Circadian rhythm dysregulation aggravates adipose tissue depletion in heart failure-induced cachexia

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Research Article

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Abstract

Background

The circadian clock is involved in lipid metabolism in adipocytes. The impairment of circadian clocks is a major cause of metabolic diseases, but the pathophysiological role of the circadian clock in adipose tissue depletion, in cachexia, remains unclear. To address this issue, we investigated the effects of circadian clock misalignment on adipose tissue metabolism in cardiac cachexia.

Methods

We produced cardiac cachexia rat models through injection of monocrotaline (MCT), which caused pulmonary hypertension-induced heart failure (HF). Cardiac function was measured by echocardiography. The histological features in fat and liver tissue were observed by H&E staining, Oil Red O staining and Picrosirius red staining. Immunohistochemical staining, Western blotting and RT-qPCR were used to detect markers of lipolysis, lipogenesis and beiging of adipose tissue in white adipose tissue (WAT) and thermogenesis in brown adipose tissue (BAT).

Results

We found that rats with MCT injection exhibited right and left ventricular dysfunction. Compared with rats in the control group, rats housed in the light: dark cycle (LD group) exhibited disrupted circadian rhythm reflected by increased BMAL1 protein and decreased REV-ERBα. Meanwhile, these rats displayed decreased adipose mass and increased ectopic lipid deposition; moreover, smaller adipocytes and reduced lipid contents as well as increased extracellular matrix were found. In WAT, rats in the LD group exhibited elevated PKA-mediated lipolysis and WAT browning, while lipid storage was decreased as lipogenesis was inhibited. Meanwhile, in BAT, PKA-mediated thermogenesis was increased. NT-proBNP levels in blood and NE and IL-6 contents in adipose tissue were higher in the LD group than in the control group. Remarkably, compared with rats in the LD group, rats with circadian misalignment in the DL group and LV-Bmal1 shRNA group exhibited aggravated lipolysis and WAT browning, inhibited lipid storage in WAT, and elevated PKA-mediated thermogenesis in BAT. Moreover, rats in the DL group and LV-Bmal1 shRNA group showed higher levels of NT-proBNP in blood and NE and IL-6 contents in adipose tissue than rats in the LD group.

Conclusion

Our study suggested that a disrupted circadian rhythm aggravated fat wasting in patients with HF-induced cachexia by increasing lipolysis, preventing lipid storage in WAT and promoting beiging/brown adipocyte thermogenesis. This result indicated that stabilizing adipose tissue rhythms may help to
combat disrupted energy homeostasis and alleviate excessive adipose tissue expenditure in HF-induced cachexia.

**Introduction**

The development and prognosis of chronic heart failure (HF) are closely associated with nutritional status. Although obesity has been regarded as a traditional risk factor for developing HF, it has been reported that overweight or class 1 obese individuals have better survival than patients with normal weight[1]. This curious phenomenon was termed the “obesity paradox”. In comparison, cachexia, which is a well-known risk factor for death in patients with end-stage chronic HF[2] is accompanied by significant muscle loss, fat wasting and bone tissue loss. The prevalence of cachexia among patients with HF ranges between 0% and 39%[3]. Melenovsky et al.[4] reported that fat wasting was more prevalent among patients with right ventricular dysfunction than among those with left ventricular dysfunction. Cachexia has long been regarded as a muscle wasting disorder, but recently, adipose tissue wasting has started to gain attention in regard to cachexia[5]. Several clinical studies have suggested that adipose tissue becomes the primary tissue consumed in cachexia rather than muscle; moreover, fat loss was predictive of adverse outcomes in advanced HF[4, 6]. Indeed, fat wasting increases susceptibility to infection and aggravates ectopic fat deposition caused by excessive lipolysis[7]. Despite the clear clinical relevance, the mechanisms by which cachexia develops remain unclear. Enhanced lipolysis and fat oxidation, decreased lipogenesis, impaired lipid storage, and increased beiging/brown adipocyte thermogenesis may be underlying causes of adipose atrophy in cachexia[8]. Additionally, studies have suggested that excessive sympathetic activity and increased proinflammatory cytokines are the main factors contributing to fat depletion in HF[3].

The circadian clock evolved as an autonomous timekeeping system that aligns behavioural patterns with the succession of day and night and supports body functions by coordinating the required metabolic program. At the molecular level, control of circadian rhythmicity is thought to involve the interaction of two major autoregulatory transcription-translation feedback loops. The central feedback loop includes circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1). CLOCK and BMAL1 interact to form a heterodimer that drives the expression of Period (PER) and Cryptochrome (CRY). The PER and CRY proteins accumulate in the cytoplasm and then translocate to the nucleus in the evening to repress their own transcription by interacting with the CLOCK:BMAL1 heterodimer[9]. Additionally, the CLOCK:BMAL1 heterodimer promotes the expression of nuclear receptor reverse ERB (REV-ERB), which negatively regulates BMAL1 expression[10]. Molecular clocks are found in adipose tissue and are known to regulate lipid metabolism in adipocytes[11].

Recently, mounting evidence has shown that a disrupted circadian rhythm leads to insulin resistance and metabolic inflammation and increases the risk of obesity and type 2 diabetes[12]. For example, chronic jet lag under abnormal lighting conditions reportedly causes obesity in both mice and shift workers[13, 14]. Deletion of Bmal1 in mice resulted in decreased capability of novo lipogenesis and fat storage[15]
and increased levels of circulating FFAs and triglycerides (TGs) due to excessive lipolysis[16]. Nevertheless, to date, evidence supporting the link between circadian misalignment and adipose tissue depletion in cachexia is lacking. In mice with cancer cachexia, induced by C26 tumours, it has been suggested that the expression of adipogenesis genes and lipogenic genes in WAT decreased and lost rhythmicity, indicating decreased lipid accumulation. Additionally, the protein levels of the lipolytic enzymes perilipin and adipose triglyceride lipase (ATGL) were increased at two time points, indicating increased lipolysis. In addition, cancer cachexia resulted in loss of rhythmicity of Rev-erbα and Per2, and the expression of Bmal1 and Cry1 increased[17]. However, whether circadian misalignment is involved in adipose tissue depletion in cachexia remains unclear. To address this issue, we generated cardiac cachexia rats by monocrotaline (MCT)-induced pulmonary hypertension and investigated the relationship between circadian rhythm disorder and adipose tissue depletion in HF-induced cachexia.

