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Suitability of a 10% fat diet for use in time-restricted feeding experiments with C57BL/6 mice

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ABSTRACT

Background: There is growing interest in the possible role of circadian rhythms in feeding behavior and their effect on diet-induced obesity. However, it is unclear whether widely used purified diets are suitable for use as normal control diets for rodents undergoing time-restricted feeding studies. In the present study, we compared the effects of 4% and 10% fat diets on body mass gain and food consumption during time-restricted feeding (TRF).

Methods: *Ad libitum*-fed male C57BL/6J mice had free access to AIN-93M (4% fat) or modified 10% fat diets, whereas TRF groups were only able to consume one of these diets twice daily, at ZT23:00–01:00 and ZT11:00–13:00, for 5 weeks.

Results: Total energy consumptions by the TRF-4% and TRF-10% fat groups were 26.5% and 18.6%, respectively, less than that of the *ad libitum*-fed groups. The body mass gains by the 4% and 10% fat diet groups fed *ad libitum* were similar, but the gain by the TRF-4% fat group was markedly lower than that of the TRF-10% fat group. In addition, whereas the body mass gain by the TRF-10% fat group was similar to that of its *ad libitum* equivalent, the gain by the TRF-4% fat group was much lower than that of the equivalent *ad libitum*-fed group.

Conclusion: We showed for the first time that a 10% fat diet (21.9% kcal from fat) is suitable for the maintenance of body mass gain during TRF of C57BL/6 mice.

Keywords: *ad libitum* feeding, fat content, mouse, time-restricted feeding

BACKGROUND

Obesity, indicated by a body mass index ≥ 30.0 kg/m^2, is recognized as unsanitary condition because of the greater risk of developing diseases, such as type 2 diabetes mellitus and cardiovascular disease [1-2]. The worldwide prevalence of obesity has been predicted to reach 18% in men and 21% in women by 2025 [3]. In addition, the economic burden of obesity-related diseases is likely to reach $50 billion/year in the US and £2 billion/year in the UK by 2030 [4]. Therefore, research regarding methods of preventing excess body mass gain should be prioritized.

Body mass gain results from a chronic energy imbalance, which occurs when energy expenditure is lower than energy intake. Therefore, the management of daily energy consumption is important for the control of body mass. Rodent models of obesity are frequently studied. The body mass of experimental rodents consuming *ad libitum* a high-energy diet, such as a high-fat diet, increases much faster than those of rodents consuming a low-energy diet [5]. The use of such diet-induced obesity models has led to the identification of many substances, such as resveratrol, capsaicin and quercetin glycosides, which can protect against excess body mass gain, and therefore obesity [5-10].

In addition to exploration of the effect of the quantity of energy fed, there is growing scientific interest in the role of circadian variations in feeding behavior on diet-induced obesity. This stems in part from interest in the effects of changes in patterns of human behavior; for example, the frequency of consumption of breakfast has declined and more calories are consumed later in the day [11]. Such evening hyperphagia (consumption of at least 25% of the daily caloric intake after the evening meal) is now recognized as a risk factor for body mass gain [12]. In addition, night shift workers, who usually consume meals during the night, have been reported to be more likely to become overweight or obese than day workers [13]. This

link between obesity and the timing of meals has provoked animal studies that have provided direct evidence that the timing of food consumption may induce obesity and obesity-related metabolic disturbances [14-15]. To study this phenomenon further, rodent models of time-restricted feeding (TRF) have been developed.

Rodents are nocturnal animals, and are therefore most active during the dark period. More specifically, monitoring of rodent food consumption under *ad libitum* feeding conditions has shown that there are two peaks of feeding, at the beginning and end of the dark period [16]. Previous TRF studies have involved access to food for 3–8 h daily [17-21], and we previously provided the purified diets, AIN-76 and AIN-93, to mice and rats daily for 4 h (2 h at both the beginning and end of the dark period). The body masses of these animals were lower than of those that had consumed the same diet *ad libitum*. For example, two groups of Sprague-Dawley (SD) rats (5 weeks old) were consumed AIN-73 diets; the first was *ad libitum* feeding and second was daily for 4 h of TRF. After 2 weeks, their body weights were increased 181.2% and 132.3%, respectively. Although similar findings have been reported previously [22], these preliminary results made us wonder whether widely used purified diets, including AIN-76 and AIN-93, are suitable for use as normal control diets in diet-induced obesity experiments, either under TRF or *ad libitum* feeding conditions.

Therefore, in the present study, we compared the effects of the basic AIN-93M diet (4% fat) and a modified diet containing 10% fat on body mass gain and food consumption during *ad libitum* feeding and TRF.

