT receptors [binding affinity MT1 \( (K_i = 22 \text{ nM} \), binding affinity MT2 \( (K_i = 34 \text{ nM} \) ] (Yalkinoglu et al., 2010). These differences between MLT and piromelatine might explain why the similar MT1 and MT2 agonistic activities of piromelatine exert an improved effect on metabolism in CF rats.

Many of MLT’s actions are mediated through its direct interactions with MT1 and MT2, which are G-protein-coupled membrane bound receptors (Masana et al., 2002), or its indirect actions via nuclear orphan receptors from the retinoid orphan receptors/retinoid Z receptors (ROR/RZR) family, which are MLT nuclear receptors functioning as transcription factors (Wiesenberg et al., 1995; Becker-Andre et al., 1994). MLT also binds to the quinone reductase II (QR2) enzyme, previously defined the MT3 receptor, with a low affinity (Mailliet et al., 2004). Melatonin also acts through non-receptor mediated mechanisms, for example serving as a scavenger for reactive oxygen species and reactive nitrogen species (Reiter et al., 2007; Reiter et al., 2001) and a broadspectrum antioxidant (Koc et al., 2003; Tomás-Zapico et al., 2005).

Piromelatine selectively binds the melatonergic MT1 and MT2 receptors over QR2/MT3 and ROR/RZR, which suggesting a crucial role of only MT1 and MT2.
receptors in neuro-endocrine mechanisms, such as normalization of HPA axis and glucose metabolism (Torres-Farfan et al., 2003; Zhou et al., 2017). In contrast, MLT additionally binding to either the QR2/MT3 or ROR/RZR possibly has the opposite effects on insulin levels and the mRNA expression of GR and other numerous genes. There is much evidence to support our hypothesis, the overexpression of RORα or treatment with an RORα-specific agonist enhances the expressions of inflammatory cytokines and increases the number of infiltrated macrophages into adipose tissue, whereas adipose tissue inflammation has been linked to metabolic diseases such as obesity and T2DM (Liu et al., 2017). The effects of MLT and its agonists on diabetes through QR2/MT3 and ROR/RZR are worth additional research.

Additionally, the food rich in energy lead to more extra fat stored in the body, finally result in obesity. Melatonin and melatonin agonists induce extra energy expenditure and limit obesity in rodents without affecting food intake and activity by a thermogenic effect on brown adipose tissue (BAT), such as stimulates growth of BAT and increases BAT activity (Jiménez-Aranda et al., 2013; Tan et al., 2011). These importance factors contributed to the effect of melatonin and melatonin agonists on obesity treatment.

In summary, our results clearly demonstrate that CF exposure induces IR as well as changes in free CORT levels and HPA axis hyperfunctioning. Piromelatine administration effectively ameliorates glucose intolerance in an IPGTT and insulin insensitivity in the HOMA-IR index as well as the associated metabolic deficits in CF rats. Mechanistic experiments indicated that the effects of piromelatine might be
related to decreases in the free CORT level and 11βHSD1 expression and the regulation of glucose metabolism-related genes. Moreover, the suppression of pro-inflammatory TNF-α and IL-6 expression in adipose tissue also possibly mediates the insulin sensitivity effect in CF rats. We conclude that piromelatine might be a potential drug for the treatment of glucose intolerance and IR. However, more studies are necessary to better understand the mechanisms of action of piromelatine on metabolic disturbance.

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Authorship contributions

Participated in the research design: Zhou, Zhang, Jia

Conducted the experiments: Zhou, Wang, Luo, Xu Jia

Performed the data analyses: Zhou, Wang, Li, Zhang, Jia

Wrote or contributed to the writing of the manuscript: Zhou, Zhang, Jia, Laudon
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Footnotes

Conflict of interest

All authors declare that they have no conflicts of interest.

Funding

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**Figure legends**

**Figure 1. Structure of MLT and piromelatine.** A) Chemical structure of MLT. B) Chemical structure of piromelatine.

**Figure 2. Effect of chronic stress and a HFD on metabolic parameters in rats.** (A) Body weight. (B) Fasting plasma glucose concentrations. (C) Plasma glucose concentrations while performing the IPGTT on the 4th week of the experiment. (D) Plasma glucose concentrations while performing the ITT on the 3rd week of the experiment. (E) Liver glycogen. (F) Muscle glycogen. (G) Blood lipid levels. (H) Blood FFA levels. (I) Plasma CORT levels. (J) Plasma insulin levels. (K) Plasma glucagon levels. (L) Plasma leptin levels. Control: normal control group; Stress: chronically stressed rats fed the same diet as the normal diet group; HFD: rats fed with a HFD; CF: chronically stressed rats fed the same diet as the HFD group (Stress + HFD). The values are expressed as the means ± S.D. (n = 8-10 per group). *P < 0.05, **P < 0.01 vs. Control group, #P < 0.05, ##P < 0.01 vs. HFD group via one-way ANOVA with Dunnett’s post hoc analyses; ^P < 0.05, ^^P < 0.01 vs. timecourse baseline via two-way ANOVA with Bonferroni post hoc analyses.

**Figure 3. Effects of piromelatine on body weight, food intake, water consumption, and blood glucose, over a 4-week treatment period.** (A) Body weight. (B) Food intake. (C) Water consumption. (D) Fasting blood glucose levels. (E) IPGTT. (F) Plasma insulin levels. (G) HOMA-IR. Control: normal control group; CF: chronically
stressed rats fed the same diet as the HFD group; MLT: CF + melatonin (20 mg·kg\(^{-1}\)) I.G.; PMT-L: CF + low-dose piromelatine group (10 mg·kg\(^{-1}\)) I.G.; PMT-H: CF + high-dose piromelatine group (20 mg·kg\(^{-1}\)) I.G.; HOMA-IR: homeostasis model assessment-index of insulin resistance. The values are expressed as the means ± S.D. (n = 8-10 per group). \(^*P<0.05\), \(^{**}P<0.01\) vs. Control group, \(^{#}P<0.05\), \(^{##}P<0.01\) vs. CF group via one-way ANOVA with Dunnett’s post hoc analyses; \(^{^*}P<0.05\), \(^{^{*^*}}P<0.01\) vs. timecourse baseline via two-way ANOVA with Bonferroni post hoc analyses.

