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Black ginger (*Kaempferia parviflora*) extract enhances circadian rhythm and promotes lipolysis in mice fed a high-fat diet

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ABSTRACT

Circadian rhythms are endogenous oscillations that regulate physiological and biochemical processes with approximately 24-h rhythms. Circadian rhythmic disorders caused by a high-fat diet (HFD) are associated with metabolic syndrome and obesity. Herein, we assessed whether black ginger (*Kaempferia parviflora*) modulates disturbances in the circadian rhythm and improves obesity caused by an HFD in C57BL/6 mice. Three study groups were created: normal diet, HFD, and HFD + *K. parviflora* extract (KPE). HFD-fed mice showed attenuated circadian locomotor activity, weight gain, and increased serum triglyceride and cholesterol levels, whereas HFD mice administered KPE showed improved circadian locomotor activity and reduced body weight and serum triglyceride levels. Moreover, following RNAi knockdown of clock genes in 3T3-L1 adipocytes, KPE was found to enhance clock gene expression and induce lipolysis-related gene expression in adipocytes. Collectively, these results suggest that KPE improves rhythm disturbances in HFD-fed mice and exhibits anti-obesity effects.

1. Introduction

Circadian rhythms are endogenous oscillations of approximately 24-h periods that regulate diel changes during physiological and biochemical processes. In mammals, circadian rhythms are regulated by the central clock located in the suprachiasmatic nucleus (SCN) of the brain and the peripheral clocks in the individual cells of most tissues. The SCN circadian clock—the master circadian pacemaker—can be entrained by light and orchestrate the peripheral clock via humoral and neuronal signalling (Albrecht, 2012; Mohawk et al., 2012). Meanwhile, the peripheral clocks can be independently attuned by food, stress, and exercise (Oike et al., 2011; Stokkan et al., 2001; Tahara et al., 2017). Approximately 10% of the mammalian transcriptome is under circadian control, with numerous circadian-regulated genes participating in biosynthetic and metabolic processes, including cholesterol and lipid metabolism, as well as glycolysis and gluconeogenesis (Green et al., 2008; Panda et al., 2002). Moreover, disruption of circadian rhythms contributes to the development of metabolic syndrome, obesity, diabetes, autoimmunity, and cancer (Bass & Lazar, 2016; Froy, 2007; Green et al., 2008). The molecular mechanism regulating circadian clocks in mammals is produced by a transcriptional-translational feedback loop. More specifically, the transcription factor heterodimer BMAL1/CLOCK induces the expression of PER and CRY and, in turn, represses BMAL1 and CLOCK expression. Positive feedback from BMAL1/CLOCK and negative feedback from PER/CRY establish a feedback loop that produces rhythms within a period of approximately 24 h. The BMAL1/CLOCK and PER/CRY dimers then regulate the expression of the nuclear receptors, REV-ERBα and RORα, which form the second clock loop. That is, RORα and REV-ERBα competitively bind to ROR/REV-ERB-response element (RORE) in the BMAL1 promoter to increase or inhibit BMAL1 transcription, respectively (Albrecht, 2012; Mohawk et al., 2012; Schroeder & Colwell, 2013). Hence, REV-ERBs and RORs are crucial for lipid metabolism and exhibit striking circadian rhythms. Other nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs), are also involved in the circadian control of metabolism. In particular, PPARα and PPARγ expression are diurnally controlled and affect the expression of circadian clock genes. In addition, PPARα promotes mitochondrial fatty acid β-oxidation in the liver, while PPARγ plays a

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; HFD, high-fat diet; IBMX, 3-isobutyl-1-methylxanthine; KP, *Kaempferia parviflora*; KPE, *Kaempferia parviflora* extract; LD, Light/dark; NCS, newborn calf serum; ND, normal diet; PMF, polymethoxyflavone; SCN, suprachiasmatic nucleus.

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key role in fatty acid synthesis and storage in white adipose tissue (Froy, 2012; Gooley, 2016).

Mice fed a high-fat diet (HFD) *ad libitum* reportedly exhibit attenuated locomotor and feeding activities compared to mice fed a regular chow diet; these changes in behavioural rhythmicity correlate with disrupted clock gene expression. Moreover, disruption of circadian rhythms leads to obesity and metabolic disorders (Froy, 2012; Kohsaka et al., 2007; Paula et al., 2020). Indeed, *Clock* mutant mice have an attenuated diurnal feeding rhythm that leads to hyperphagia and obesity as well as development of metabolic syndromes, such as hyperleptinemia, hyperlipidaemia, hepatic steatosis, hyperglycaemia, and hypoinsulinemia (Turek et al., 2005; Williams & Schwartz, 2005). Meanwhile, timed HFD can lead to decreased body weight, cholesterol, and TNFα levels and improved insulin sensitivity compared with mice fed HFD *ad libitum*. In addition, a timed HFD improves the metabolic pathway function and oscillations of the circadian clock and target gene expression (Hatori et al., 2012; Sherman et al., 2012). Certain small molecules, including functional food factors, have been reported to directly affect circadian rhythms, making them potential candidates for reducing the disruption of circadian rhythms and improving clock-associated diseases (Z. Chen, Yoo, et al., 2018; Cheng et al., 2021; Gloston et al., 2017). Nutrients and food factors have been shown to modulate disrupted circadian rhythms caused by HFD in mice and ameliorate obesity and metabolic syndrome. For instance, resveratrol restores circadian rhythm disorders of lipid metabolism induced by HFD in mice. Resveratrol can modify the rhythmic expression of the clock and clock-controlled lipid metabolism-related genes (Sun et al., 2015). Meanwhile, apple polyphenol extract (APE) has been reported to improve the hepatic biological clock and lipid homeostasis in HFD-fed mice (Cui, Yin, Li, Xie, et al., 2022). Additionally, caffeine enhances the circadian rhythm of peripheral clocks, maintains low lipogenesis gene expression and serum lipid levels, and suppresses body weight gain induced by HFD in mice. Moreover, caffeine intake at the beginning of the active period effectively suppressed HFD-induced body weight gain (Haraguchi et al., 2022). Taken together, these reports suggest that obesity and metabolic syndrome caused by circadian rhythm disruption in HFD-fed mice can be ameliorated by regulating the rhythm with specific food ingredients.