**Materials and methods**

**Animals and reagents**

A total of 50 specific pathogen-free (SPF) adult male Wistar rats weighing 260–280 g were purchased from Beijing Vital River Corporation (Permit No. SCXK(JING) 2021-0011). All rats were housed in separate cages under a 12 h light-dark cycle at 23 ± 1°C and 50% relative humidity, and food and water were available ad libitum. The animal study was approved by the Experimental Animal Ethics Committee of Shandong University of Traditional Chinese Medicine (Licence No. 2021-30).

After 1 week of adaptive feeding, 40 rats were intraperitoneally injected with monocrotaline (60 mg/kg, Lot No. MUST-21111217, Chengdu Must Bio-Technology Co., Ltd) to induce cardiac cachexia. MCT-induced pulmonary hypertension is an experimental model of cardiac cachexia since it leads to progressive HF and cachexia very rapidly[18, 19]. After 4 weeks of MCT injection, we measured body weight and heart function. Meanwhile, rats in the control group were intraperitoneally injected with 3 ml/kg solvent (saline: alcohol = 8:2). Subsequently, the rats with cachexia were randomly assigned to four groups: the LD group, DL group, LV-Control shRNA group and LV-Bmal1 shRNA group. Rats without MCT treatment were assigned to the control group. Rats in the DL group were housed in a dark:light 8 h:16 h cycle to mimic a rotating shiftwork schedule, with lights off at 7 A.M and lights on at 3 P.M. Rats in the other three groups were housed in a light:dark 12 h:12 h cycle, with lights on at 7 A.M. and light off at 7 P.M. In addition, rats in the LV-Bmal1 shRNA group received a focal point injection of lentivirus carrying Bmal1 shRNA in inguinal fat and epididymal fat, and 1.5×10⁷ TU was injected at each point. Rats in the LV-Control shRNA group were injected with empty lentiviral vehicle. Rats in the control group and DL group, LV-Bmal1 shRNA group and LV-Control shRNA group were fed in pairs to match the daily food intake of the LD group[20].

**Construction of the lentivirus LV-Bmal1 shRNA**
Lentivirus-expressing short hairpin RNA (shRNA) targeting the sequence of the Bmal1 gene (5'-ATCCTCAATTATAGCAATG-3') and a negative control (5'-TTCTCCG AACGTGTCACGT-3') were constructed and synthesized by GenePharma Biotech (Shanghai, China). Correct insertion of shRNA cassettes was confirmed by direct DNA sequencing. The titer of LV-Bmal1 shRNA was 2E + 9 TU/mL.

**Ultrasound cardiography**

After the rats were anaesthetized at the 4th week after MCT injection, indicators reflecting heart function, including left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) as well as right ventricular fractional area change (RV-FAC), were recorded. RV-FAC was calculated by RV-FAC= (end-diastolic area (EDA)-end-systolic area (ESA))/EDA×100%.

**Tissue collection**

At the end of the 8th week after injection of MCT, each rat was anaesthetized with pentobarbital sodium (40 mg/kg, i.p.). Blood samples collected from the inferior vena cava were centrifuged at 3000 rpm for 15 min at 4°C to separate the serum and preserved at -80°C for ELISA. White adipose tissue (WAT), including epididymal fat and inguinal fat, and brown adipose tissue (BAT) in the scapula were removed as quickly as possible on ice and recorded as the total fat weight. The liver tissue, epididymal fat and brown fat in the scapula were immediately stored in liquid nitrogen for subsequent experimental testing[20].

**Histological analysis**

Epididymal fat and brown fat were fixed in 4% paraformaldehyde for 12 h, embedded in paraffin, cut into 4-µm thick sections and subsequently stained with HE staining and Oil Red O staining (Servicebio, Wuhan, China). Additionally, epididymal fat was stained with picrosirius red (PSR). Finally, tissue sections were viewed under a Nikon microscope (Nikon Eclipse E100, Nikon, Tokyo, Japan). Images were obtained from three random regions of each slice. The analysis was performed with a Nikon DS-U3 (Nikon, Tokyo, Japan).

**Immunohistochemical staining**

Paraffin-embedded sections were cut into 4 µm thick sections, deparaffinized with xylene and incubated with hydrogen peroxide to block endogenous peroxidase. The sections were sequentially incubated with 5% bovine serum albumin. Then, they were incubated with anti-uncoupling protein-1 (UCP-1) (1:200, Cat No. GB112174, Servicebio, Wuhan, China) primary antibodies overnight at 4 °C and subsequently incubated with goat anti-rabbit secondary antibody (1:200, Cat No. G1213-100UL, Servicebio, Wuhan, China). Sections were counterstained with haematoxylin before being mounted. The stained slides were observed under a Nikon microscope (Nikon Eclipse E100, Nikon, Tokyo, Japan). Images collected from three random fields per slice were finally analysed using a Nikon DS-U3 (Nikon, Tokyo, Japan).

**Enzymelinked immunosorbent assay (ELISA)**
The levels of TGs, FFAs and NT-proBNP in blood and interleukin-6 (IL-6) and norepinephrine (NE) in both WAT and BAT were measured using high-sensitivity ELISA kits (TG (Cat No. JYM0046Ra), FFA (Cat No. JYM0208Ra), NT-proBNP (Cat No. CSB-E07972r), IL-6 (Cat No. JYM0646Ra), NE (Cat No. JYM0587Ra)). All these products were purchased from Colorfulgene Biological Technology, Wuhan, China. All testing steps were performed according to the product instructions attached to the product.

**Realtime quantitative PCR (qRT–PCR)**

Total RNA was extracted from the frozen epididymal fat and brown fat in the scapula by the TRIzol method using SparkZol Reagent (Cat No. AC0101, SparkJade, Jinan, China) and reverse transcribed by using the SPARKscript II RT Plus Kit (Cat No. AG0304, SparkJade, Jinan, China). Gene expression was analysed by quantitative PCR performed using Light Cycler 480 SYBR Premix Ex Taq II (Roche, Basel, Switzerland).