METHODS

Institutional approval of the study protocol

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miyazaki (No. 2015-013-01-03). This study was conducted in accordance with the Japanese Law for the Humane Treatment and Management of Animals (Law No. 105, 1973) defined as animal experimentation as the use of animals for scientific purposes with the consideration of 3Rs.

Animal experiments

Twenty-eight male C57BL/6JJmaSlc mice (9 weeks old) were obtained from Japan SLC (Shizuoka, Japan), and three were placed in each cage (width 235 × depth 165 × height 125 mm) in an air-conditioned room (temperature: 23 ± 1°C; humidity: 55 ± 5%) under a 12-h dark/light cycle (lights on between 9:00 and 21:00), with free access to deionized water and powdered AIN-93M diet (Table 1).

Table 1. Composition of the experimental diets.

Constituent	4% fat diet (AIN-93M) (%)	10% fat diet (%)
β-corn starch	46.57	40.57
α-corn starch	15.50	15.50
Casein	14.00	14.00
Soybean oil	4.00	10.00
Sucrose	10.00	10.00
Cellulose	5.00	5.00
Vitamin mixture	1.00	1.00
Mineral mixture	3.50	3.50
L-Cystine	0.18	0.18
Choline bitartrate	0.25	0.25
tert-Butylhydroquinone	0.0008	0.0008
Energy[1]	3.80 kcal/g diet	4.10 kcal/g diet
% kcal from fat	9.5	21.9
% kcal from carbohydrate	75.8	64.4
% kcal from protein	14.7	13.6

[1]Energy values were calculated using Atwater factors: 4 kcal/g for carbohydrate and protein, and 9 kcal/g for lipid.

After 1 week of acclimation, the mice were divided into two groups: an *ad-libitum* feeding group and a TRF group. The former was group-housed and fed *ad libitum*, while the latter mice were singly housed in cages of the same size and acclimated to TRF for 1 week, during which they all consumed standard AIN-93M. These mice were provided with food only between Zeitgeber time (ZT) 23:00 and 01:00, and ZT 11:00 and 13:00, where ZT00:00 represents the time when the lights were turned on. Thus, the light period was between ZT00:00 and ZT12:00, and the dark period was between ZT12:00 and ZT24:00. After the TRF acclimation period, when the mice were 11 weeks old, each group was further subdivided into two, one of which received 4% fat-containing AIN-93M diet, and the other received 10% fat-containing diet.

After 5 weeks of TRF with these diets, the mice were anesthetized with isoflurane (1.5%), and then decapitated at ZT11:00, which was just before starting the daily consumption and meaning after fasting for 10 h. The *ad libitum*-fed groups were sacrificed at the same time point (ZT11:00). Here, the fasting was not conducted, because in order to evaluate the biological variations under the ordinary life condition (*ad libitum* feeding). Trunk blood was collected into serum Capiject tubes (Terumo Medical Corporation, Somerset, NJ, USA). Serum was obtained by centrifugation (3,500 × *g*, 90 sec) after the blood had been left to stand at room temperature for 30 min, and was stored at −80°C until analyzed.

Blood chemistry

Serum biochemical parameters (triglycerides, total cholesterol, and phospholipids) were analyzed using test kits obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All standards were used in accordance with the manufacturer's instructions.

Statistical analysis

Results are presented as mean ± standard deviation. Statistical analysis was undertaken using StatView for Windows (version 5.0, SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was used, followed by Fisher's PLSD method for multiple comparisons between groups. Results were considered significant if $P < 0.05$.

RESULTS

Effects of TRF on food intake

The food intakes of mice consuming the 4% and 10% fat diets *ad libitum* were similar (Table 2), as were the mean total daily intakes by the TRF groups. However, the amount of food consumed at ZT11:00–13:00 was much higher than at ZT23:00–1:00 in both TRF groups. In addition, the total energy consumptions by the TRF-4% fat and TRF-10% fat groups were 26.5% and 18.6%, respectively, less than those of the equivalent *ad libitum* feeding groups.

Table 2. Food consumption.