Black ginger, the rhizome of *Kaempferia parviflora* (KP), is used in traditional Thai medicine to treat gout, asthma, allergies, gastrointestinal disorders, and diabetes. Several studies have demonstrated the biological activities of polymethoxyflavones (PMFs) in KP extract (KPE), including anti-oxidant, anti-inflammatory, anti-allergic, anti-tumour, cardioprotective, and anti-obesity properties (Chen, Li, et al., 2018; Horigome et al., 2017; Kobayashi et al., 2016; Tewtrakul & Subhadhirasakul, 2008). In a previous study, we found that PMFs of KP can enhance the amplitude of circadian clock gene expression and modulate circadian rhythms, thus, highlighting the potential of KP to accelerate re-entrainment, shift light/dark cycles, and improve circadian rhythm disruption caused by jet lag syndrome, including social jet lag and shift work disorders (Yoshida et al., 2020). In the current study, we investigated whether KPE exerts anti-obesity effects by improving rhythm disturbances caused by HFD in mice. Moreover, we have attempted to elucidate the relationship between the enhancement of clock gene expression by KPE and its anti-obesity effects using RNAi technology to knockdown clock genes in 3T3-L1 adipocytes.

2. Material and methods

2.1. Materials

Dried KP, of the red-leaf type variety, was obtained from the Yumeshima Tokashiki Association in Tokashiki Village (Okinawa, Japan). Mouse 3T3-L1 pre-adipocytes were obtained from the Health Science Resources Bank (Osaka, Japan). C57BL/6J mice were obtained from Japan SLC, Inc. (Shizuoka, Japan).

2.2. Extraction from KP

The dried KP rhizomes were pulverized and KP powder (100 g) was extracted with 1 L of 95% ethanol for 6 days at approximately 25 °C. After extraction, the ethanol layer was evaporated and a 95% ethanol extract of KP (KPE) was obtained (extraction yield: 6.8%). The major active polymethoxyflavones components obtained by fractionation of KPE were follows; 3,5,7,3′,4′-pentamethoxyflavone (13.4%), 5,7-dimethoxyflavone (12.5%), 5,7,4′-trimethoxyflavone (13.3%), 3,5,7-trimethoxyflavone (2.6%), 3,5,7,4′-tetramethoxyflavone (6.9%), 5-hydroxy-7-methoxyflavone (2.3%) and 5-hydroxy-7,4′-dimethoxyflavone (1.5%) (Yoshida et al., 2020).

2.3. Animals, behavioural analysis, and metabolic analysis

Eight-week-old male C57BL/6J mice were divided into three groups: normal diet (ND; CE-2, CLEA Japan, Tokyo, Japan), high-fat diet (HFD; 60 kcal% fat, HFD-60, Oriental Yeast CO., Ltd., Tokyo, Japan), or HFD supplemented with KPE (100 mg/kg/day; HFD + KPE). The mice were individually housed in cages equipped with running wheels under 12-h:12-h light/dark (L/D) conditions (light intensity in the light phase was 50 lx) and provided with food (ND, HFD, or HFD + KPE) and tap water *ad libitum*. Wheel running activities were monitored continuously and analysed using the ClockLab system (Actimetrics, Evanston, IL, USA). After 12 weeks of the locomotor activity test, mice were euthanized by cardiac blood sampling under anaesthesia with pentobarbital (25–35 mg/kg, ip) at ZT 5, and the liver, visceral fat, and blood were obtained. Serum triglyceride (TG) and total cholesterol (TC) levels were measured using commercial kits (E-test Wako, FUJIFILM Wako Chemicals, Tokyo, Japan). The liver and visceral fat were frozen in liquid nitrogen and stored at −80 °C till further use.

The animals were treated according to the guidelines for animal experiments established by the Japanese Association for Laboratory Animal Science. The experimental protocol was approved by the Animal Care and Use Committee of the Japan Food Research Laboratories (JFRL-ST4A-2015-4).

2.4. Cell culture and adipocyte differentiation

The 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% newborn calf serum (NCS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C under 5% CO_2. The 3T3-L1 cells were seeded in a 24-well plate (2.5×10^4 cells/well) and maintained in DMEM supplemented with 10% NCS until they reached confluence. After 4 days of incubation, cell differentiation was induced by replacing DMEM with 10% foetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) containing IBMX, dexamethasone, and insulin (Adipogenesis Assay Kit; Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. After the cells were maintained in the differentiation medium for 3 days, the medium was replaced with DMEM supplemented with 10% FBS for an additional four days.

2.5. siRNA transfection

Per2 siRNA (s211368, Ambion-Life Technologies, Carlsbad, CA, USA) and *Bmal1* siRNA (s62621, Ambion-Life Technologies) were introduced into cells by reverse transfection with Lipofectamine RNAiMAX (Invitrogen Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. 3T3-L1 adipocytes were transfected with siRNAs on Day 2 or 6 of differentiation. After 24 h of incubation, the sample was added to siRNA-transfected cells. The cells were further incubated for 24 h before assessing the knockdown efficiency and target gene abundance. Cells transfected with negative control siRNA (D-001810-10-05; Dhamacon, Lafayette, CO, USA) were used as controls.

2.6. RNA extraction and real-time quantitative reverse transcription (qRT)-PCR

Total RNA was extracted from the mouse liver and 3T3-L1 cells using the RNeasy Lipid Tissue Mini Kit and RNeasy Mini Kit (Qiagen, NV, Netherlands), respectively, according to the manufacturer's instructions. cDNA was synthesized using random primers and PrimeScript Reverse Transcriptase (Takara Bio, Shiga, Japan). An aliquot of cDNA was used as a template for qRT-PCR using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The target cDNAs were amplified with Fast SYBR Green Master Mix (Applied Biosystems) together with the following gene-specific primers: *Per2* (MA122369, Takara Bio), *Cry1* (MA127578, Takara Bio), *Bmal1* (MA132390, Takara Bio), *Clock* (MA101095, Takara Bio), *Dec1* (MA122909, Takara Bio), *Dbp* (MA105148, Takara Bio), *Rev-erbα* (MA041774, Takara Bio), *PPARα* (MA029805, Takara Bio), *PPARγ* (MA029808, Takara Bio), *Lpl* (MA120682, Takara Bio), *Hsl* (MA062172, Takara Bio), *Acox1* (MA061629, Takara Bio), *Cpt1a* (MA031170, Takara Bio), *Cebpa* (MA024950, Takara Bio), *Cd36* (MA157745, Takara Bio), *Ap2* (MA117397, Takara Bio), *Atgl* (forward: 5′-CAACGCCACTCACATCTACGG-3′; reverse: 5′-TCACCAGGTTGAAGGAGGGAT-3′), *Fasn* (forward: 5′- GTCACCACAGCCTGGACCGC-3′; reverse: 5′-CTCGCCATAGGTGCCGCCTG-3), *Dgat1* (forward: 5′-TCCGTCCAGGGTGGTAGTG-3′; reverse: 5′-TGAACAAAGAATCTTGCAGACGA-3′), *Hmgcr* (forward: 5′-ATGGCTGGGAGCATAGGCGG-3′; reverse: 5′-CTGCATCCTGGCCACATGCG-3′), *Cyp7a1* (forward: 5′- ACAGAGTGCTGGCCAAGAGCTC-3′; reverse: 5′-GATGCACTGGAGAGCCGCAGA-3′) and *36B4* (MA167177, Takara Bio). The relative expression level of each mRNA was normalized to that of the housekeeping gene *36B4*.