The mRNA expression levels of hormone sensitive lipase (Hsl), Atg1, Peripilin, carnitine palmitoyltransferase 1 (Cpt1), acyl-CoA, stearoyl-CoA desaturase 1 (Scd1), fatty acid synthase (Fas), monoacylglycerol acyltransferase (Mgat), diacylglycerol acyltransferase-2 (Dgat2), Cd137, Tbx-1, Zinc finger protein 1 (Zic-1), Ucp-1, CD36, Fatty Acid Transport Protein-1 (Fatp-1), Peroxisome Proliferator-Activated Receptor Gamma (Ppary), proliferator-activated receptor-γ coactivator 1α (Pgc-1α), Clock, Bmal1, and Rev-erba were detected, and β-actin was used for normalization. The relative gene expression was analysed using the comparative Ct method formula, $2^{-\Delta\Delta CT}$ [21]. The sequences of the forwards/reverse primers (synthesized by SparkJade China) are listed in Table 1.
<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>Forward 5’-CCCATCTATGAGGGTTACGC’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TTTAATGTCACGCACGATTTC’</td>
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<tr>
<td>Hsl</td>
<td>Forward 5’-GTCCCTCATCGCCCTCAAAGAAG’</td>
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<tr>
<td></td>
<td>Reverse 5’-GCCAGCCACAACCTAGCAGAAC’</td>
</tr>
<tr>
<td>Atgl</td>
<td>Forward 5’-TGGATGAAGGAGCAGACAGGTAGC’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-AGTGGACAGACGGGAGAC’</td>
</tr>
<tr>
<td>Periphilin</td>
<td>Forward 5’-ACCCTCAAGTTCGTCAGAGTTTC’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TTCATGCTCAGCGACTTTTC’</td>
</tr>
<tr>
<td>Cpt1</td>
<td>Forward 5’-CAGGAGAGTGCAGCAGGTATAG’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TGCCGAAAGAGTCAAATGGAAG’</td>
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<tr>
<td>Scd1</td>
<td>Forward 5’-TGTCAAGAAGAGGCGGAAAGG’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-CAGGATGAAGACATGAGAGG’</td>
</tr>
<tr>
<td>Fas</td>
<td>Forward 5’-GTCTCTGCTCTCTGCTTTGC’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TTCAGCAGGCTCTCTCTCACT’</td>
</tr>
<tr>
<td>Dgat2</td>
<td>Forward 5’-CCGCAGCGGAAACAAAGAAA’</td>
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<tr>
<td></td>
<td>Reverse 5’-GACGGACCTGAGACTGAGTAC’</td>
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<tr>
<td>Mgat</td>
<td>Forward 5’-GCAGCCAGACCCGAGGAGT’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TCCAGATCAGTCCACTACCACAG’</td>
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<td>Cd137</td>
<td>Forward 5’-AAGCCTTGGCTCTCTACCCA’</td>
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<td></td>
<td>Reverse 5’-CGTCTCAAGCCACAGTT’</td>
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<td>Tbx-1</td>
<td>Forward 5’-CGGTGAAGAAGACCCAAAG’</td>
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<td></td>
<td>Reverse 5’-TCCACAGGCACAAAGTCCA’</td>
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<td>Zic1</td>
<td>Forward 5’-GTCTCTTTTCAGGGCTACAG’</td>
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<tr>
<td></td>
<td>Reverse 5’-GCTGGTGTGCGGTGTTGT’</td>
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<tr>
<td>Ucp-1</td>
<td>Forward 5’-GGCATCCAGAGGCAATCA’</td>
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<tr>
<td></td>
<td>Reverse 5’-GTCAAGGGCCAGCCGAGA’</td>
</tr>
<tr>
<td>Clock</td>
<td>Forward 5’-ACGGCGGAACTTGGCTATGAG’</td>
</tr>
</tbody>
</table>
**Western blot analysis**

Protein was collected from the epididymal fat and brown fat in the scapula using the Minute™ Animal Adipose Tissue Protein Extraction Kit (Invent, Minnesota, USA). Adipose tissue homogenate was extracted using ice-cold RIPA buffer, and protein concentrations were determined using an enhanced bicinchoninic acid (BCA) protein assay kit (Cwbio, Jiangsu, China). The protein concentration determined by the BCA kit was finally prepared into a normalized protein system with a concentration of 2 µg/µL. Then, 20 µg of protein from each group was separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA) for blotting. After incubation with 5% nonfat milk in TBST for 1 h at room temperature. Subsequently, the immunoblots were washed and then probed with primary antibodies overnight for 8 h at 4°C against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000, Cat No. ab181602, Abcam, Cambridge, United Kingdom), protein kinase A (PKA) (1:1000, Cat No. ab32390, Abcam, Cambridge, United Kingdom), phospho-PKA (p-PKA) (1:500, Cat No. Bs-3725r, Bioss, Beijing, China), HSL (1:1000, Bioss, Cat No. Bs-0455r), ATGL (1:2000, Bioss, Cat No. Bs-3831r), REV-ERBα (1:1000, Cat No. bs-38157r), PPARγ (1:1000, Cat No. bs-45567r), PGC-1α (1:500, Cat No. bs-3831r), and LPL (1:2000, Cat No. bs-3725r). After incubation with the secondary antibodies, immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) detection system and exposed using film. The intensity of the protein bands was quantified using a densitometry system.
Santa Cruz, Cat No. sc-393215) and BMAL1 (1:1000, Abcam, Cat No. ab235577). Then, the membranes were washed and incubated with the secondary antibody of the corresponding species. Then, the protein bands were visualized with a Sensitivity ECL Chemiluminescence Detection Kit (Proteintech, Wuhan, China). Finally, the protein content was analysed by densitometric quantification using FluorChem Q 3.4 (ProteinSimple, California, USA).

