

Regular Article

Diurnal Variation of Sitagliptin-Induced Pharmacological Effects in C57BL/6J Mice

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Chronopharmacology is the study of the varying responses of drugs to changes in biological timing and endogenous periodicities. The dipeptidyl peptidase-4 inhibitor sitagliptin is a globally prescribed anti-hyperglycemic drug. Although dipeptidyl peptidase-4 inhibitors are usually administered once, the specific intake time is generally not mentioned. Therefore, this study aimed at investigating the diurnal effects of sitagliptin-induced anti-hyperglycemia in high-fat diet (HFD)-induced obesity in mice. Five-week-old male C57BL/6J mice were fed normal (control) diet or HFD for 10 weeks. During the last 2 weeks, the mice were administered saline or sitagliptin (10 mg/kg, *per os*) in the light or dark phase, respectively. At the end of the experiment, the mice were euthanized after an 18 h fasting period, and plasma and tissue samples (liver, kidney, and epididymal white adipose tissues) were collected, or the oral glucose tolerance test was performed. Sitagliptin administration in the light phase significantly decreased plasma glucose levels, insulin levels, hepatic steatosis, and restored the glucose tolerance compared with the HFD group. In contrast, these parameters remained unchanged in the dark phase-treated mice. Our data therefore suggests that sitagliptin portrays definite chronopharmacology, which may provide valuable information on the importance of drug administration timing for maximum pharmacological effects.

Key words chronopharmacology; sitagliptin; hyperglycemia; obesity

INTRODUCTION

Type 2 diabetes (T2D) mellitus is a chronic progressive disease characterized by hyperglycemia, resulting from defects in insulin secretion and/or action. It is responsible for life-long multi-organ failure and premature deaths.^{1,2} Recently, there has been a surge in the number of T2D people, with the current global estimates of 400 million expected to increase to 600 million by 2035.³ Nutrition and exercise therapies are recommended as initial T2D treatment options. When these fail (failure to restore glucose and hemoglobin A1c), medications such as oral anti-hyperglycemic drugs and insulin are prescribed.

Various classes of oral anti-hyperglycemic drugs exist, most of which act by increasing insulin secretion or improving the insulin sensitivity of target tissues such as the liver, adipose tissues, and skeletal muscle.⁴ The more recently developed anti-hyperglycemic drugs include those targeting the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP).

Sitagliptin (Sit), a dipeptidyl peptidase-4 (DPP-4) inhibitor, is approved for monotherapy and combination (with other anti-hyperglycemic medicines) therapy in adult T2D treatment in over 130 countries worldwide. It improves glycemic control by inhibiting DPP-4 inactivation, and increasing the levels of

the endogenous incretin hormones GLP-1 and GIP. GLP-1 and GIP enhance the release of glucose-dependent insulin and the decrease in glucagon response. GLP-1 suppresses glucagon response after the uptake of a meal.⁵ DPP-4 inhibitors such as Sit are essentially well tolerated and not known to cause hypoglycemia, hence, Sit is popularly prescribed globally.⁶

Circadian rhythms are endogenous 24h oscillations in biological and behavioral patterns common to all living organisms. The circadian clock drives oscillations in a diverse set of biological processes, including locomotion, sleep, blood pressure, blood hormone levels, and body temperature.^{7,8} These differences directly affect disease frequency. For example, there are higher occurrences of asthma at midnight and myocardial ischemia in the morning.^{9,10} Moreover, it is well reported that circadian time-dependent differences change the pharmacokinetics of medications, such as anticancer drugs and antibiotics.^{11,12} It is therefore, necessary to consider the chronobiology of such medications during treatment.

Continuous glucose monitoring systems are available for diabetic patients for checking glucose readings in real-time or over a period of time.¹³ Hence, they can be used to monitor the chronopharmacology of antidiabetic drugs, which are of particular interest in insulin-based therapy. Conversely, chronopharmacological reports on oral anti-hyperglycemic drugs (such as metformin) in experimental animals are very limited.¹⁴ More so, because DPP-4 inhibitors (anti-hyperglycemic drugs) are usually prescribed as a single dose and without a specified intake time, we considered the possibility of DPP-4 inhibitor-induced pharmacological effects varying with chang-