2.7. Statistical analysis

Data were analysed using one-way ANOVA, followed by Tukey's test or Dunnett's test, and an unpaired *t*-test with Welch's correction using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A $P < 0.05$ was considered statistically significant.

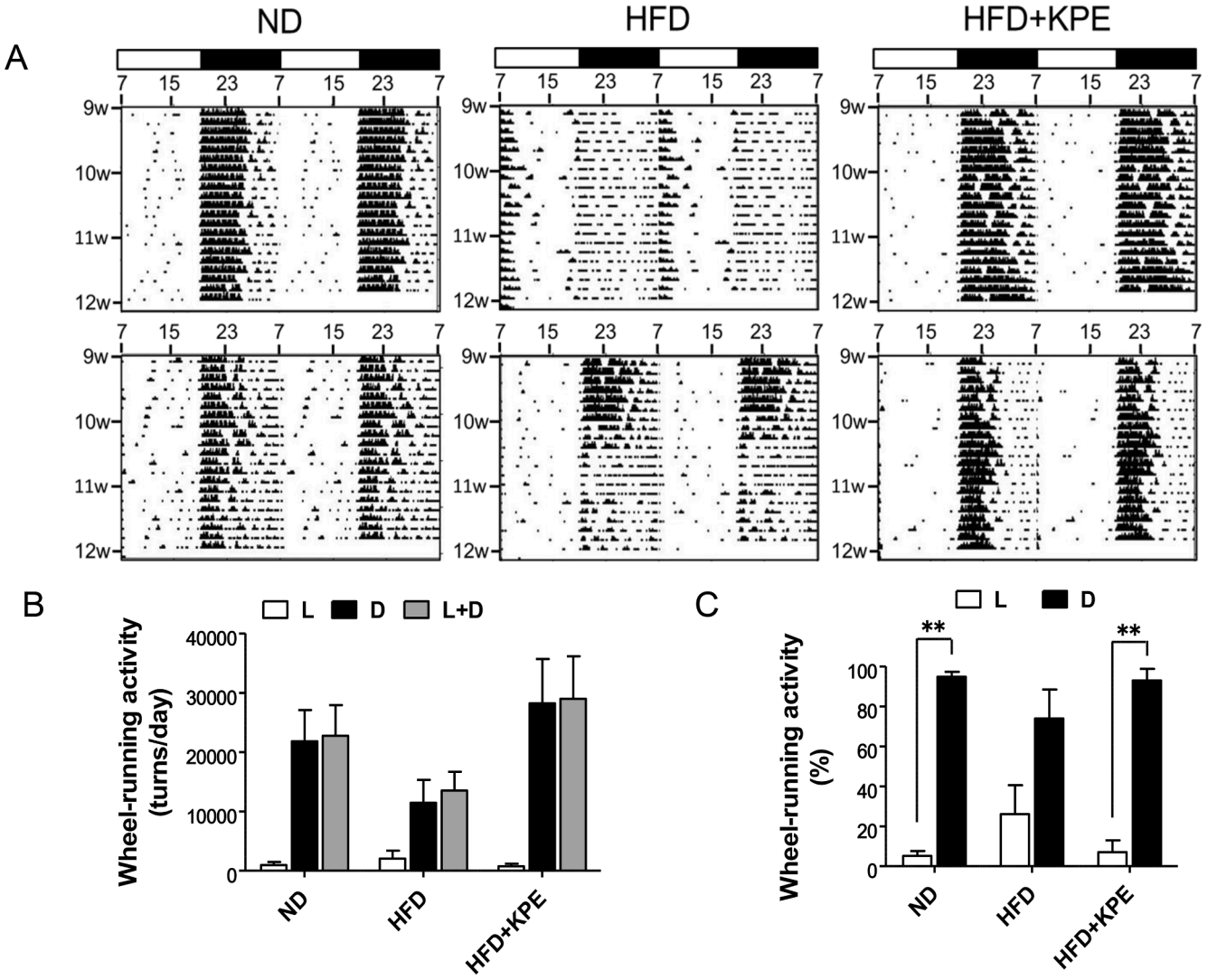

Fig. 1. Effects of *Kaempferia parviflora* extract (KPE) on locomotor activity rhythms in high-fat diet (HFD)-fed mice (A) Double-plotted actograms for 9–12 weeks of two representative mice in normal diet (ND)-, HFD-, and HFD + KPE-fed grouos. White and black bars indicate light and dark cycles, respectively. (B) Daily wheel-running activities in the light period (L), the dark period (D) and the light and dark period combined (L + D); values were calculated from 9- to 12-week actograms of ND-, HFD- and HFD + KPE- fed mice, and are expressed as the mean + SEM ($n = 5$). (C) Diurnal rhythm of wheel-running activity in ND-, HFD-, and HFD + KPE-fed mice. Values are expressed in the light period (L) or the dark period (D) as a percentage of total 24-hour activity and are expressed as the mean + SEM ($n = 5$). Significantly differences between the L and D groups were analysed using an unpaired *t*-test with Welch's correction (**$P < 0.01$).

3. Results

3.1. Effects of KPE on disturbed circadian rhythm in HFD-fed mice

To investigate the effects of KPE on circadian rhythmic disorders caused by HFD, the locomotor activity rhythms of mice fed ND, HFD, or HFD + KPE under 12-h:12-h L/D conditions were measured individually for 12 weeks. The actograms of mice fed the ND, HFD, and HFD + KPE from 9 to 12 weeks are shown in Fig. 1A. An HFD attenuated the diurnal rhythm of wheel-running activity compared to ND, whereas HFD + KPE restored the weakened rhythm to approximately the ND level. Daily wheel-running activities during the light (L), dark (D), and light and dark (L + D) periods are shown individually in ND-, HFD-, and HFD + KPE-fed mice (Fig. 1B). The diurnal rhythm of wheel-running activity weakened with HFD, however, was restored to the ND level by the addition of KPE to the HFD ($P < 0.01$) (Fig. 1C).