**Statistical Analysis**

The obtained data were statistically analysed by GraphPad Prism 8.0. Data are presented as the mean and standard deviation (SD). Differences were analysed using the single-factor analysis of variance method (ANOVA) or nonparametric Kruskal-Wallis test according to the variances. \( P < 0.05 \) was defined as statistically significant.

**Results**

1. **MCT injection induced progressive ventricular dysfunction**

At the end of the 4th week after MCT injection, as shown in Fig. 1, left and right ventricular function were significantly decreased, as demonstrated by reduced indicators, including LVEF, LVFS and RV-FAC (Fig. 1A, C, E, \( P < 0.01 \)). This indicates that rats treated with MCT exhibited significant HF.

2. **Disrupted clock gene expression was observed in HF-induced cachexia**

We tested the expression of circadian clock genes in the context of cachexia. According to our results, in both WAT and BAT, compared with healthy rats in the control group, the protein expression of REV-ERB\( \alpha \) was increased (Fig. 2A, C, G, I, \( P < 0.01 \)), and BMAL1 was suppressed in the LD group, although there was no significant difference (Figure. 2A, B, G, H, \( P > 0.01 \)). Consistently, the mRNA levels of Clock and Bmal1 in both WAT and BAT of rats treated with MCT were decreased, while the mRNA levels of Rev-erb\( \alpha \) were increased compared to those in healthy rats in the control group (Fig. 2D-F, J-L, \( P < 0.01 \)). As expected, compared with HF-induced cachexia rats housed in a normal diurnal cycle, disrupting the light: dark cycle and suppressing Bmal1 expression via injection of LV-Bmal1 shRNA suppressed the mRNA and protein levels of Clock and Bmal1 expression while upregulating Rev-erb\( \alpha \) expression (\( P < 0.01, P < 0.05 \)). This result suggested that dysregulated expression of circadian clock genes occurred under conditions of HF-induced cachexia.

3. **Circadian rhythm disruption aggravated adipose tissue loss and ectopic lipid deposition in HF-induced cachexia**
During the experimental period, rats that were administered MCT in the LD group exhibited reduced body weight after 2 weeks of MCT injection compared with rats that were not administered MCT in the control group, although there was no significant difference. Rats in both the DL group and LV-Bmal1 shRNA group exhibited significant weight loss compared with rats in the control group at the 3rd week and 5th week, respectively (Fig. 3A, P < 0.05). At the 8th week, we evaluated total adipose tissue weight. As expected, rats in the LD group exhibited a significant decrease compared with rats in the control group (Fig. 3B, C, P < 0.01). Compared with rats in the LD group, rats housed in a disrupted light: dark cycle and injected with LV-Bmal1 shRNA exhibited more severe adipose loss (P < 0.01).

As described previously, cachexia leads to dyslipidaemia and fatty liver attributed to FFAs released from excessive lipolysis deposits in ectopic tissue[22]. In the present study, we found that MCT-injected rats housed in the light: dark cycle exhibited increased blood FFA and TG levels compared with rats in the control group; importantly, disrupted circadian rhythm resulted in higher blood levels of both FFA and TG in the DL group (Fig. 3D, E, P < 0.01). Consistently, rats in the LD group exhibited increased lipid deposition in the liver compared with healthy rats in the control group (Fig. 3F, G, P < 0.01); moreover, rats in both the DL group and LV-Bmal1 shRNA group exhibited more lipid deposition than rats in the LD group (P < 0.01). These results indicated that circadian rhythm dysregulation through disruption of the light: dark cycle and inhibition of Bmal1 expression aggravated ectopic lipid deposition.

4. Circadian rhythm disruption exacerbated adipocyte atrophy and increased extracellular matrix in adipose tissue under conditions of HF-induced cachexia

Using HE staining and Oil Red O staining, we observed that rats treated with MCT in the LD group exhibited smaller adipocytes (Fig. 4A, B, G, H) and decreased lipid accumulation (Fig. 4C, D, I, J) in both WAT and BAT compared with rats in the control group (P < 0.01). This observation was more significant when circadian rhythm balance was disrupted, with rats in both the DL group and LV-Bmal1 shRNA group having less lipid accumulation than MCT-injected rats housed in normal circadian rhythm, as shown by Oil Red O staining (P < 0.01). Moreover, rats in the DL group had smaller adipocytes in both WAT and BAT than rats in the LD group (P < 0.01). It indicated that circadian rhythm disruption exacerbated adipocyte atrophy. Additionally, we revealed that there was increased extracellular matrix deposition in the WAT of rats in the LD group after induction of cardiac cachexia by injection of MCT compared with rats in the control group (Fig. 4E, F, P < 0.01). Moreover, disrupting circadian rhythm balance further aggravated extracellular matrix deposition (P < 0.01). This result indicated that WAT in cachexia exhibited fibrosis, which could be more severe due to disordered circadian rhythm.

5. Circadian rhythm disruption exacerbates WAT consumption by promoting lipolysis and beiging of WAT and reducing lipid storage in HF-induced cachexia

To test the effect of circadian rhythm disorder on WAT lipolysis in cachexia, we measured the protein expression of protein lipase (PKA) signalling and its downstream enzymes primarily responsible for the hydrolysis of lipid droplets, including ATGL and HSL. According to our results, we found that the expression of ATGL, HSL, PKA and p-PKA increased under conditions of HF-induced cachexia (Fig. 5A, C-
These results were more obviously due to dysregulated circadian rhythm, with rats in the DL group showing higher protein expression of ATGL, HSL, PKA and p-PKA (P < 0.05, P < 0.01). Additionally, rats in the LV-Bmal1 shRNA group exhibited higher protein levels of ATGL, HSL, PKA and p-PKA, although only ATGL was significantly different (P < 0.05). Consistently, the mRNA expression levels of lipase genes (Hsl, Atgl and Peripilin) (Fig. 5H-J) and FFA β-oxidation genes (Cpt1 and acyl-CoA) (Fig. 5K, L) increased under conditions of HF-induced cachexia (P < 0.05). These genes exhibited higher expression levels in the rats that experienced disrupted circadian rhythm compared with MCT-injected rats housed in a normal circadian rhythm cycle environment (P < 0.01, P < 0.05). This result indicated that circadian rhythm disruption aggravated the lipolysis process in WAT under conditions of HF-induced cachexia.