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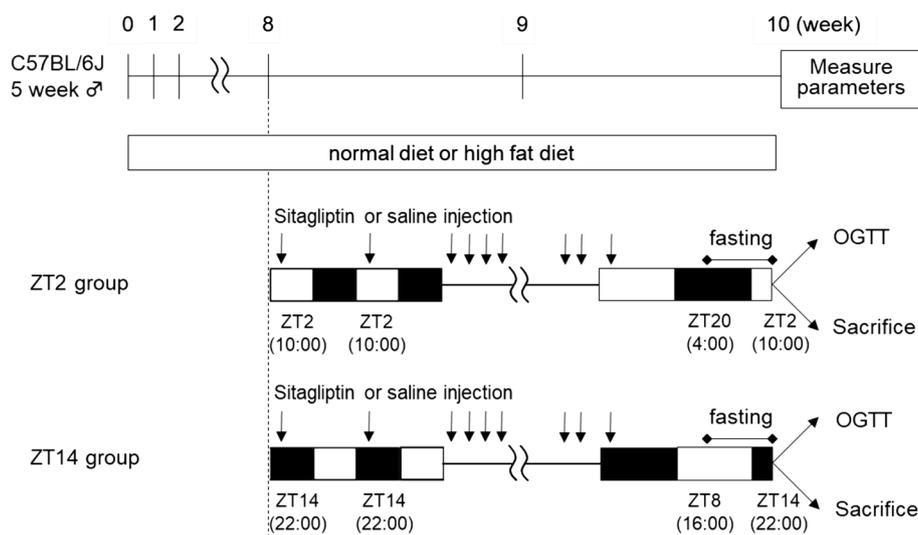


Fig. 1. Schematic Experimental Design

Mice received CE-2 or HFD for 10 weeks. For the last 2 weeks (weeks 8 to 10), mice were also administered Sit (10 mg/kg, *per os*) or saline at ZT2 or ZT14. Then, mice in each group were fasted for 18 h. Thereafter, they were euthanized or performed OGTT, respectively.

es in administration time. In this study, we therefore investigated the circadian variations in Sit-induced pharmacological effects, on T2D mellitus-related parameters in experimental C57BL/6J mice.

MATERIALS AND METHODS

Animal Treatment Four-week old C57BL/6J mice (males) were purchased from CLEA Japan Inc. (Tokyo, Japan). The mice were maintained in a controlled environment: temperature ($24 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$), and light cycle (12 h light [08:00–20:00]: 12 h [20:00–08:00] dark). Food and water were given *ad libitum*, and the mice were allowed to acclimatize to laboratory conditions for a week. The mice were 5-weeks-old (18–21 g) at the start of experiments. All experimental procedures were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University (No. 158).

Experimental Protocol C57BL/6J mice were randomly divided into 6 groups (6–12 mice per group). Two groups were fed with the normal diet (CE-2 [CLEA]; 3.59 kcal/g; 24.9% protein [soybean waste, whitefish meal, and yeast], 51.0% carbohydrate [wheat flour, corn, Milo], 4.6% fat [cereal germ and soybean oil], and other excipients), and 4 groups (obesity model) were fed with HFD 60 from Oriental Yeast Co. (Tokyo, Japan): (HFD 60, 5.06 kcal/g; 23% protein [casein and L-cysteine]; 25.3% carbohydrate [corn, maltodextrin, and sucrose], 35.0% fat [lard and soybean oil], and other excipients). All 6 groups were fed for a total of 10 weeks. After 8 weeks, the mice were further divided into the vehicle-treated groups (control and HFD) which received saline, and the Sit-treated groups (HFD + Sit) which received 10 mg/kg (0.05 mL/kg) Sit purchased from Cayman Chemical Company (MI, U.S.A.). Saline or Sit treatments were administered by oral gavage once daily for 2 weeks. Treatments were administered at 2 specific time points (clock time: 10:00 or 22:00), described here as 2 different zeitgeber times (ZT): ZT2 (control, HFD, and HFD + Sit) as morning or ZT14 (control, HFD, and HFD + Sit) as evening. Body weights were measured weekly for 10 weeks. After the 2 week treatment, mice from each

group were allowed to fast for 18 h (ZT2 group is starting at ZT20 (4:00) and ZT14 is at ZT8 (16:00)) and randomly divided into 2 groups. In one group, the mice were euthanized using pentobarbital, and bled to obtain plasma samples, which were stored at -80°C until use. Epididymal adipose tissue, liver, and kidney weights were also determined. Separate liver samples were immediately-frozen in liquid nitrogen and subsequently kept at -80°C , or fixed in 15% neutral buffered formalin (pH 7.4). In the other group, an oral glucose tolerance test (OGTT) was carried out. Experimental procedure is described in Fig. 1.