3.2. Effects of KPE on body weight, visceral fat, and serum lipid profile in HFD-fed mice

Eight-week-old mice were fed ND, HFD, or HFD + KPE for 12 weeks. The body weights of mice in the three groups are shown in Fig. 2A. After 4 weeks, HFD-fed mice gained more body weight than ND-fed mice, whereas KPE suppressed the HFD-induced body weight increase, even though HFD- and HFD + KPE-fed mice consumed the same amount of energy over the 12-week study period (Fig. 2B). After 12 weeks of feeding, we investigated the amount of visceral fat, serum TG, and TC levels. Visceral fat content was significantly higher in HFD-fed mice than in ND-fed mice ($P < 0.01$). This increase was suppressed by the addition of KPE to a level similar to that in ND-fed mice ($P < 0.05$) (Fig. 3A). Similarly, the increase in serum TG levels due to HFD feeding was suppressed by KPE ($P < 0.05$); however, its suppressive effect on serum TC was not significant (Fig. 3B, C).

3.3. Effects of KPE on the expression of clock genes, lipolysis-related genes, and lipogenesis-related genes in livers of HFD-fed mice

To investigate the effects of KPE on lipid metabolism in obese mice, we examined the expression of circadian clock genes and lipid metabolism-related genes in the livers of ND-, HFD-, and HFD + KPE-fed mice. Livers were obtained from mice at ZT5 under 12-h:12-h L/D conditions for 12 weeks. The expression of *Per2*, *Cry1*, *Dec1,* and *Dbp* at ZT5—the trough of the circadian rhythm—was higher in HFD-fed mice than in ND-fed mice (*Per2*; $P < 0.01$, *Dec1*; $P < 0.05$), however, the expression was reduced by the addition of KPE (*Dec1*; $P < 0.05$). In contrast, the expression of *Bmal1* and *Clock1*, which have phases opposite to those of *Per2* and *Cry1*, was attenuated in the HFD-fed mice and increased with the addition of KPE (Fig. 4A). The expression of *PPARα* and *PPARγ*—transcription factors involved in lipid metabolism—was upregulated in HFD-fed mice compared to ND-fed mice (*PPARα*; $P < 0.01$, *PPARγ*; $P < 0.05$), however, the expression was downregulated by the addition of KPE (*PPARα*; $P < 0.05$, *PPARγ*; $P < 0.05$) (Fig. 4B).

Moreover, the expression of lipolysis-, lipogenesis-, and cholesterol metabolism-related genes in the livers of mice fed the ND, HFD, and HFD + KPE was investigated. The expression of *Atgl* and *Lpl* tended to be attenuated in HFD-fed mice compared to ND-fed mice and was restored in HFD + KPE-fed mice. *Hsl* expression also increased with the addition of KPE to HFD (Fig. 4C). Meanwhile, lipogenesis-related gene expression was upregulated in the HFD-fed mice compared with the ND-fed mice (*Fasn*; $P < 0.01$, *C/EBPα*; $P < 0.05$). This HFD-induced increase was suppressed by the addition of KPE (*Fasn*; $P < 0.05$) (Fig. 4D). The expression of *Hmgcr*—rate-limiting enzyme for cholesterol biosynthesis—was higher in HFD-fed mice than in ND-fed mice ($P < 0.05$). However, *Hmgcr* expression was not affected by the addition of KPE to HFD. The expression of *Cyp7a1—a* rate-limiting enzyme in the conversion of cholesterol to bile acids—increased with the addition of KPE to the HFD (Fig. 4E).

3.4. Relationship between clock genes and lipid metabolism in 3T3-L1 adipocytes

The effects of KPE on lipid accumulation were investigated in 3T3-L1 cells. Although KPE did not suppress lipid accumulation during 3T3-L1 differentiation into adipocytes (early stage), it did restrict lipid accumulation (middle and late stages; Supplementary Fig. 1). The middle stage represents the period after adipocyte differentiation, while the late stage is the process of adipocyte hypertrophy from differentiated small adipocytes. To investigate whether a relationship exists between the enhancement of clock gene expression by KPE and suppression of fat accumulation in the middle or late stages, we knocked down the clock genes, *Per2* and *Bmal1,* on Day 2 or Day 6 of differentiation in 3T3-L1 cells using the RNAi technique.

We transfected *Per2* and *Bmal1* siRNAs into 2-day-differentiated 3T3-L1 cells resulting in a substantial decrease in *Per2, Bmal1,* and *Rev-erbα* expression (*Per2*; $P < 0.05$, *Bmal1*; $P < 0.01$, *Rev-erbα*; $P < 0.01$)

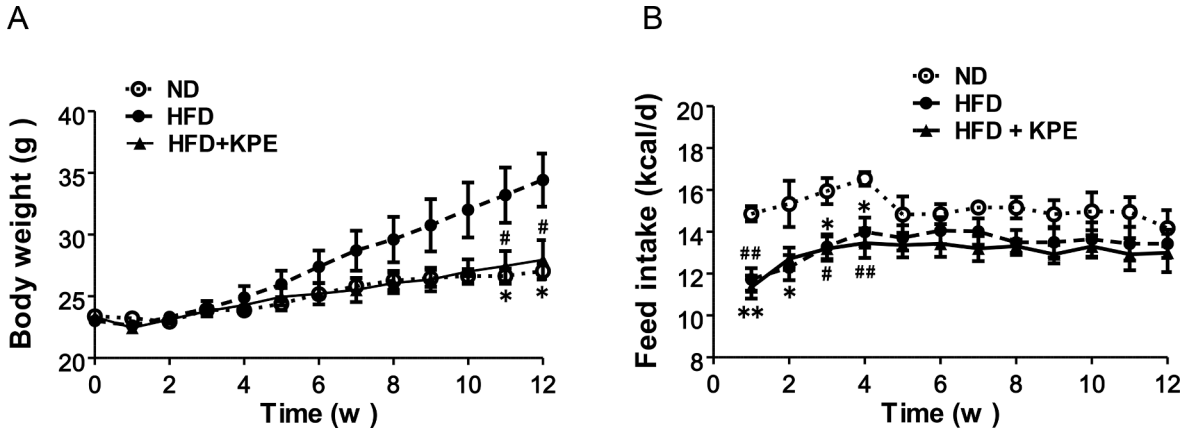

Fig. 2. Effects of *Kaempferia parviflora* extract (KPE) on body weight and feed intake in high-fat diet (HFD)-fed mice. (A) Body weight progression from the first day of feeding to the 12th week of feeding in ND-, HFD- and HFD + KPE- fed mice. Values are expressed as the mean ± SEM ($n = 5$). Significance was determined using one-way ANOVA, followed by Tukey's test. Differences between HFD and ND ($^{*}P < 0.05$); Differences between HFD and HFD + KPE ($^{\#}P < 0.05$). (B) Feed intake progression from the first week of feeding to the 12th week of feeding in ND-, HFD- and HFD + KPE- fed mice. Values are expressed as the mean ± SEM ($n = 5$). Significance was determined using one-way ANOVA, followed by Tukey's test. Differences between ND and HFD ($^{*}P < 0.05$, $^{**}P < 0.01$); Differences between ND and HFD + KPE ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