A previous study suggested that clock genes are involved in adipogenesis by directly and indirectly regulating transcription factors involved in lipogenesis processes[23]. In the present study, we found that the expression of the lipogenic genes Scd1, Fas, Mgat and Dgat2 decreased in HF-induced cachexia (Fig. 5M-P, P < 0.01). Circadian misalignment resulted in further decreased lipogenesis in white adipocytes, reflected by decreased mRNA expression of Scd1, Fas, Dgat2 and Mgat in rats in the DL group and decreased mRNA expression of Fas and Dgat2 in rats in the LV-Bmal1 shRNA group (P < 0.01). These results indicated that circadian rhythm disorder is associated with decreased lipid storage in white adipocytes under conditions of HF-induced cachexia.

Beiging of WAT is regarded as a driver of fat expenditure in cachexia[24]. As described previously in cancer-associated cachexia[25], we found that beige adipocyte markers, including the mRNA expression of Cd137, Tbx-1, Ucp-1 and Zic-1 (Fig. 5Q-T, P < 0.01) as well as the protein expression of UCP-1 (Fig. 5B,G, P < 0.01), were increased compared with those in rats without HF-induced cachexia. Circadian misalignment aggravated beiging of WAT in both the DL group and LV-Bmal1 shRNA group, as shown by the increased protein expression of UCP-1 and mRNA expression of Cd137, Tbx-1, Ucp-1 and Zic-1 in both the DL group and LV-Bmal1 shRNA group compared with the LD group (P < 0.01).

6. Circadian rhythm disruption promoted PKA-mediated thermogenesis in BAT in HF-induced cachexia

In brown adipocytes, activated PKA signalling initiates thermogenesis by upregulating thermogenesis genes, such as Pparγ and Pgc-1α to induce UPC-1 expression. During this process, FFAs derived from white adipocyte lipolysis are transported into brown adipocytes by transport proteins such as fatty acid transport protein (FATP) and CD36 and then transported to mitochondria via CPT-1 to serve as substrates for mitochondrial thermogenesis[20]. As described previously, BAT exhibits elevated thermogenesis in cancer-induced cachexia[26]. In our present study, we found that HF-induced cachexia exhibited elevated thermogenesis in brown adipocytes exhibiting increased protein levels of PKA, p-PKA and UCP-1 (Fig. 6A-E, P < 0.01, P < 0.05), although p-PKA had no significant difference. Meanwhile, thermogenesis genes (Ucp-1, Pparγ and Pgc-1α) and lipid transportation genes (Cd36) increased (Fig. 6F-H, I, P < 0.01). Compared with rats housed in a normal light: dark cycle, rats with a disrupted light: dark cycle exhibited
upregulated protein expression of PKA, p-PKA and UCP-1 (P < 0.01) and genes including Ucp-1, Pparγ, Pgc-1α, Cd36, Fatp-1 and Cpt-1 (P < 0.01). Similarly, suppressing the key clock gene Bmal1 also increased UCP-1 protein levels and upregulated thermogenesis genes (Ucp-1, Pparγ and Pgc-1α) and lipid transportation genes (Cd36, Fatp-1 and Cpt-1). Altogether, our study showed activated thermogenesis in BAT in conditions of HF-induced cachexia, contributing to enhanced adipose expenditure. More importantly, circadian rhythm misalignment aggravated thermogenesis in BAT.

7. Circadian rhythm disruption elevated factors contributing to fat depletion, including NT-proBNP, IL-6 and NE

Studies have suggested that increased NT-proBNP, IL-6 and NE contribute to the prevalence of cachexia[27–29]. In our study, we observed that rats treated with MCT in the LD group exhibited higher levels of NT-proBNP in blood (Fig. 7A, P < 0.01) and IL-6 and NE in adipose tissue (Fig. 7B-E, P < 0.01, P < 0.05). Disrupting the circadian rhythm by reversing the light: dark cycle or injecting LV-Bmal1 shRNA further elevates the blood levels of NT-proBNP and adipose tissue levels of IL-6 and NE. This result indicated that circadian rhythm dysregulation is associated with increased NT-proBNP, IL-6 and NE, which further aggravate fat depletion in HF-induced cachexia.

Discussion

Adipose tissue represents a critical component in healthy energy homeostasis. It plays important roles in the modulation of whole-body lipid storage and utilization. Adipose dysfunction is tightly linked to metabolic disorders, including obesity, metabolism and cachexia[30]. While numerous studies have addressed the causes and consequences of obesity-associated adipose dysfunction, the critical molecular mechanisms underlying adipose tissue expenditure in cachexia are far less understood. Here, our study explored the relationship between circadian rhythm dysregulation and adipose tissue expenditure in the context of cardiac cachexia. We first illustrated that a disrupted circadian clock resulted in more serious fat depletion in cardiac cachexia.

Recently, the majority of experimental in vivo studies on cachexia have relied on animal models of cancer cachexia. A reliable and appropriate model for cardiac cachexia has not yet been established. As described in a previous study, we induced cardiac cachexia via injection of MCT. Although MCT treatment is mainly a model of pulmonary hypertension[31], it is also considered an appropriate model for cardiac cachexia since MCT-induced pulmonary hypertension leads to progressive HF and cachexia very rapidly. MCT-treated animals usually exhibit marked weight loss and decreased food intake[18]. In our experiment, 4 weeks after a single MCT injection, we found decreased right ventricular and left ventricular function. This is consistent with a previous study, which showed that rats exhibited significant right ventricular dysfunction 4 weeks after the first MCT injection[32, 33]. Moreover, MCT-treated rats exhibited a significant decrease in body weight and reduced fat mass[33], which is consistent in our study, reflected by mitigated weight gain and decreased total adipose tissue weight compared with healthy rats in the control group.
Accumulating evidence has confirmed that the circadian clock modulates lipid metabolism and is closely related to metabolic diseases[11]. However, the relationship between the circadian clock and adipose tissue expenditure in cachexia has not yet been elucidated. A previous study performed using C26 tumour-induced cachexia mice showed reduced amplitudes in the mRNA expression of Rev-erba and Per2 and a parallel increase in Bmal1 and Cry1, which indicated alterations in core clock regulation during cancer cachexia[17]. Nevertheless, whether perturbation of the circadian clock further aggravates fat wasting was not investigated in this study. The transcription factor Bmal1 is regarded as a core component of the mammalian circadian clock, and animals lacking Bmal1 exhibit abolished circadian behaviours and altered metabolic features[34, 35]. In our present study, we first revealed decreased expression of Clock and Bmal1 and increased Rev-erba in both WAT and BAT in HF-induced cachexia, suggesting that an imbalanced Clock:Bmal1/Rev-erba circuit is involved in adipose tissue expenditure in cardiac cachexia.