Plasma Biochemical Analysis The plasma glucose concentrations were determined enzymatically, using a commercially available assay kit (glucose CII-test) from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), following the manufacturer's instructions and a previously described protocol.¹⁵⁾ Plasma insulin levels were measured using a commercially available kit from Morinaga Co. (Tokyo, Japan), following the manufacturer's instructions and a previously described protocol.¹⁵⁾ Calibration curves were obtained using the respective standard solutions. Fasting glucose and insulin levels were used to estimate homeostasis model assessment of insulin resistance (HOMA-IR).

Evaluation of Glucose Metabolism OGTTs were performed after 10 weeks (including 2 weeks of saline- or Sit-treatment) of feeding with CE-2 or HFD. The mice were allowed to fast for 18 h (ZT2 group is starting at ZT20 (4:00) and ZT14 is at ZT8 (16:00)), and a basal blood sample (0 min) was collected from their tail veins. Blood samples were drawn 15, 30, 60, 90, and 120 min after oral administration of glucose (2 g/kg) from FUJIFILM Wako Pure Chemical Corporation. The blood samples were centrifuged at $3000 \times g$ for 10 min at 4°C , and plasma samples measured immediately. Plasma glucose levels were determined as described above, and the area under the curve (AUC) for blood glucose was calculated.

Histopathological Analyses Liver tissues obtained from each mouse were formalin-fixed, and embedded in paraffin. Paraffin-embedded liver blocks were cut into $4 \mu\text{m}$ sections. These sections were dewaxed in xylene and rehydrated in a

graded ethanol series. The sections were then rinsed under running tap water, stained with Mayer's hematoxylin solution from Nacalai Tesque (Kyoto, Japan), and 0.1% eosin solution (containing acetic acid) from FUJIFILM Wako Pure Chemical Corporation. Finally, the sections were dehydrated, cleared, and mounted with a cover glass. Then, histopathological features were observed under a light microscope.¹⁶⁾

RNA Isolation and Quantitative Real-Time PCR Assay
Total RNA was extracted from liver using the ISOGEN II kit (Nippon Gene, Tokyo, Japan). Five hundred nanograms of

total RNA from each sample was reverse-transcribed using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and was incubated for 15 min at 37°C; the reaction mixture containing synthesized cDNA was diluted three times with Tris-ethylenediaminetetraacetic acid (EDTA) buffer. For the quantitative (q) real-time RT-PCR, 2 μ L of diluted cDNA product was amplified using an Applied Biosystems 7300 system (Applied Biosystems, Foster City, CA, U.S.A.) in a reaction mixture containing Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, U.S.A.) and 0.2 μ M of each primer. PCR conditions were as follows: initial denaturation at 95°C for 3 min, and 40 cycles of 95°C for 5 s and 60°C for 27 s. The amount of each target mRNA quantified was normalized against that of mRNA encoding β -actin. The oligonucleotide sequences of the primers used are: mouse β -actin (NM_007393) sense, 5'-GCA ACG AGC GGT TCC G-3', and antisense, 5'-CCC AA GAAG GAA GGCT GGA-3'; mouse brain muscle arnt-like 1 (*Bmal1*) (NM_007489) sense, 5'-TTC TCC AGG AGG CAA GAA GA-3', and antisense, 5'-TTG CTG CCT CAT CGT TAC TG-3'; mouse circadian locomotor output cycles kaput (*Clock*) (NM_007715) sense, 5'-TGCCAG CTC ATG AAA AGA TG-3', and antisense, 5'-CGCTGCTCTAGCTGGTCTTT-3'.

Statistical Analyses Data are presented as the mean \pm standard deviation (S.D.). Statistical significances were determined using one-way ANOVA or two-way ANOVA with repeated measures, followed by the Tukey-Kramer's *post*