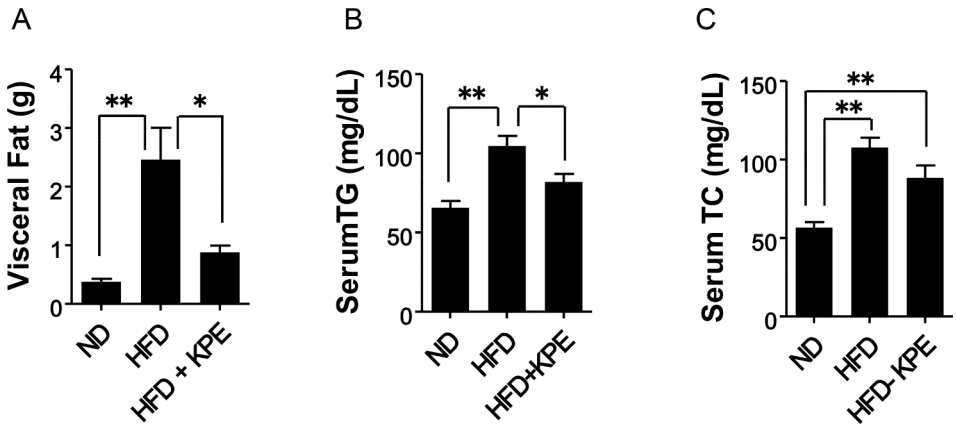

Fig. 3. Effects of *Kaempferia parviflora* extract (KPE) on visceral fat, serum triglyceride and total cholesterol in high-fat diet (HFD)-fed mice. (A) Visceral fat weight, (B) serum triglyceride (TG) level and (C) serum total cholesterol (TC) level in mice fed normal diet (ND), HFD-, and HFD + KPE for 12 weeks. Values are expressed as the mean + SEM ($n = 5$). Significance was determined using one-way ANOVA with Tukey's test (*$P < 0.05$ and **$P < 0.01$).

(Fig. 5A). KPE did not increase the expression of lipogenesis-related genes (Fig. 5B); however, it did increase the expression of the lipolysis-related genes, *PPARα* and *Atgl* (*PPARα*; $P < 0.05$, *Atgl*; $P < 0.05$) (Fig. 5C). Meanwhile, knocking down the clock genes prevented the KPE-induced enhancement of *PPARα* and *Atgl* expression (*PPARα*; $P < 0.05$, *Atgl*; $P < 0.05$).

Next, we transfected *Per2* and *Bmal1* siRNAs into 6-day-differentiated 3T3-L1 cells and confirmed the significant downregulation of *Per2*, *Bmal1* and *Rev-erbα* expression (*Per2*; $P < 0.01$, *Bmal1*; $P < 0.01$, *Rev-erbα*; $P < 0.01$) (Fig. 6A). KPE increased the expression of *PPARα*, *Atgl* and *Acox1* (*PPARα*; $P < 0.05$, *Atgl*; $P < 0.05$, *Acox1*; $P < 0.05$), however, this effect was lost following clock gene knockdown (*PPARα*; $P < 0.05$, *Atgl*; $P < 0.05$, *Acox1*; $P < 0.05$) (Fig. 6B).

4. Discussion

The circadian clock regulates the activities of enzymes involved in cholesterol, amino acid, lipid, glycogen, and glucose metabolisms. In addition, many hormones involved in metabolisms such as insulin, glucagon, adiponectin, corticosterone, leptin, and ghrelin exhibit circadian oscillations. Disruption of circadian rhythms is involved in the development of metabolic syndromes, obesity, diabetes, autoimmunity, and cancer (Bass & Lazar, 2016; Froy, 2007; Green et al., 2008). HFD-fed mice have been shown to exhibit attenuated diurnal rhythms of locomotor activity and a higher percentage of total activity during light periods. Changes in these behavioural rhythms are associated with disrupted clock gene expression in the hypothalamus, liver, and adipose tissues, as well as with hormonal cycles and lipid and carbohydrate metabolism (Froy, 2012; Kohsaka et al., 2007; Paula et al., 2020). In addition, HFD-fed mice exhibit an increase in the hepatic expression of PPARα and PPARγ, clock-interacting nuclear receptors, during the light period, corresponding with increased locomotor activity and feeding during this period (Kohsaka et al., 2007). They also reported that the expression of the downstream lipogenic genes *Fasn* and *Acc* increased during the light period in the livers of HFD-fed mice compared to that in ND-fed mice.

In the current study, we showed that KPE administration improved the circadian rhythm of wheel-running activity, which was attenuated in HFD-fed mice, reducing the proportion of higher activity during the light period compared to that of ND-fed mice. These changes in behavioural rhythms, which are associated with clock gene expression in various tissues, might improve the disrupted hormonal cycles, as well as lipid and carbohydrate metabolism caused by HFD. Additionally, we reported that KPE administration decreased the hepatic expression of *PPARα*, *PPARγ*, and *Fasn*, which had been increased by HFD at ZT5. Hence, KPE might ameliorate metabolic abnormalities in HFD-fed mice. Similar results were reported for nobiletin (NOB), a major PMF in citrus peels, which greatly increases wheel-running activity in HFD-fed mice relative to the control treatment, consistent with its effect on body weight (He et al., 2016). Moreover, serum and liver TG and TC levels were significantly reduced by NOB. In contrast, NOB did not improve lipid homeostasis in *Clock* mutant mice. These results indicate the *Clock*-dependent efficacy of NOB against metabolic syndrome. In addition, they identified ROR nuclear receptors as the molecular targets of NOB, which enhances circadian clock gene transcription in the core CLOCK: BMAL1 transcriptional feedback loop; enhanced circadian amplitude ameliorates metabolic disorders. Meanwhile, we previously identified 3,5,7,3′,4′-pentamethoxyflavone, 5,7,4′-trimethoxyflavone, and 5,7-dimethoxyflavone as active components in KPE that enhance the expression of circadian clock genes (Yoshida et al., 2020). Hence, the PMFs in KPE may exhibit anti-obesity effects by improving rhythm disturbances caused by HFD, in the same manner as NOB. However, whether the enhancement of clock gene expression by KPE is directly related to its anti-obesity effects remains unclear.

