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Research Article

Food, not the photoperiod, entrains the circadian rhythms in the liver of Winstar albino rats

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Abstract
The central oscillator is located in mammals' suprachiasmatic nucleus (SCN). The liver is the non-photic organ and the centre for metabolic activities. Food could be a potential zeitgeber for the liver as the timing of feeding is precise in animals. The present study hypothesized that the food provided at a different time of the day (consistently delay of 6 hours) could lead to the desynchronization of daily rhythms in clock genes in liver tissues. The Winstar albino rats were divided into three groups and were exposed to a daily light-dark cycle (12L:12D; 12h light and 12h dark). The Group 1 (Control group) had food *ad libitum*, Group 2- second group- 6h food group had daily food availability of 6h (night fed group). In contrast, Group 3- T30 group was provided food for 6 hours but delayed by 6h from the previous day's food timing. After 30 days, animals were sacrificed at six-time points and the expression of clock genes was studied in the liver. Food cycle's effect was observed on body mass, and it was significantly ($P < 0.05$) reduced in the T30 group. The circadian clock persisted in both food ad libitum and night fed groups but changed in phase and amplitude. However, it lost daily rhythm in clock genes in liver tissues of the T30 group. These results are significant as they suggest that the food's timing is critical for synchronizing the circadian clock in the metabolic center, i.e., the liver.

Keywords: Circadian clock, Daily rhythms, Hepatic, Oscillation, Peripheral

INTRODUCTION

Circadian rhythms are ubiquitous and persist in the absence of environmental cues. Mammals synchronize their daily activity primarily to the light-dark cycles of the environment. The retinal photoreceptors transmit signals to the suprachiasmatic nuclei (SCN) in the hypothalamus via the retinohypothalamic tract (RHT). *The (SCN) is the central pacemaker in mammals (Cox and* Takahashi, 2019). Signals from the SCN cause the synchronization of independent circadian clocks throughout the body to appropriate phases (Partch *et al*., 2014). These circadian clocks are not restricted to the nervous tissue alone, but many peripheral tissues also contain endogenous circadian clocks that are critically involved in the coordination of physiology and metabolism (Abe *et al*., 1989; Balsalobre *et al*., 2000; Damiola *et al*., 2000; Yamazaki *et al*., 2000; Yoo *et al*., 2004). The liver is the center of metabolism. There are circadian rhythms in the expression of genes involved in nutrient metabolism, heme and glutamine biosynthesis, and drug detoxification (Kornmann *et al*., 2001; Reddy, *et al*., 2006).

A similar clock mechanism operates in the SCN and the peripheral oscillators, consisting of a network of transcriptional-translational feedback loops that drive rhythmic, 24-h expression patterns of core clock components. The primary feedback loop, the positive elements, consists of members of the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription factor family, *Clock* (Circadian Locomotor Output Cycles Kaput), and *Bmal1* (Brain and Muscle arnt-like 1). CLOCK and BMAL1 heterodimerize and initiate transcription of target genes containing E-box-

regulatory enhancer sequences, Period (in mice, *Per1*, *Per2*, and *Per3*) and Cryptochrome (*Cry1* and *Cry2*). On the other hand, the negative feedback loop is operated by PER:CRY heterodimers that translocate back to the nucleus to repress their own transcription by acting on the CLOCK:BMAL1 complex. In secondary regulatory loop CLOCK:BMAL1 heterodimers activate retinoic acid-related orphan nuclear receptors, *Rev-Erbα* and *Rorα* (Retinoid-related orphan receptor alpha). Subsequently, REV-ERBα and RORα proteins compete to bind retinoic acid-related orphan receptor response elements (RORES) of the *Bmal1* promoter. The autoregulatory feedback loops take 24 h to complete a cycle and constitute a circadian molecular clock *(Cox and* Takahashi, 2019). This transcriptional feedback loop operates in the SCN and in almost all other tissues (Dibner *et al*., 2010). Along with the core clock genes, several other genes are circadian in different tissues, known as clock-controlled genes. These clock-controlled genes may be involved in diverse gene pathways in different tissues.

The effect of time-restricted food availability on the peripheral clock has been studied. Nocturnal mammals having food access only during the daytime leads the activity in anticipation of meal-time. It shows the phase advances of the circadian rhythms of gene expression in the peripheral tissues and some brain structures, uncoupling them from the SCN whose entrainment to the light remains intact (Damiola *et al*., 2000; Stokkan *et al*., 2001; Hara *et al*., 2001; Wakamatsu *et al*., 2001; Cassone and Stephan, 2002). It is possible that the possibility that in intact animals, the SCN synchronizes peripheral clocks primarily through temporal feeding patterns imposed through behavioral rest-activity cycles.