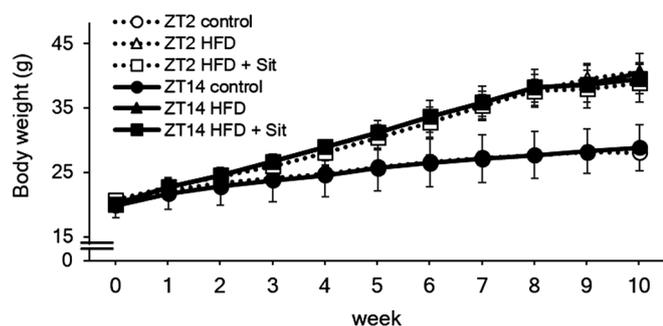


Fig. 2. Diurnal Effect of Sit on Body Weights of HFD Mice

Animals were treated as described in Fig. 1. We measured body weight per one week at ZT2 or ZT14, respectively. The data represent the mean \pm S.D. of results of 6–12 mice per group. “○”: control group at ZT2; “●”: control group at ZT14; “△”: HFD group at ZT2; “▲”: HFD group at ZT14; “□”: HFD + Sit at ZT2; and “■”: HFD + Sit at ZT14.

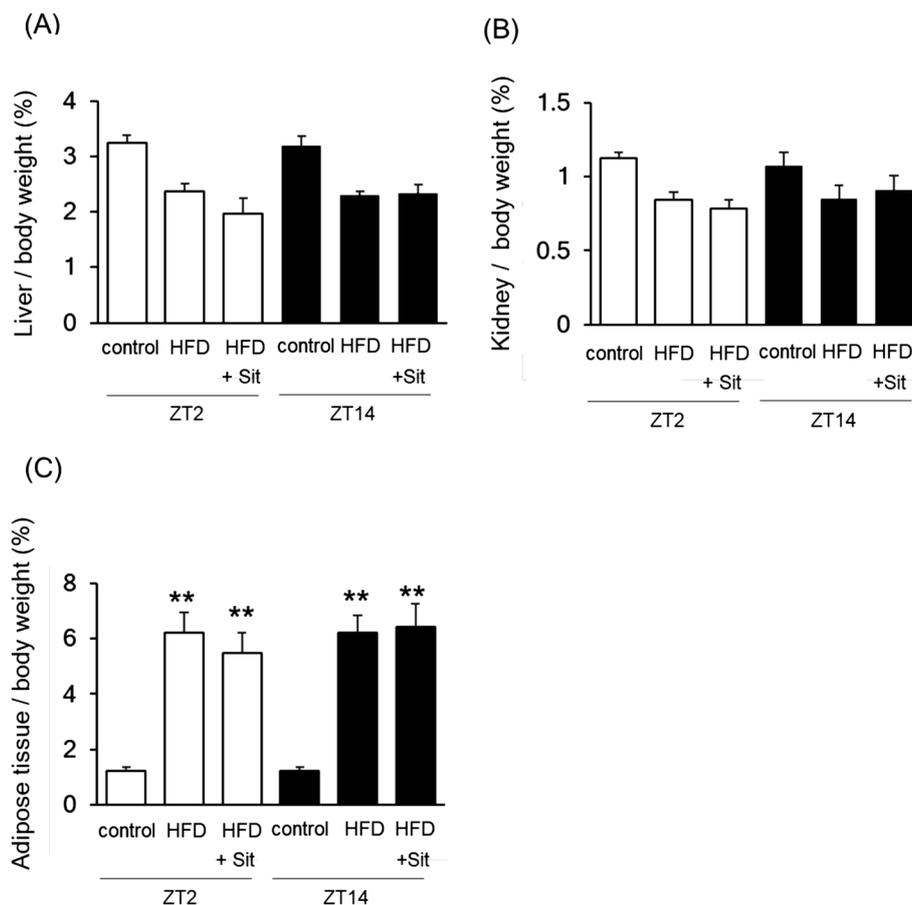


Fig. 3. Sit-Induced Diurnal Effects on Organ Weights of HFD Mice

Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18 h. Thereafter, they were euthanized, and their liver, kidney, and epididymal white adipose tissues were isolated and weighed. Panels indicate the percentage of (A) liver tissues/body weight, (B) kidney tissue/body weight, and (C) epididymal white adipose tissues/body weight. The data represent the mean \pm S.D. of results of 6–12 mice per group. ** p < 0.01 vs. the control group at same time.