KPE and its active components, 3,5,7,3′,4′-pentamethoxyflavone and 5,7,4′-trimethoxyflavone, up-regulate the gene expression levels of adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) and enhance lipolysis in mature 3T3-L1 adipocytes, thus preventing adipocyte hypertrophy (Okabe et al., 2014). Moreover, KPE and its components, 3,5,7,4′-tetramethoxyflavone and 3,5,7,3′,4′-pentamethoxyflavone, strongly enhance the expression of PPARγ, C/EBPβ and C/EBPδ, while inducing differentiation of 3T3-L1 preadipocytes to adipocytes. The PMFs in KPE also increase adiponectin mRNA levels and the release of adiponectin into the 3T3-L1 medium. Thus, the functions of KP and PMFs, which enhance adipogenesis and secretion of adiponectin, are involved in the mechanisms of anti-metabolic disorder effects (Horikawa et al., 2012). Recent studies have shown that KPE and its component PMFs activate browning in white adipocytes and lipolysis of white adipocytes, which may effectively treat obesity-related dysfunction of lipid metabolism (Huang et al., 2022; Toda et al., 2016).

Lipolysis and the release of free fatty acids and glycerol in the adipose tissue have diurnal rhythms that are altered in *Clock* mutant mice, along with decreased lipolysis and increased adiposity (Onder & Green, 2018; Shostak et al., 2013). In this study, we investigated the relationship between the enhancement of clock gene expression by KPE and its anti-obesity effects using 3T3-L1 adipocytes in which clock genes were knocked down. Consequently, we demonstrated for the first time that the anti-obesity effect of KPE-induced lipolysis is directly regulated by clock genes.

The circadian rhythm can be altered by inputs from external zeitgebers, such as feeding, while modifications in the biological clock might lead to metabolic changes and subsequent alterations in cell-

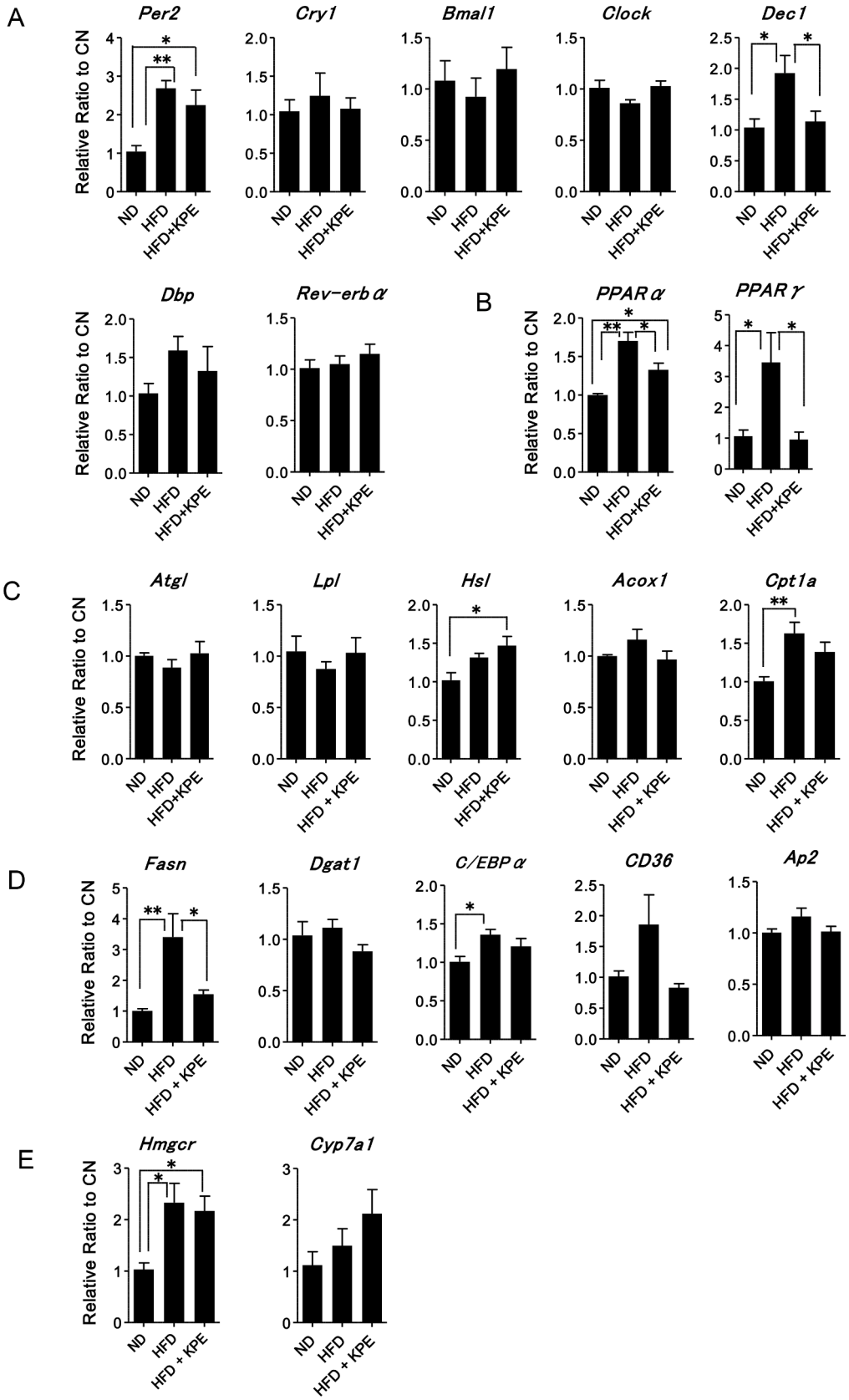

Fig. 4. Effects of *Kaempferia parviflora* extract (KPE) on the expression of circadian clock genes and lipid metabolism-related genes in the liver of high-fat diet (HFD)-fed mice. Expression of (A) circadian clock genes, (B) transcription factors involved in lipid metabolism, (C) lipolysis-related genes, (D) lipogenesis-related genes and (E) cholesterol metabolism-related genes in the liver of mice fed normal diet (ND), HFD, and HFD + KPE. The livers were obtained from the mice at ZT5 after 12 weeks of the locomoter activity tests under a 12-h:12-h L/D condition. Values are expressed as the mean + SEM ($n = 5$). Significance was determined using one-way ANOVA with Tukey's test (*$P < 0.05$, **$P < 0.01$).