**Figure 4. Effects of piromelatine on blood lipid levels over 4 weeks of treatment.**

The values are expressed as the means ± S.D. (n = 8-10 per group). \(^*P<0.05\), \(^{**}P<0.01\) vs. Control group, \(^{#}P<0.05\), \(^{##}P<0.01\) vs. CF group via one-way ANOVA with Dunnett’s post hoc analyses. TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

**Figure 5. Effects of piromelatine and MLT on HPA axis function over 4 weeks of treatment.** (A) Plasma CORT levels. (B) Urinary CORT levels. (C) The relative expression of GR and 11\(\beta\)HSD1 mRNA in adipose tissue was measured through a real-time quantitative RT-PCR analysis. The values are expressed as the means ± S.D. (n = 8-10 per group). \(^*P<0.05\), \(^{**}P<0.01\) vs. Control group, \(^{#}P<0.05\), \(^{##}P<0.01\) vs. CF group via one-way ANOVA with Dunnett’s post hoc analyses.

**Figure 6. Effects of piromelatine and MLT on diabetes-related genes in adipose tissue.**
tissue over 4 weeks of treatment. The relative expression of adiponectin (A), leptin (B), the adiponectin/leptin ratio (C), TNF-α (D), IL-6 (E), GLUT-4 (F), and PPAR-γ (G) mRNAs in adipose tissue were measured by real-time quantitative RT-PCR analysis. The values are expressed as the means ± S.D. (n = 8-10 per group). *P < 0.05, **P < 0.01 vs. Control group, #P < 0.05, ##P < 0.01 vs. CF group via one-way ANOVA with Dunnett’s post hoc analyses.

Figure 7. Effects of piromelatine and MLT on adipose GR and 11βHSD-1 protein expression in CF rats over 4 weeks of treatment. Relative expression of the GR and 11βHSD-1 proteins in adipose tissue was measured by Western blotting. The band intensities were quantified by densitometry. The values are expressed as the means ± S.D. (n = 8-10 per group). *P < 0.05, **P < 0.01 vs. Control group, #P < 0.05, ##P < 0.01 vs. CF group via one-way ANOVA with Dunnett’s post hoc analyses.
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<td></td>
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</table>
Figure 1.
Figure 2.

A

Body weight (g)

Control
HFD
CS
CF

* *
**
*
##
###
*
^^
^^
^^
^
^^
^

Time (week)

B

Fasting blood glucose (mmol/L)

Control
HFD
CS
CF

* *
#
*
#
*
#
*
#^
^^
^^
^^^
^

Time (week)
**Figure C**

- Blood glucose (mmol/L) vs. Time after glucose injection (min)
- Control, HFD, CS, CF groups

**Figure D**

- AUC (mmol/L×h) for Control, HFD, CS, CF groups

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**Figure I**
Blood lipid levels (mmol/L) for TC, TG, HDL-C, and LDL-C.

**Figure J**
FFA content (mmol/L) for FFA.
Figure 3.

(A) Body weight (g) over time (weeks) for different groups: Control, CF, MLT, PMT-L, and PMT-H. Significant differences are indicated by asterisks (*) and double asterisks (**) for comparisons between groups.

(B) Food intake (g/100gBW) over time (weeks) for different groups: Control, CF, MLT, PMT-L, and PMT-H. Significant differences are indicated by symbols (#) and double asterisks (**) for comparisons between groups.
Figure C: Water consumption (g/100gBW) over time (week).

Figure D: Blood glucose levels (mmol/L) over time (week).
**Figure G**

Insulin levels (μIU/ml)

**Figure H**

HOMA-IR

Legend:
- Control
- CF
- MLT
- PMT-L
- PMT-H

Statistical symbols:
- 
- 
- 

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**Figure 4.**

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<th>TC</th>
<th>TG</th>
<th>HDL-C</th>
<th>LDL-C</th>
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<td>PMT-H</td>
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</table>

Blood lipid levels (mmol/L)

- TC: Total Cholesterol
- TG: Triglycerides
- HDL-C: High-Density Lipoprotein Cholesterol
- LDL-C: Low-Density Lipoprotein Cholesterol

- **: Significant difference compared to control
- #: Significant difference compared to CF
- **: Significant difference compared to MLT
- #**: Significant difference compared to PMT-L
Figure 5.

A

Plasma CORT levels (μg/dl)

Control
CF
MLT
PMT-L
PMT-H

B

Urinary CORT levels (μg/dl)

Control
CF
MLT
PMT-L
PMT-H
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Figure 6.

(A) Relative mRNA expression of Adiponectin for Control, CF, MLT, PMT-L, and PMT-H groups. Error bars indicate standard error of the mean.

(B) Relative mRNA expression of Leptin for Control, CF, MLT, PMT-L, and PMT-H groups. Error bars indicate standard error of the mean.
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Figure 7.

**Relative mRNA Expression**

- **β-actin**
- **GR**
- **11β-HSD1**

<table>
<thead>
<tr>
<th></th>
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<td><strong>GR</strong></td>
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<tr>
<td><strong>11β-HSD1</strong></td>
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*Significance levels:*

- *: p < 0.05
- **: p < 0.01
- ##: p < 0.001

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