Fat droplets can account for 95% of the total volume of white adipocytes and are mainly composed of TGs. In the lipolysis process, TGs stored within lipid droplets are hydrolysed into FFAs and released as fuel in peripheral tissues according to metabolic demand. In the hypermetabolism state of cachexia, excessive TGs are hydrolysed, causing not only adipocyte atrophy but also increased FFA ectopic deposition in peripheral tissue[20]. As described previously, fatty liver and elevated blood levels of FFAs were found in cachexia caused by cancer and severe burn injury[36]. Similarly, our results revealed that HF-induced cardiac cachexia resulted in increased ectopic lipid accumulation reflected by elevated blood levels of TG and FFA as well as excessive fat accumulation in the liver. Additionally, rats bearing HF-induced cachexia exhibited reduced adipocyte size and lipid contents in adipocytes, as reflected by HE staining and Oil Red O staining in both WAT and BAT. More interestingly, perturbation of the circadian clock further reduced adipocyte size and lipid contents in fat droplets and aggravated ectopic lipid deposition in the hypermetabolic state during the progression of cachexia.

In obesity, persistent inflammation induces an accumulation of extracellular matrix protein in adipose tissue, which limits the pliability and capacity of adipose tissue to remodel itself in response to a changing metabolic landscape[37]. Similarly, in cancer cachexia, fibrotic areas were observed in adipose tissue with overexpression of extracellular matrix elements[38]. We consistently observed enriched deposition of extracellular matrix in HF-induced cachexia; moreover, it seems that perturbation of the circadian clock markedly aggravated the fibrosis in WAT, since rats with a disrupted diurnal cycle or downregulated Bmal1 expression elicited pronounced accumulation of extracellular matrix compared with HF-induced cachexia rats with cachexia housed in a normal diurnal cycle. A recent study has already demonstrated that a disrupted circadian clock is involved in adipose tissue remodelling, which has shown that a chronic circadian shift leads to adipose tissue macrophage infiltration and extracellular matrix remodelling[39]. One of the most common mechanisms underlying fibrosis could be attributed to disrupted clock genes leading to macrophage-induced inflammatory signals, which further exacerbate extracellular matrix deposition in adipose tissue[40].
WAT stores energy, in the form of TGs, at times of high energy levels and then serves as fuel in times of need[10]. Stimulation of FFA release from TG was thought to be primarily mediated via PKA, which phosphorylates enzymes primarily responsible for hydrolysis, including perilipin and HSL, and indirectly influences ATGL[22]. FFAs produced by hydrolysis are transported into mitochondria for FFA β-oxidation under the control of Cpt-1 and acyl-CoA[41]. As key enzymes of lipolysis, HSL and ATGL play a critical role in the process of cancer cachexia. In patients with cachexia, the activities of HSL and ATGL are increased[42]. Moreover, in a murine model of cancer cachexia, depletion of ATGL and HSL was sufficient to protect against loss of WAT, with HSL knockout conferring less protection[42]. Adipocyte clocks regulate the lipolysis process by regulating the local transcription of lipolysis enzymes in a circadian manner. CLOCK:BMAL1 heterodimers controlled the transcription of ATGL and HSL. In both ClockΔ19 and Bmal1-/- circadian mutant mice, the circadian variations in ATGL and HSL were disrupted[16]. As shown in our study, PKA signalling-mediated lipid droplet hydrolysis was markedly stimulated in HF-induced cachexia. Remarkably, perturbation of the circadian clock markedly increased lipolysis and FFA β-oxidation in white adipocytes, as reflected by upregulated protein expression of PKA, p-PKA, ATGL and HSL and mRNA levels of Atgl, Hsl, Perlipin, Cpt1 and acyl-CoA. This result indicated that a disrupted circadian clock is an important contributor to promoting lipolysis in HF-induced cachexia.

Adipocytes robustly synthesize FFAs from carbohydrates through the de novo lipogenesis pathway, which contributes to TG storage in lipid droplets. DGAT2 is a rate-limiting enzyme that catalyses TG synthesis using diacylglycerol and fatty acyl CoA as substrates. FAS and SCD1 are key enzymes for de novo lipogenesis in adipocytes[43]. SCD1 interacts with DGAT2 by producing substrates (fatty acylCoA) for DGAT enzymes. MGAT is an enzyme that catalyses the synthesis of diacylglycerol and interacts with DGAT to promote TG synthesis[44]. Several studies have suggested a critical function of clock genes, especially BMAL1, in regulating lipid synthesis and storage in white adipocytes[15, 45, 46]. Depletion of Bmal1 caused a reduction in lipogenesis genes (Acc1, Fas, Scd1, and Gpat (glycerol-3-phosphate acetyltransferase)) in the liver. Conversely, Bmal1 overexpression was sufficient to elevate the mRNA expression of lipogenic enzymes[47]. Here, in our study, we focused on whether perturbed circadian rhythm influences lipogenesis capability in white adipocytes in cachexia. According to our study, we found that HF-induced cachexia demonstrated lower lipogenesis capability and decreased lipid storage in white adipocytes; furthermore, the phenomenon was exacerbated when the circadian rhythm was disrupted by altering the diurnal cycle and downregulating Bmal1 expression. Thus, we speculated that an imbalanced circadian clock interferes with lipogenesis and lipid storage in white adipocytes by suppressing lipogenesis genes, which is an unfavourable factor for lipid storage in cachexia.