Circadian rhythms of gene expression occur widely in peripheral organs. However, it is unclear how these multiple rhythms are coupled together to form a coherent system. The present study investigated the effects of rotating cycles of food availability (6h) on the liver's rhythms of clock gene expression to study such coupling. The study hypothesized that the food timing is critical for the entrainment of the metabolic centre, i.e., the liver and loss of fixed food timing will result in loss of expression of daily rhythms of clock genes in liver tissue.

MATERIALS AND METHODS

Four to five months old adult inbred (animal house facility, Mizoram University) male Wistar albino rats (n= 90), weighing (107± 12 gm), were used in this study. The animals were housed in polypropylene cages (size 10 x 7.5 x 5.5 cm having two animals per cage). Rats were maintained under a photoperiod of 12L: 12D (light on:06:00; light off: 18:00) and were given a dry pellet diet. Water was available *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (MZUIAEC) of Mizoram University. Rats were randomly divided into three groups (30 rats/ group). Group 1 (control) was given access to food *ad libitum*; Group 2 (6h food; Night-fed) was allowed access to food for only 6 hours at dark phases starting from 18:00 - 00:00 (first 6h of dark phase; Light on 06:00 and light off 18:00), they were starved the whole day, and food was given at night at the same time as mentioned above. Group 3 (T30) was provided food for 6 hours (18:00 - 00:00 first day), and they were starved for 24 hours, and food was given again for 6 hours (00:00 am - 6:00 am) and 24 hours of restriction and presented again at 6:00 am - 12:00 pm and so on manifesting rotational food-cycle which kept going on for 30 days. The animals' initial and final weights were recorded at the beginning and end of the experiment, respectively. After completing the feeding cycle of 30 days, sampling for clock genes expression was done for six-time points at 4h intervals beginning 1h after lights on (ZT= 0; light on timing), i.e., ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21. Liver tissue was excised and stored in RNAlater solutions (Thermo Fisher Scientific, USA, AM 7020) overnight at 4 ^{0}C and then at -80 ^{0}C until RNA was extracted.

RNA Isolation, cDNA Synthesis

Tri Reagent solution (Ambion AM9738; USA) was used to extract RNA. NanoDrop One spectrophotometer (Thermo Electron Scientific Instruments, LLC; USA) was used for RNA quantification, and 1-µg RNA was used to prepare cDNA. RQ1 DNase (Promega M6101; Wisconsin, USA) kit was applied to remove genomic DNA contamination. cDNA synthesis kit (Thermo Scientific, K1622; Lithuania, Europe) was used to synthesize cDNA.

Gene expression

mRNA expression of genes coding for clock genes (*Bmal1*, *Clock*, *Per1*, *Cry1*, *rRora,* and *rRev-Erbα*) was measured in the liver tissue of each animal, as described in our previous publications (Renthlei *et al*.,

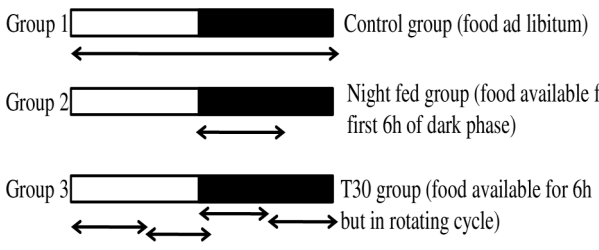

Fig.1. *Schematic diagram of the protocol followed to feed the animals*

2019; Renthlei and Trivedi, 2019; Borah *et al*., 2020).

Quantitative (Real-time) RT-PCR (qPCR)

Primers for clock and clock-controlled genes (*Bmal1*, *Clock*, *Per1*, *Cry1*, *rRora,* and *rRev-Erbα*) were used from previously published work (Kamphuis *et al*., 2005). The QuantStudio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) system was used to conduct qPCR amplification, as described in previous publications (Renthlei *et al*., 2019; Renthlei and Trivedi, 2019; Borah *et al*., 2020). The ΔΔCt method (Livak and Schmittgen, 2001) using beta-2-microglobulin (B2M) as a control gene was used to determine the relative expression of genes.