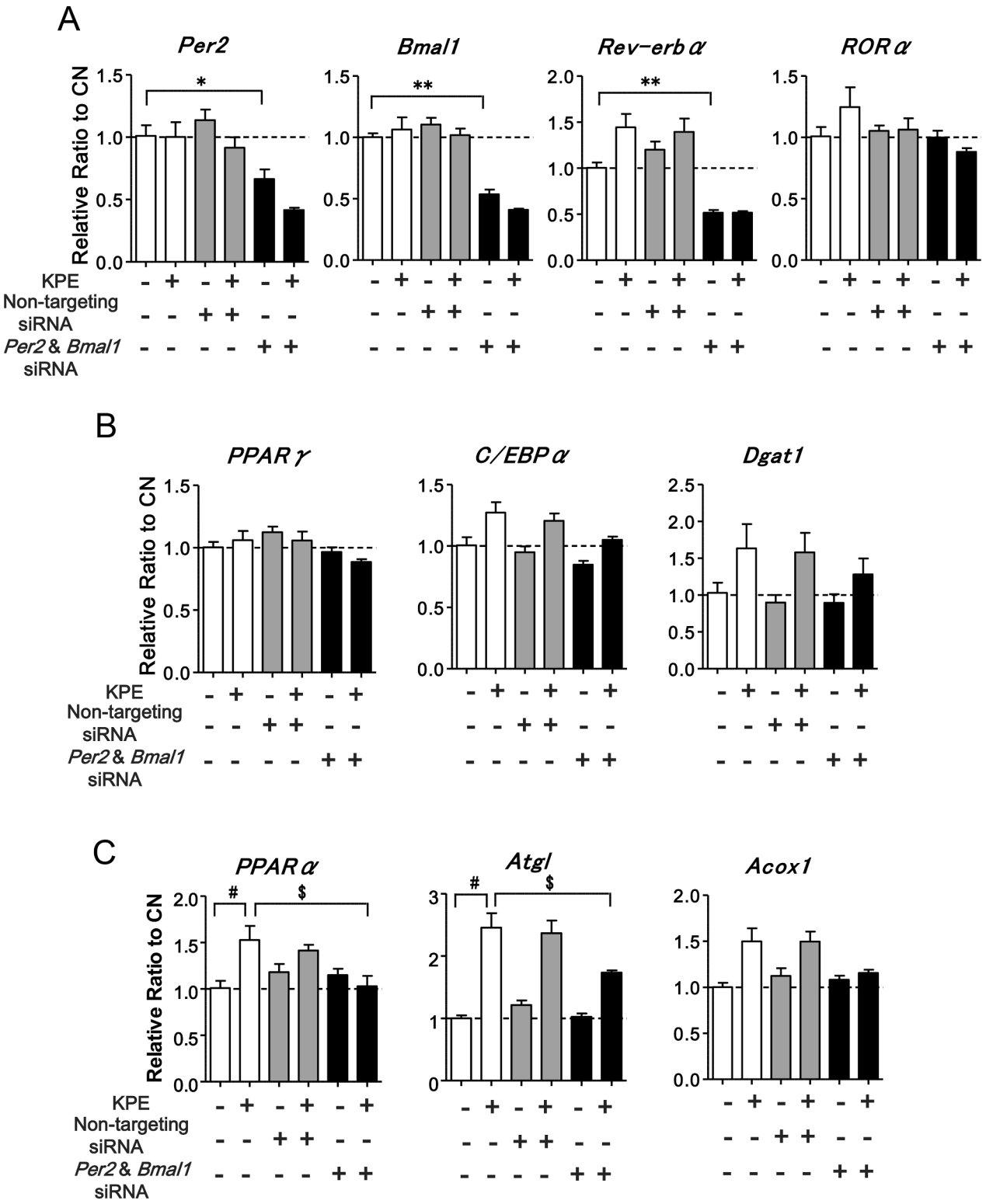

Fig. 5. Effects of siRNA-mediated double knockdown of *Per2* and *Bmal1* on the expression of lipogenesis- and lipolysis-related genes following *Kaempferia parviflora* extract (KPE) treatment in differentiated 3T3-L1 cells. *Per2* and *Bmal1* siRNAs were transfected in the 2-day-differentiated 3T3-L1 cells. After 24 h incubation, the sample was added to the siRNA-transfected cells. Cells were further incubated for 24 h and total RNA was extracted. Expression of (A) circadian clock genes, (B) lipogenesis-related genes (C) lipolysis-related genes in 3T3-L1 adipocytes. Values are expressed as the mean ± SEM ($n = 3$). Significantly differences between KPE(−) non-targeting siRNA(−)*Per2* & *Bmal* siRNA(−) and KPE(−)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(+) were analysed using an unpaired *t*-test with Welch's correction ($^{*}P < 0.05$, $^{**}P < 0.01$). Significantly differences between KPE(−)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(−) and KPE(+)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(−) were analysed using an unpaired *t*-test with Welch's correction ($^{\#}P < 0.05$). Significantly differences between KPE(+)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(−), KPE(+)non-targeting siRNA(+)*Per2* & *Bmal* siRNA(−) and KPE(+)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(+) were analysed using one-way ANOVA, followed by Dunnett's test ($^{\$}P < 0.05$).