	Ad libitum feeding		Time-restricted feeding	
	4% fat	10% fat	TRF-4% fat	TRF-10% fat
	Mass consumed (g/mouse/day)			
Total	3.9	3.4	2.81 ± 0.20^{a}	2.73 ± 0.46^{a}
ZT23:00–01:00	-	-	1.30 ± 0.09^{ab}	1.27 ± 0.23^{a}
ZT11:00–13:00	-	-	1.54 ± 0.14^{a}	1.50 ± 0.26^{a}
	Energy consumption (kcal/mouse/day)			
Total	14.7	14.0	10.8 ± 0.8^{a}	11.4 ± 1.9^{a}
ZT23:00–01:00	-	-	4.9 ± 0.3^{ab}	5.2 ± 0.9^{b}
ZT11:00–13:00	-	-	5.9 ± 0.6^{a}	6.1 ± 1.1^{a}

The AIN-93M diets used contained 4% fat or 10% fat. *Ad libitum* feeding groups had permanent free access to one of these diets. Time-restricted feeding (TRF) groups had one of these diets available only during ZT23:00–01:00 and ZT11:00–13:00. Values are mean ± standard deviation (n=2 for *ad libitum*-fed groups; n=7 for time-restricted feeding groups). Different alphabetical superscripts "a" and "b" indicate significantly difference on TRF groups (P<0.05, Fisher's PLSD).

Effects of TRF on body mass gain

The body masses of the mice undergoing TRF were measured four times daily, just before (at ZT11:00 and ZT23:00) and just after (at ZT13:00 and ZT01:00) feeding. Over the 5 weeks of the study, there were similar body mass gains in mice consuming the 4% fat and 10% fat diets *ad libitum* at each time point (Figure 1A–D). In contrast, the body mass gain of the TRF-4% fat group was much lower than that of the TRF-10% fat diet group at all the time points from 3 weeks of the study onwards (Figure 1E–H). In particular, TRF-4% restricted mass gain at the time points prior to feeding (ZT11:00 and ZT23:00) more effectively than TRF-10% at 25 and 30 days of the study. Body mass gain by the TRF-10% fat groups and the equivalent *ad libitum* feeding group at the final time point was similar at ZT01:00 and ZT13:00 (after feeding; Table 3), but the gain by the TRF-4% fat group was much lower than that by the equivalent *ad libitum*-fed group.

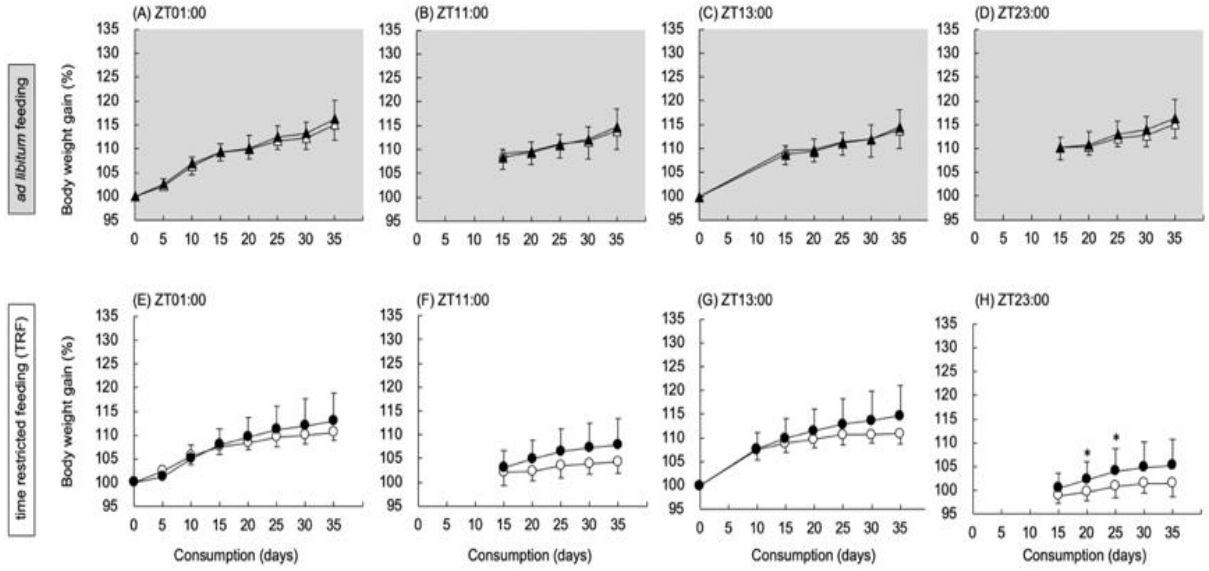

Figure 1. Effect of time-restricted feeding on body mass gain.