Statistical analysis

One-way analysis of variance (1-way ANOVA) followed by Newman-Keuls post hoc test was applied to detect the significance of difference among the six-time points of the daily profile. Cosinor analyses based on unimodal cosinor regression [y=A+(B.cos(2π(x-C)/24))], where A, B, and C denote mean level (mesor), amplitude, and acrophase of the rhythm, respectively (Cuesta *et al*., 2009) was used to determine daily variation. Two-way analysis of variance (2-way ANOVA) was employed when two factors (factor one: food and factor two: time) were considered together. Bonferroni post hoc test was used if 2-way ANOVA showed differences. Statistical analysis was done using graph pad prism version 8.

RESULTS

Effect of food cycle was observed on body mass. After 30 days of respective food cycle treatment resulted in change in body mass (treatment: $F_{(2,64)}$= 3.470, P = 0.0371; time: $F_{(1,64)}$= 2.149, P = 0.1475; interaction of treatment x time: $F_{(2,64)}$= 2.925, P = 0.0609; 2-way ANOVA). Body mass of rats receiving food in rotating food cycle (T30 group) was significantly reduced in comparison to control and 6h fixed food timing group (P< 0.05; Bonferroni test Fig. 2). All clock genes studied showed daily oscillation in liver tissue of rat of control group (*Per1*: $F_{(5,29)}$= 2.863, P = 0.0450; *Cry1*: $F_{(5,29)}$ = 8.896, P = 0.0002; *Bmal1*: $F_{(5,29)}$= 4.442, P = 0.0090*;Clock*: $F_{(5,29)}$= 14.32, P < 0.0001; *Rora*: $F_{(5,29)}$= 4.125, P = 0.0450 and *Rev-Erba*: $F_{(5,29)}$= 23.69, P < 0.0001; one-way ANOVA; Fig. 3). Peak expression timing (cosinor analyses) of *Per1* transcripts was observed late dark hours (ZT 21.4; Table 1), *Cry1* at early light phase (ZT 5.8; Table 1), *Bmal1* at late light phase (ZT 10.2; Table 1), *Clock* at during middle of light phase (ZT 5.4; Table 1), *Rorα* at early dark phase (ZT 13.8; Table 1) and *Rev-Erbα* during middle of light phase (ZT 6.5; Fig. 3; Table 1). All clock genes except *rRora* had significant daily variations in the liver of rats getting 6h food but fixed time at night (*Per1*: $F_{(5,29)}$= 3.490, P = 0.0222; *Cry1*: $F_{(5,29)}$= 4.598, P = 0.0071; *Bmal1*: $F_{(5,29)}$= 3.385, P = 0.0249; *Clock*: $F_{(5,29)}$= 6.691, P = 0.0011; *Rora*: $F_{(5,29)}$=0.8363, P = 0.5410 and *Rev-Erba*: $F_{(5,29)}$= 7.833, P = 0.0005; one-way ANOVA; Fig. 3). Peak expression timing of *Per1* and *Cry1*was during the middle of dark phase (*Per1* ZT 18.2 and *Cry* ZT 18.9; Table 1), *Bmal1* was during late light phase (ZT 8.1; Table 1), *Clock* during early light phase (ZT 4.6; Table 1) and *Rev-Erba* during middle of light phase (ZT

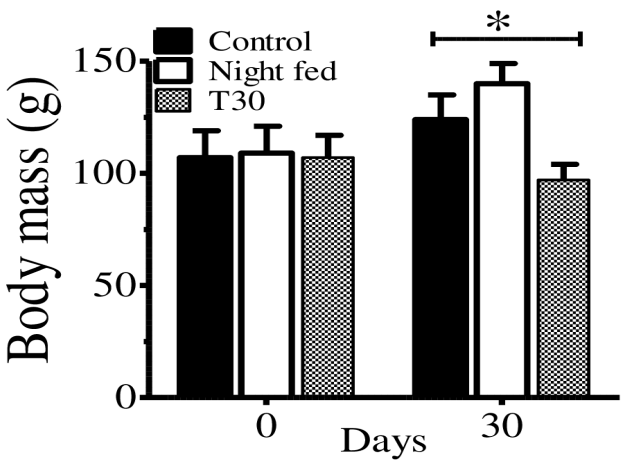

Fig. 2. *Showing means (± SE) body mass on day 0 and day 30 of the experiment. * indicates a significant difference in body mass between the groups (p < 0.05, 2- Way ANOVA)*

Table 1. Rhythm parameters of all six genes in different tissues under different food conditions, as determined by Cosinor analyses