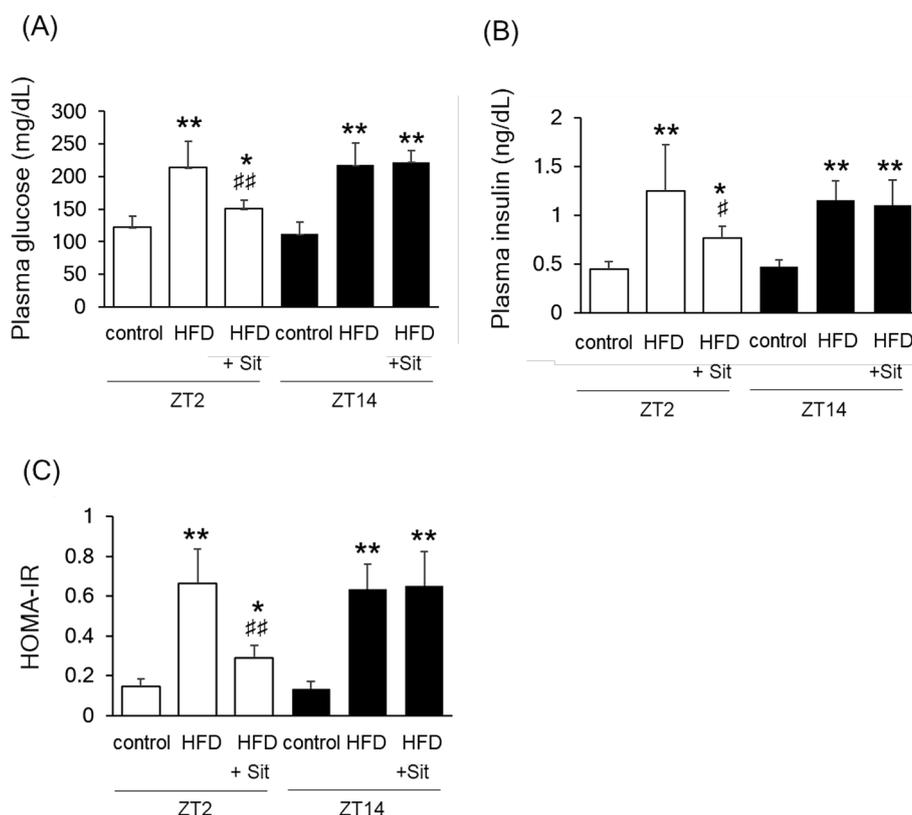


Fig. 4. Diurnal Effects of Sit on Plasma Biochemical Parameters

Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18h, euthanized, and bled to obtain plasma. Levels of plasma (A) glucose, (B) insulin, and (C) HOMA-IR are shown. The data represent the mean \pm S.D. of results of 6–12 mice per group. * p < 0.05 and ** p < 0.01 vs. the control group at same time; and # p < 0.05, and ## p < 0.01 vs. the HFD group at same time.

hoc test for multi group comparisons. All statistical analyses were performed using the SPSS software (version 25.0) from IBM Corp. (Armonk, NY, U.S.A.). p values < 0.05 were considered statistically significant.

RESULTS

Sit Administration at ZT2 Prevents Adipocyte Hypertrophy Tendency Our initial investigations involved monitoring the effect of Sit (at ZT2 or ZT14 as morning vs. evening) on obesity-related abnormalities. Mice were fed CE-2 (control) or HFD for 10 weeks and administered saline or Sit at ZT2 or ZT14 for the last 2 weeks. Changes in body weights are shown in Fig. 2: control group (CE-2 + vehicle) at ZT2 (○); control group at ZT14 (●); HFD group (HFD + vehicle) at ZT2 (△); HFD group at ZT14 (▲); HFD + Sit group at ZT2 (□); and HFD + Sit group at ZT14 (■). Body weights of HFD mice were greater than those of control mice at both times. Although the changes in body weights at ZT2 and ZT14 in the HFD + Sit group were not significantly different from the changes observed in the respective HFD groups, there was a higher tendency for body weights to decrease at ZT2 (40.66 vs. 38.87g) compared with at ZT14 (40.37 vs. 39.55g).

To elucidate the cause of body weight alterations (Sit-induced or HFD-induced body weight gain), we determined the weights of epididymal white adipose tissue (WAT), liver, and kidney (Figs. 3A–C). At ZT2, the HFD + Sit group had slightly lighter epididymal WAT, liver, and kidney tissues, compared with the respective tissues in the HFD group. Conversely, this trend was not observed at ZT14. The tendency

for body weights to increase in HFD mice at ZT2 after Sit administration can be attributed partially to Sit's effects on the epididymal WAT, liver, and kidney tissues.