Enlarged beiging of WAT was discovered in conditions of hypermetabolism, such as severe burn injury and cancer, which convert lipids stored in white adipocytes into heat via thermogenesis, resulting in increased fat wasting in cachexia. Recently, limited studies have reported beiging adipocytes in the WAT of HF animals[48, 49]. Additionally, in our study, we found beiging markers, including Ucp-1, Cd137, Tbx-1 and Zic-1, indicating increased beiging of WAT in HF-induced cachexia by MCT injection. This was consistent with our previous study using a salt-sensitive hypertension-induced cardiac cachexia rat study.
Moreover, our study showed, for the first time, that disrupted circadian rhythm aggravates beiging of WAT in HF-induced cachexia.

Brown adipose tissue is a metabolically active organ distinguished by its unique capacity for adaptive thermogenesis in response to cold and adrenergic stimuli[50]. The conversion of energy into heat in BAT is achieved by β-oxidation or through uncoupling of mitochondrial proton transport from energy production by UCP-1[51]. NE released from sympathetic nerve terminal active β3-adrenergic receptors in brown adipocytes results in G-protein controlled activation of the PKA pathway and further thermogenetic genes, including Ucp-1, Pparγ and Pgc-1α. At the same time, this pathway activates ATGL to produce FFAs, which are taken up into brown adipocytes via FFA binding and transport proteins such as CD36 and FATP-1 and then FFAs are transported into mitochondria through CPT, serving as substrates for mitochondrial thermogenesis[22]. Similar to WAT, the lipid metabolism and thermogenesis process of BAT is also modulated by the circadian clock[52]. A study performed by van den Berg et al.[53] revealed that BAT displayed a pronounced daily rhythm in FFA uptake, which was synchronized with the light/dark cycle and was highest upon waking. BMAL1 is required for normal lipid metabolism in brown fat. Mice lacking Bmal1 in brown adipocytes revealed increased lipid accumulation and larger lipid droplets accompanied by dysregulation of genes involved in lipid metabolism and adaptive thermogenesis[54]. Rev-erbα promotes brown adipogenesis and thermogenesis by upregulating UCP-1 expression[55, 56]. Nevertheless, the effects of the circadian clock on BAT in the context of cachexia are less clear. In tumour-induced cachexia, it has been suggested that perturbed diurnal expression patterns of lipid uptake/accumulation/utilization and thermogenesis pathways in brown adipocytes are likely linked to fat expenditure[26]. However, the direct causal relationship between the circadian clock and increased fat expenditure by BAT thermogenesis in cachexia has not been explored. As shown in our present study, we found that PKA signalling-mediated thermogenesis was increased in HF-induced cachexia, as reflected by the upregulated protein levels of PKA and UCP-1 and the mRNA levels of thermogenesis genes (Ucp-1, Pparγ and Pgc-1α) and FFA binding and transport genes (Cd36, Fatp-1 and Cpt-1), indicating increased thermogenesis in the BAT of HF-induced cachexia mice. Remarkably, inhibited expression of Bmal1 caused by disruption of the light:dark cycle and injection of LV-Bmal1 shRNA further promoted PKA signalling-mediated thermogenesis. Our results demonstrate, for the first time, that a disrupted circadian clock serves as a contributor to elevated thermogenesis in BAT, which produces an unfavourable effect in HF-induced cachexia.

Additionally, it is well known that sympathetic nervous system activation and inflammation are the two main upstream mechanisms participating in adipose tissue expenditure in cachexia[3]. Studies have shown that patients with chronic HF who developed cachexia showed higher levels of catecholamines[57]. Moreover, in severe burn injury and cancer, increased catecholamine is released from overactivated sympathetic nerves and stimulates beiging of WAT by acting on the β3-adrenergic receptor of white adipocytes[58, 59]. The circadian clock regulates rhythmic catecholamine synthesis and secretion. Thus, oscillations in catecholamine signals have the potential to influence various cellular targets expressing adrenergic receptors[60]. For example, prolonged day length decreased sympathetic input into BAT and reduced β3-adrenergic intracellular signalling, resulting in impaired BAT activity[61].
Additionally, HF is characterized by chronic inflammation activation. The proinflammatory cytokine IL-6 is a key inflammatory mediator that upregulates the expression of UCP-1, which mediates beiging/browning thermogenesis; moreover, IL-6 suppresses lipid synthesis and accelerates lipolysis in white adipocytes[62, 63]. The circadian clock controls inflammation and immune function by modulating the expression of chemokines and cytokines. Circadian clock dysregulation is associated with metabolic inflammation in adipose tissue[12]. The dark-light cycle increases the IL-6-mediated inflammatory reaction[64]. NT-proBNP is a clinical marker that reflects the severity of HF. Studies have demonstrated that NT-proBNP stimulates white adipocyte lipolysis and promotes beiging of white adipocytes, contributing to fat wasting in cachexia[27]. It has been reported that blood levels of NT-proBNP exhibit a diurnal rhythm, although the direct connection between the circadian clock and NT-proBNP has not been clearly determined[65]. As seen in our study, we showed that HF-induced cachexia exhibited elevated levels of NT-proBNP in blood and NE and IL-6 in adipose tissue. Furthermore, challenges with perturbed circadian rhythm resulted in higher levels of NT-proBNP in blood and NE and IL-6 in adipose tissue. Thus, we deduced that the disturbed circadian rhythm impairs diurnal variations in the synthesis and release of NT-proBNP, NE and IL-6, which contribute to cachexia fat wasting by increasing lipolysis while decreasing lipid storage and elevating beiging/brown adipose tissue thermogenesis. Further studies are needed to elucidate the other detailed mechanisms underlying the association between adipose tissue expenditure and the circadian clock.

Food intake can influence body weight and fat weight. However, pair feeding was used during this study to minimize the effect of food intake. Therefore, statistical analysis of food intake was not performed. Moreover, the activity factors of the rats were not recorded. Additionally, we did not observe gene and protein expression at different time points. Further study should observe the dynamic alteration of genes and proteins involved in lipid metabolism at different time points.