	Control (Group 1)			6h food (Group II)		
Gene	**Mesor**	**Amplitude**	**Phase**	**Mesor**	**Amplitude**	**Phase**
Per1	1.5	1.2	21.4	1.9	1.4	18.2
Cry1	1.0	1.0	5.8	0.6	0.6	18.9
Bmal1	1.4	0.4	10.2	1.0	0.6	8.1
Clock	1.2	1.3	5.4	1.5	1.3	4.6
Rorα	3.1	3.2	13.8	3.8	1.5	-
Rev-Erα	2.2	2.6	6.5	1.4	1.5	5.7

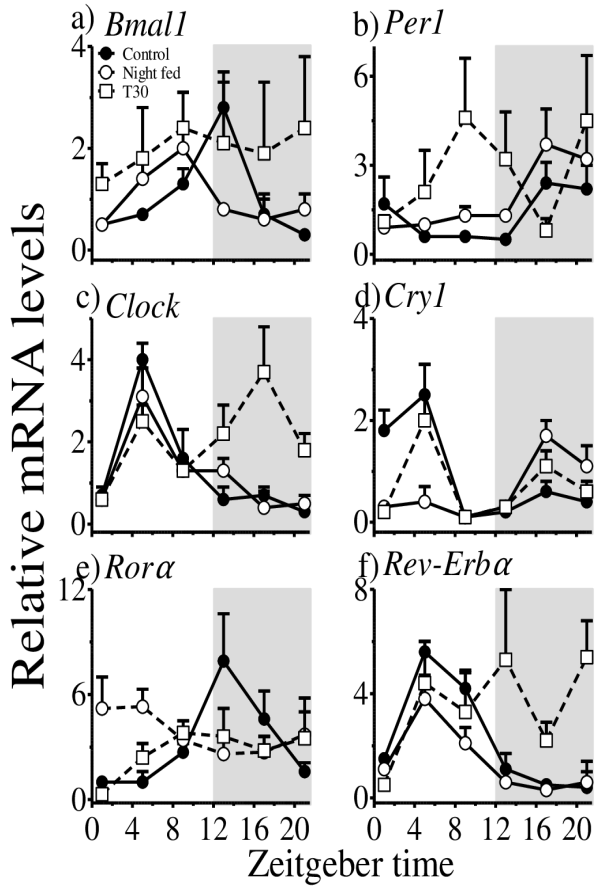

Fig. 3. *Clock genes expression in the liver of rat in the group fed with three different feeding regime. Control group (food ad libitum; solid circle), night fed group (food available for first 6h of dark phase; hollow circle) and T30 group (food available for 6h but every day at different time; hollow rectangle) (a) Bmal1 (b) Per1 (c) Clock (d) Cry1 (e) Rorα, and (f) Rev-Erbα. Solid line represents rhythmic oscillation while dotted line represents loss of daily rhythm in the expression of transcripts*

5.7; Table 1). However, the clock genes transcripts have lost the daily variations in their expression in the group (T30) received food in pattern of rotating cycle (*Per1*: $F_{(5,29)}$= 1.092, P = 0.4003; *Cry1*: $F_{(5,29)}$= 2.199, P = 0.0996; *Bmal1*: $F_{(5,29)}$= 0.1485, P = 0.9779; *Rora*: $F_{(5,29)}$= 1.601, P = 0.2103 and *Rev-Erba*: $F_{(5,29)}$= 1.574, P = 0.2175; one-way ANOVA; Fig. 3) clock transcript (*Clock*: $F_{(5,29)}$= 2.963, P = 0.0501; one-way ANOVA; Fig. 3).

DISCUSSION

The present study hypothesized that the timing of food availability influence energy regulation and consequently results in altered metabolism. The study supported it and showed that food timing differently affects the food's ingestion/digestion/absorption/metabolism, as reflected by the change in body mass (Fig.2). The entrainment of peripheral clocks by feeding- fasting cycles allows peripheral tissues to anticipate food supply and potentially optimizing processes required for food digestion, metabolism, and energy storage and utilization (Vera *et al*., 2007). In comparison to control and fixed 6h timing of food availability, a significant reduction in body mass was observed in the group that received food for 6h but rotating cycle (T30 group; Fig.2). Several previous studies showed that the timing of food intake affects metabolism. However, most of these studies found that having late food hours leads to obesity (Garaulet and Gómez-Abellán, 2014; McHill *et al*., 2017).