Sit Administration at ZT2 Decreases the Levels of Plasma Biochemical Parameters To investigate if Sit reduces obesity-induced hyperglycemia, we determined fasting plasma glucose (Fig. 4A), insulin levels (Fig. 4B), and HOMA-IR (Fig. 4C). The HFD groups showed increased plasma glucose levels when compared with the control groups-at ZT2 and ZT14. At ZT2 but not ZT14, the HFD + Sit group had significantly decreased plasma glucose levels compared with the HFD group (Fig. 4A). The same trend was observed with plasma insulin levels (Fig. 4B) and HOMA-IR (Fig. 4C). These results indicate that at ZT2, Sit administration decreased HFD-induced hyperglycemia and hyperinsulinemia, thereby improving glucose metabolism.

Sit Administration at ZT2 Restores Glucose Tolerance To further analyze the effects of Sit, we performed OGTT 2 weeks after Sit administration. Plasma glucose levels in the HFD group were significantly higher at ZT2 and ZT14 compared with the levels in the control group, after glucose (2g/kg) administration (Figs. 5A, C). At ZT2, glucose levels in the HFD + Sit group were reduced compared with the levels in the HFD group. Likewise, the *AUC* was lower in the HFD + Sit group compared with the HFD group (Fig. 5B). Conversely, glucose tolerance levels was slightly restored in the HFD + Sit group at ZT14, though not as high as the levels at ZT2.

Sit Administration at ZT2 Decreases Lipid Formation in the Liver Next, we conducted histopathological stud-

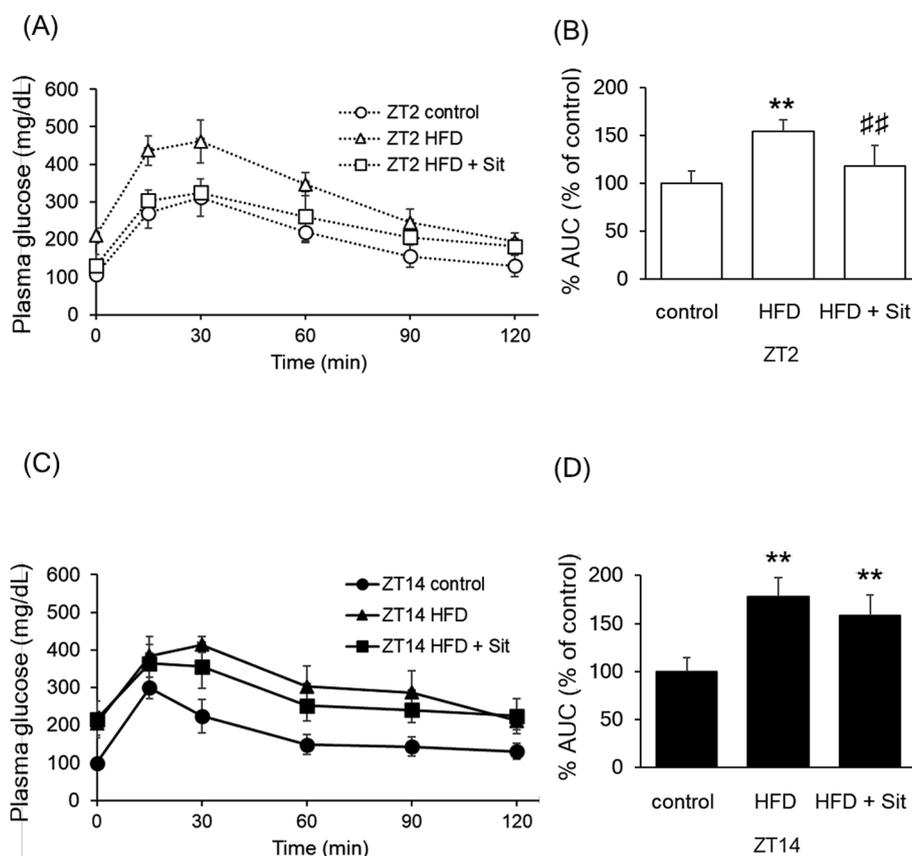


Fig. 5. Diurnal Effects of Sit on Glucose Tolerance

Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18h, and their OGTT tested. Panels indicate plasma glucose levels at (A) ZT2 and (C) ZT14. Data indicate the AUC for plasma glucose at (B) ZT2 and (D) ZT14. Data are plotted as mean \pm S.D. ** $p < 0.01$ vs. the control group; and ## $p < 0.01$ vs. the HFD group.