**Conclusion**

In conclusion, our present study first provided evidence that circadian rhythm dysregulation aggravated fat wasting in HF-induced cachexia by promoting lipolysis, decreasing lipid storage in WAT and elevating beiging/brown adipocyte thermogenesis. We deduced that one of the molecular mechanisms underlying these results is attributed to the circadian clock directly or indirectly regulating the local transcriptome in a circadian manner, including key enzymes of lipogenesis, lipolysis and thermogenesis in both white and brown adipocytes. Additionally, the circadian clock controls the synthesis and release of NT-proBNP, NE and IL-6, which promote adipocyte lipolysis and beiging/brown adipocyte thermogenesis and is another molecular mechanism underlying excessive adipose tissue wasting in conditions of HF-induced cachexia. From a clinical point of view, our study indicates that stabilizing adipose tissue rhythms may help to combat disrupted energy homeostasis and alleviate excessive adipose tissue expenditure in HF-induced cachexia.

**Abbreviations**
CLOCK circadian locomotor output cycles kaput

BMAL1 brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1

PER Period

CRY Cryo/chromosome

REV-ERB nuclear receptor reverse ERB

TGs triglycerides

ATGL adipose triglyceride lipase

MCT monocrotaline

RV-FAC right ventricular fractional area change

EDA end-diastolic area

ESA end-systolic area

WAT White adipose tissue

BAT brown adipose tissue

IL-6 interleukin-6

NE norepinephrine

Cpt1 carnitine palmitoyl transferase 1

Scd1 stearoyl-CoA desaturase 1

Fas fatty acid synthase

Mgat monoacylglycerol acyltransferase

Dgat2 diacylglycerol acyltransferase-2

Zic-1 Zinc finger protein 1

Fatp-1 Fatty Acid Transport Protein-1

Pparγ Peroxisome Proliferator-Activated Receptor Gamma

Pgc-1α proliferator-activated receptor-γ coactivator 1α
PKA protein kinase A
p-PKA phospho-PKA

Declarations

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Ethical Approval and consent to participate

The animal study was approved by the Experimental Animal Ethics Committee of Shandong University of Traditional Chinese Medicine (Licence No. 2021-30).

Consent for publication
No applicable

Competing interests
All of the authors declare that there are no conflicts of interest for this manuscript.

Authors’ contributions
Dufang Ma, Yong Wang designed the study and wrote the article; Dufang Ma and Yiwei Qu performed experimental study, data analysis and manuscript preparation; Tao Wu analyzed the data; Lu Cai contributed to the literature survey. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

References


Figures
**Figure 1**

*Figure 1*

**MCT injection induced decreased right and left ventricular function.** (A) Left ventricular ejection fraction (EF). (B) Representative images of echocardiography reflecting left ventricular function. (C) Left ventricular fractional shortening (FS). (D, F) Representative images of echocardiography reflecting right ventricular function. (E) Right ventricular fractional area change. ******P < 0.0001. n=6.**
Figure 2

Disrupted clock gene expression was observed in HF-induced cachexia. **(A-C)** Protein expression of BMAL1 and REV-ERBα measured by Western blot in WAT. **(D-F)** mRNA expression of Clock, Bmal1 and Rev-erba measured by qRT–PCR in WAT. **(G-I)** Protein expression of BMAL1 and REV-ERBα measured by Western blot in BAT. **(J-L)** mRNA expression of Clock, Bmal1 and Rev-erba measured by qRT–PCR in BAT. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n=6.
Figure 3

Circadian clock misalignment aggravated body weight loss and total fat mass and caused increased lipid ectopic deposition in HF-induced cachexia. (A) Changes in body weight through the duration of the 8-week study. (B) Representative image of epididymal fat volume. (C) Total adipose tissue weight. (D) Plasma FFA levels. (E) Plasma TG levels. (F, G) Representative images of Oil O staining and positive area of liver tissue. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n=6.
Figure 4

Circadian rhythm disruption exacerbated adipocyte atrophy and increased extracellular matrix in adipose tissue under conditions of HF-induced cachexia. (A, B) Representative images of HE staining and fat cell inner diameter of WAT. (C, D) Representative images of Oil O staining and positive area of WAT. (E, F) Representative images of picrosirius red staining and positive area of WAT. (G, H) Representative images of HE staining and fat cell inner diameter of BAT. (I, J) Representative images of Oil O staining and positive area of BAT. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n=3.
Figure 5

Circadian rhythm disruption exacerbated WAT consumption by promoting lipolysis and beiging of WAT and reducing lipid storage in HF-induced cachexia. (A, C-F) Protein expression of BMAL1, REV-ERBa, PKA and p-PKA measured by Western blot in WAT. (B, G) Immunostaining and positive areas of UCP-1 in WAT. (H-J) mRNA expression of Hsl, Atgl and Periplin in WAT. (K, L) mRNA expression of FFA β-oxidation genes, including Cpt1 and acyl-CoA. (M-P) mRNA expression of lipogenic genes, including Scd1, Fas, Mgat and
Dgat2, Q-T, mRNA expression of beige adipocyte markers, including Cd137, Tbx-1, Ucp-1 and Zic-1. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n=6.

Figure 6

Circadian rhythm disruption promoted PKA-mediated thermogenesis in BAT in HF-induced cachexia. (A-C) Protein expression of PKA and p-PKA measured by Western blot in BAT. (D, E) Immunostaining and
positive areas of UCP-1 in BAT. (F-H) mRNA expression of thermogenesis genes, including Ucp-1, Pparγ and Pgc-1α. (I-K) mRNA expression of lipid transportation genes, including Cd36, Fatp-1 and Cpt-1. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n=6.

Figure 7

Circadian rhythm disruption elevated factors contributing to fat depletion, including NT-proBNP, IL-6 and NE. (A) Plasma levels of NT-proBNP. (B) IL-6 content in WAT. (C) IL-6 content in BAT. (D) NE content in WAT. (E) NE content in BAT. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n=6.