This study's main objective was to examine the effects of timing of food availability on clock gene expression in liver tissue. In the present results, the clock genes (*Per1, Cry1, Bmal1, Clock, Rora*, and *Rev-Erba*) showed daily oscillations in the hepatic tissue and are consistent with the literature available for fish, amphibian, and avian systems (Hernández-Pérez *et al*., 2017; Renthlei *et al*., 2019; Borah *et al*., 2020). Both the control and night fed group had daily variations in the transcripts of clock genes in the liver. However, acrophase of expression timing differed between control and night fed food timing groups, and it was advanced in night fed food-restricted rats ranging from 0.8 to 11.2h and is consistent with the previous report (Mendez *et al*., 2011). Interestingly, although the animals were exposed to a fixed light-dark cycle (12L:12D) regime; except *Rora* the rotating food cycle (T30) group has lost the daily oscillation in clock genes in liver tissue, suggesting that not the photoperiod but the food cycle could be a dominant zeitgeber for the entrainment of the circadian rhythms in the liver tissues; a center for metabolism. The previous study has demonstrated the entrainment of the circadian clock in the liver by the feeding cycle (Stokkan *et al*., 2001).

In mammals, the suprachiasmatic nuclei (SCN) are the central circadian oscillator and are predominantly entrained by the solar light-dark cycle to run within a period of a solar day, that is, 24 h synchronizes various peripheral clocks located in the body's cells and tissues accordingly. Under experimental conditions, rats exposed to continuous bright light become behaviourally arrhythmic. Still, the restricted feeding rapidly entrains the liver, suggesting that feeding fasting cycles can entrain the liver independently of the central oscillator (SCN) and the light cycle (Stokkan *et al*., 2001). Circadian transcription in hepatic tissue is considered to be driven by the liver clock and is influenced mainly by the feeding cycle. In SCN lesioned rats (SCNx), a feeding schedule can affect the entrainment of the clock and clock-controlled (CCG) genes in the liver tissue (Sabath *et al*., 2014). The clock genes and the clock-controlled

genes Rev-Erb*a* and peroxisome proliferator-activated receptor alpha (*Ppara*) in food-scheduled intact and SCNx show a robust diurnal differential expression persisting after a 24 h fast in the liver (Sabath *et al*., 2014). Restricted food availability with a periodicity of 24 h leads to the entrainment of behavioral activity in rats (Challet *et al*., 2003; Coleman *et al*., 1982), mice (Abe *et al*., 1989; Sharma *et al*., 2000; Krizo et al., 2018), and hamsters (Mistlberger, 1994). Further, the timed restricted food cycle can entrain the locomotor activity rhythms that were free-running in constant darkness (Honma et al., 1983), having a long tau in LL (Mistlberger, 1993; Sharma *et al*., 2000), or behaviourally arrhythmic due to SCNx (Stephan *et al*., 1979; Stephan *et al*., 1981). A restricted feeding regime can also restore the rhythm in pineal melatonin levels in SCN-lesioned rats (Feillet *et al*., 2008) and affect the rats' corticosterone levels maintained under LL conditions (Ventura *et al*., 1984).

In present study, the rotating food cycle of 6h failed to entrain the circadian rhythms in liver tissue as reflected by the loss of rhythm in the expression of clock genes suggests that in the absence of the food cycle, the light -dark cycle alone was insufficient to entrain the liver clock. The study demonstrated that the rotating food cycle could influence the clock gene expression in the liver tissues to raise the question of whether the food-entrainable oscillators are present in the digestive system? Many species under captive conditions anticipate delivering a scheduled daily meal, showing an increase in locomotor activity and core body temperature (Mistlberger, 1994; Stephan, 2001; Breno et al., 2012). The circadian clock controls this food-anticipatory activity and persists when animals are food-restricted (Davidson *et al*., 2001). The present study did not record the locomotor activity rest pattern; however, there was an effect of food restriction and rotation of the food cycle on the expression pattern of clock genes in liver tissue. As 6h restricted food cycle groups showed alteration in phase and amplitude of expression of clock genes, it further suggests that timing of food availability was critical for the entrainment and phase resetting of the liver clock. However, the rotating cycle caused a more severe effect, and hepatic tissue lost track of timing as the clock gene expression became arrhythmic. Altogether this study suggests that food-restricted for 6h with different timing leads to the loss of body mass and expression of daily clock genes in liver tissue.

Conclusion

The present study demonstrated that exposure to the rotating food cycle significantly reduced body mass. Food restricted to 6h during the early light phase alters the expression of clock genes expression in liver tissues. Light alone fails to entrain the liver circadian clock in the absence of consistent food timing in Winstar albino rats.

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Conflict of interest

The authors declare that they have no conflict of interest.

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