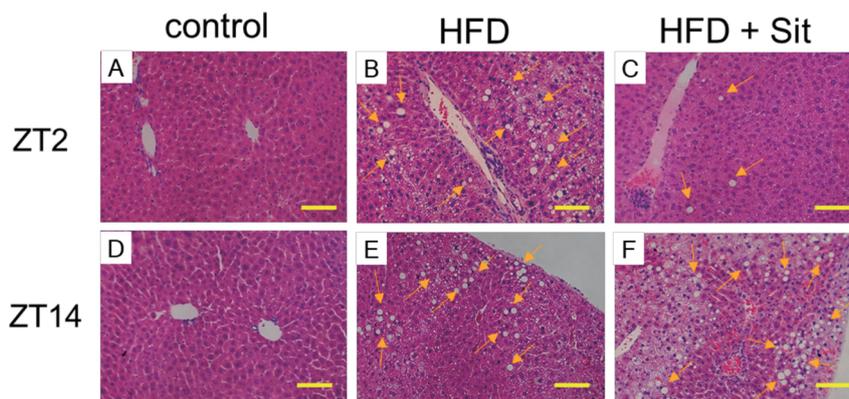


Fig. 6. Diurnal Effect of Sit on Hepatic Steatosis

Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18h, euthanized, and their hepatic tissues harvested. The tissues were fixed and processed by standard methods, and the sections were stained with H&E. Panels indicate (A) control group at ZT2, (B) HFD group at ZT2, (C) HFD + Sit group at ZT2, (D) control group at ZT14, (E) HFD group at ZT14, and (F) HFD + Sit group at ZT14. Black arrows indicate lipid globules; scale bar, 100 μ m.

ies on hepatic tissues since Sit is known to improve hepatic steatosis.¹⁷⁾ At ZT2 and ZT14, numerous large lipid globules were observed in the liver tissues of HFD mice (Figs. 6B, E). This number was reduced in the HFD + Sit group at ZT2 (Fig. 6C). Conversely, lipid globules remained unchanged in the HFD + Sit group at ZT14. These observations indicate that at ZT2, Sit administration attenuates obesity-induced hepatic steatosis.

Sit Administration at ZT2 Partially Increases Clock Gene in the Liver Finally, we measured two clock genes

(*Bmal1* and *Clock*) expression alternation in the liver (Fig. 7). Both clock genes at ZT2 were significantly higher than ZT14 in the control mice. Additionally, HFD-fed mice in the *Bmal1* and *Clock* levels significantly decreased in the both time, respectively. Sit administration at ZT2 partially recovered in the *Bmal1* levels, while this trend was not confirmed at ZT14 (Fig. 7A). As for the *Clock* gene, although recovery tendency was observed by administrated with Sit, these levels were not significant and same trend was confirmed at both groups (Fig. 7B).

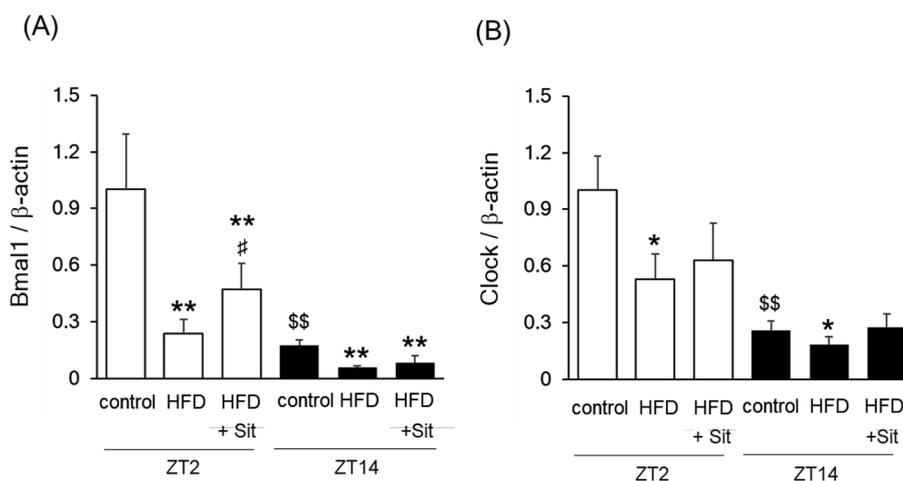


Fig. 7. Diurnal Effect of Sit on Hepatic Clock Gene Expression Levels

Animals were treated as described in Fig. 1. Then, mice in each group were fasted for 18h, euthanized, and total RNA was isolated from the liver samples. The hepatic mRNA levels of genes encoding for *Bmal1* (A) and *Clock* (B) are shown. The mRNA levels of β -actin were used as the internal control. Data are represented as mean \pm S.D. * p < 0.05 and ** p < 0.01 vs. the control group at same time; # p < 0.05 vs. the HFD group at same time; and \$\$ p < 0.01 vs. the control group at ZT2.