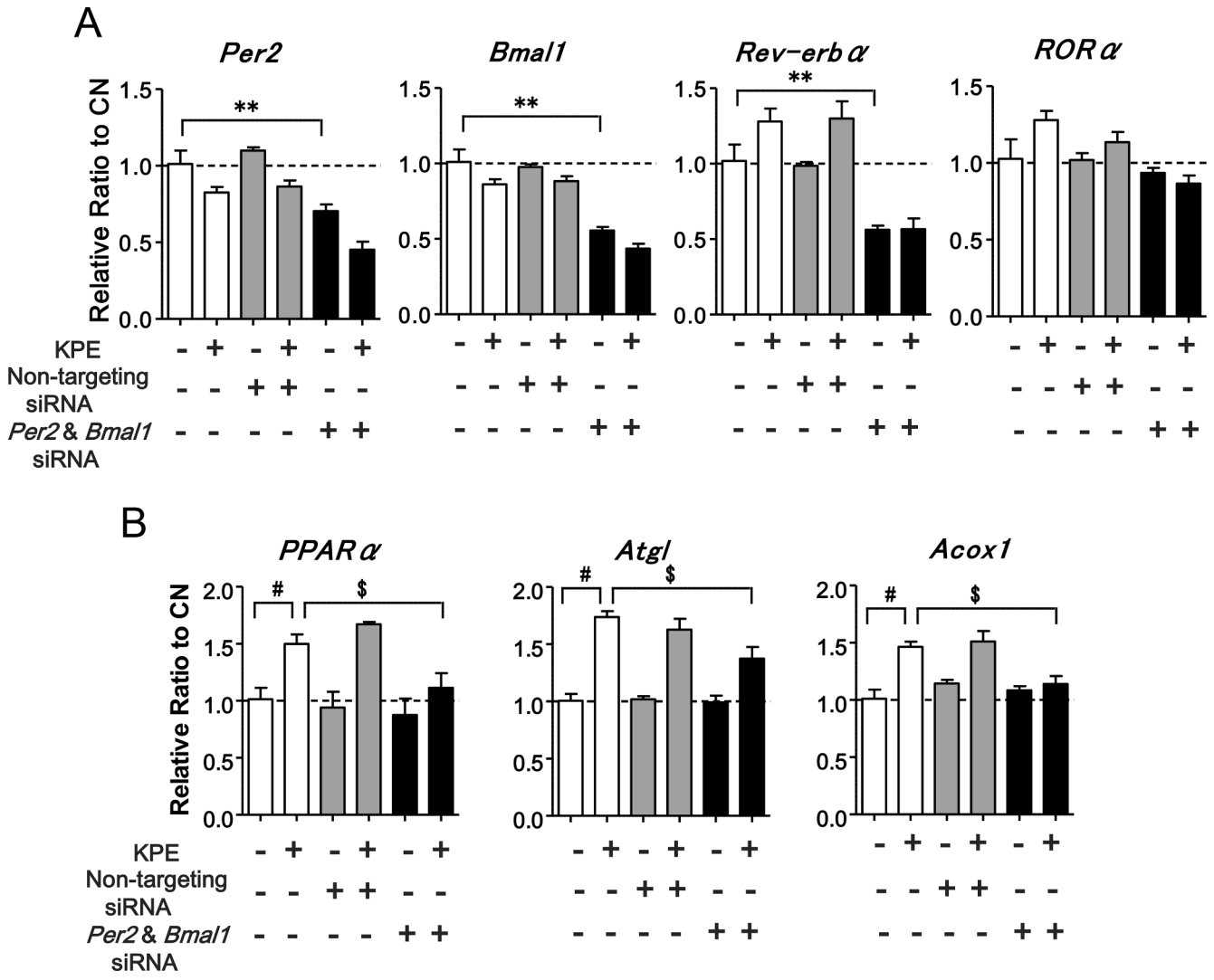

Fig. 6. Effects of siRNA-mediated double knockdown of *Per2* and *Bmal1* on the expression of lipolysis-related genes by *Kaempferia parviflora* extract (KPE) in differentiated 3T3-L1 cells. *Per2* and *Bmal1* siRNAs were transfected in 6-day-differentiated 3T3-L1 cells. After 24 h incubation, samples were added to the siRNA-transfected cells. Cells were further incubated for 24 h and total RNA was extracted. Expression of (A) circadian clock genes, (B) lipolysis-related genes in 3T3-L1 adipocyte were examined. Values are expressed as the mean ± SEM ($n = 3$). Significantly differences between KPE(−)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(−) and KPE(−)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(+) were analysed using an unpaired *t*-test with Welch's correction ($^{**}P < 0.01$). Significantly differences between KPE(−)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(−) and KPE(+)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(−) were analysed using an unpaired *t*-test with Welch's correction ($^{\#}P < 0.05$). Significantly differences between KPE(+)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(−), KPE(+)non-targeting siRNA(+)*Per2* & *Bmal* siRNA(−) and and KPE(+)non-targeting siRNA(−)*Per2* & *Bmal* siRNA(+) were analysed using one-way ANOVA, followed by Dunnett's test ($^{\$}P < 0.05$).

autonomous peripheral clocks. These peripheral clocks provide feedback to the central clock of the SCN, thereby forming a feedback loop. Hence, feeding serves as an important signal for adjusting the central and peripheral clocks (Onder & Green, 2018; Paula et al., 2020). An HFD influences the biological clock, and the consequences of increased body fat from such diets translate into factors associated with chrononodisruption that alter homeostasis and health. Recently, HFDs were found to impact different metabolites and metabolic pathways in the SCN (Tognini et al., 2020). The circadian reorganization of metabolic pathways in the SCN is accompanied by corresponding changes in gene expression in the master clock.

Previously, we confirmed that PMF is absorbed into the liver and brain of mice (Yoshida et al., 2020). We indicated one possibility that PMFs in the KP modulate the peripheral clock in the liver or other organs and subsequently synchronize the SCN oscillator via hormonal signals or neural connections. Moreover, we indicated the other possibility that PMFs directly entrain the SCN oscillator and synchronize the peripheral clocks. Accordingly, in the current study, we hypothesized that the application of PMFs might improve the disrupted circadian rhythm caused by HFD by acting on the central or peripheral clocks.

Recently, the gut microbiota has been shown to exhibit diurnal oscillations driven primarily by the food intake rhythms of the host organism, leading to rhythmic composition and functional profiles of the intestinal bacteria. Moreover, the gut microbiome and daily feeding/fasting cycles influence host metabolism and contribute to obesity and metabolic diseases (Broussard & Devkota, 2016; Gutierrez Lopez et al., 2021; Zarrinpar et al., 2014). Indeed, the diurnal variation in gut microbes in HFD-fed mice affects the host circadian clock function. An HFD induces changes in bacterial composition and circadian oscillations as well as in metabolites produced by the gut bacteria. These disruptions in microbial metabolite oscillations are associated with host circadian rhythms and metabolism (Leone et al., 2015). The authors proposed that bidirectional communication between the gut microbiota and host clock might contribute to diet-induced obesity. Meanwhile, APE—a clock-regulating natural compound—can modulate bile acid (BA) metabolism and gut microbiota, thereby protecting against circadian

disruption in HFD-fed mice (Cui, Yin, Li, Wu, et al., 2022). Similarly, NOB has been shown to alter the abundance of microbial taxa that modulate BA production and improve cholesterol and BA homeostasis in HFD-fed mice in clock-dependent manner (Nohara et al., 2019). Hence, KPE might also modulate circadian rhythms to improve BA metabolism and gut microbiota composition and exhibit anti-obesity effects similar to those of APE and NOB. However, further investigation is required to verify this hypothesis.

5. Conclusion

In this study, we demonstrated for the first time that the mechanism underlying the anti-obesity action of KPE involves the promotion of clock gene expression. We found that KPE enhances the amplitude of circadian clock gene expression in attenuated circadian rhythms in HFD-fed mice and induces the expression of lipolysis-related genes, which are controlled by circadian clock genes, in adipocytes and the liver. These results suggest that KPE supplementation might alleviate circadian rhythm disruption caused by HFD and improve metabolic syndrome and obesity. However, it remains unclear whether KPE directly affects the circadian clock in the hypothalamus, liver, and adipose tissue, or indirectly modulates BA metabolism and gut microbiota composition by regulating the circadian clock. Although further investigation is warranted to elucidate the precise underlying mechanism, we have comprehensively demonstrated that KPE protects against circadian rhythm disturbances and metabolic disorders.

Ethics statement

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the guidelines for animal experiments established by Japanese Association for Laboratory Animal Science. We indicated it in my manuscript.

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CRediT authorship contribution statement

Izumi Yoshida: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Takashi Mishima:** Formal analysis, Investigation, Methodology. **Momochika Kumagai:** Formal analysis. **Yushi Takahashi:** Methodology. **Kazuhiro Fujita:** Project administration, Supervision, Writing – review & editing. **Tomoji Igarashi:** Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105649.

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