DISCUSSION

In this study, we investigated the circadian variations in Sit-induced pharmacological effects using diet-induced obesity mice. Elaborate reports indicate that circadian rhythms control drug metabolism and transport, as well as DNA repair, apoptosis, and cell-cycle progression in experimental animals and humans.^{12,18} Since differences in administration times can potentially change drug pharmacokinetics and pharmacodynamics, chronopharmacological reports have been developed for many medications.^{11,12,19}

Day time Sit administration decreased body weight gain in HFD C57BL/6 mice.^{20,21} The tendency of weight loss was highly observed in Sit administration in the light phase (ZT2) compared with the dark phase (ZT14). Body weight alterations were reflected by the decreased weights of epididymal WAT, liver, and kidney tissues. These results suggest that Sit may exert its weight loss effect in various organs, specifically in the mornings.

We observed that HFD-induced plasma glucose, insulin, and hepatic steatosis were improved in the light phase (ZT2) but remained unchanged in the dark phase (ZT14). We also observed that plasma glucose and insulin levels were decreased by Sit in the light phase, but not in the dark phase. The different Sit-induced glucose metabolism responses observed at different times of the day (morning and evening) suggests that the mechanisms responsible for Sit's pharmacological effects varies according to the time of the day.

The most probable hypothesis for the diurnal variation observed in Sit-induced pharmacological effects is it is the effect of circadian rhythm. Clock genes such as *Bmal1*, *Clock*, *Cryptochrome (Cry)*, and *Period (Per)*, play important roles in maintaining the circadian rhythm.²² *Bmal1* and *Clock*, which are transcriptional activators, stimulate the activation of *Cry* and *Per* genes. *Cry* and *Per* are translated into proteins in the cytoplasm and then transported back into the nucleus after interacting with each other. Subsequently, they stop their transcription by binding to *Bmal1* and *Clock*. Thus, these clock genes are rhythmically expressed over a 24h period. Disruption of *Bmal1* and *Clock* is known to cause hyperinsulinemia

and diabetes.^{23,24} *Bmal1* knockout mice are reported to have high blood glucose and low insulin levels.²⁵ Moreover, HFD is also known to disrupt clock gene.²⁶ Our present results are consistent with this manuscript. Therefore, these clock genes are important for insulin and glucose homeostasis. Ando *et al.* reported that, transcription levels of *Bmal1* were inversely correlated with hemoglobin A1c level.²⁷ Additionally, we showed that HFD-induced *Bmal1* suppression was significantly recovered by Sit administration at ZT2, while not at ZT14. This result suggest that Sit administration at ZT2 may exert *Bmal1* recovery against HFD-induced clock gene disruption. Although further investigation is necessary, *Bmal1* may be associated with diurnal variations in Sit administration.

A second probable hypothesis will be food intake, which is highly interconnected to circadian clocks.²⁸ Several medications (such as antibiotics) are known to change their pharmacological effects by changing their absorption efficiency, *AUC*, and *T_{max}*.¹¹ Our investigations showed that daily food intake caused no significant change at ZT2 or ZT14 (data not shown). Moreover, Sit-induced *AUC* and *T_{max}* were similar during fasting and after a meal.²⁹ Since food intake is thought to affect circadian clocks, it can be said that the food intake conditions were slightly affected. However, this hypothesis may not be very efficient as this study did not consider the fasting time. Further experiments may be necessary to investigate the effect of diet.

In conclusion, we have demonstrated that mice are sensitive to Sit-induced anti-hyperglycemic effects when administered during the light-phase (ZT2). Thus, Sit exhibits definite chronopharmacology in mice. At this time, direct extrapolation to human is difficult since mice are nocturnal habit and human is not. Therefore, more chronopharmacology studies should be carried out on anti-hyperglycemic medications in the basic research and human research, respectively. Now, we are starting the survey in the human research focused on the shift workers since they are known to disrupt clock genes and time of taking. We propose that these researches may provide valuable information on the quality of life in the treatment of T2D and other related diseases.

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Conflict of Interest The authors declare no conflict of interest.

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