Four percent and 10% fat-containing AIN-93M diets were used. *Ad libitum*-fed groups (A–D) consumed the 4% fat diet (-△-) or the 10% fat diet (-▲-). Time-restricted feeding (TRF) groups (E–H) were provided with the 4% fat diet (-○-) or the 10% fat diet (-●-) twice daily at ZT23:00–01:00 and ZT11:00–13:00. Body mass was measured at ZT01:00 (A and E), ZT11:00 (B and F), ZT13:00 (C and G), and ZT23:00 (D and H). Values are mean ± standard deviation (n=6 for *ad libitum* mice; n=7 for TRF mice). *Significantly different *vs*. 4% fat diet group (P<0.05).

Table 3. Body mass gain after 5 weeks of diet consumption.

Time	*Ad libitum* feeding		Time-restricted feeding	
	4% fat	10% fat	4% fat	10% fat
	Body mass gain (%)*			
ZT01:00	114.9 ± 3.0	116.3 ± 3.8	110.5 ± 1.7	112.9 ± 6.0
ZT11:00	113.9 ± 4.0	114.7 ± 3.7	104.4 ± 2.5	107.9 ± 5.6
ZT13:00	113.9 ± 3.9	114.6 ± 3.6	110.8 ± 2.2	114.7 ± 6.4
ZT23:00	115.1 ± 3.0	116.3 ± 4.0	101.4 ± 2.8	105.2 ± 5.6

AIN-93M diets were fed that contained either 4% or 10% fat. *Ad libitum*-fed groups had one of these diets available permanently, whereas the time-restricted feeding (TRF) groups were provided with either the 4% or 10% fat diet daily at ZT23:00–01:00 and ZT11:00–13:00. Body mass was measured at ZT01:00, ZT11:00, ZT13:00, and ZT23:00 after 5 weeks of the diet regimen. *Body mass gain (%) was set on the basis of day 0 (100%). Values are mean ± standard deviation (n=6 for *ad libitum*-fed mice; n=7 for TRF mice).

Effects of TRF on serum lipid concentrations

Serum samples were obtained at ZT11:00, after 10 h of fasting, and used to measure triglyceride, total cholesterol, and phospholipid concentrations, which were similar in the TRF-4% fat and TRF-10% fat groups (Figure 2). The concentrations were also similar in serum obtained from unfasted mice fed the 4% or 10% fat diets *ad libitum*: triglyceride, 83.4±9.5 mg/dL and 85.8±14.6 mg/dL; total cholesterol, 116.8±5.2 mg/dL and 123.0±8.9 mg/dL; and phospholipid 175.7±14.7 mg/dL and 187.8±12.0 mg/dL, respectively.

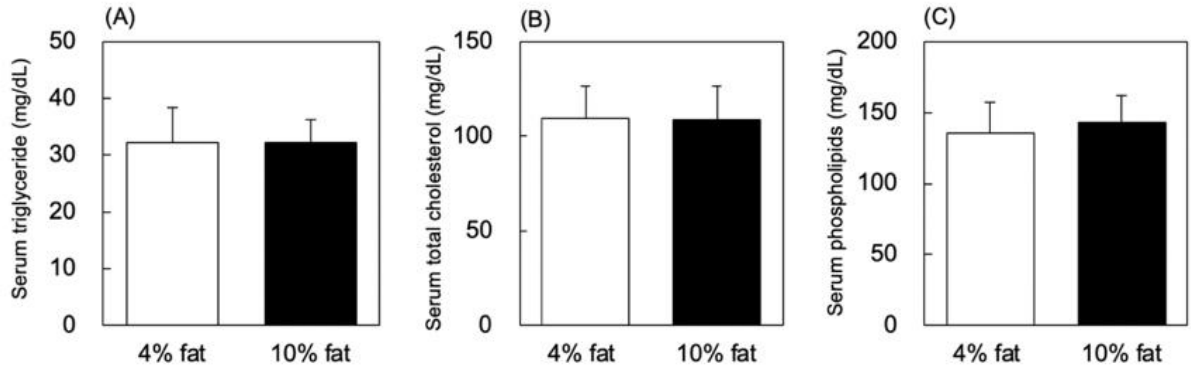

Figure 2. Effect of time-restricted feeding on serum lipid concentrations.

Four percent and 10% fat-containing AIN-93M diets were used. Time-restricted feeding (TRF) groups were provided with the 4% fat diet or the 10% fat diet twice daily at ZT23:00–01:00 and ZT11:00–13:00 for 5 weeks. Values are mean ± standard deviation (n=7).

DISCUSSION

Many rodent studies use an AIN-93-based purified diet, the composition of which has been published by the American Institute of Nutrition (AIN) [23]. This diet was formulated to improve the performance of experimental models, and comes in two versions: AIN-93G for growth, pregnancy, and lactation, and AIN-93M for adult maintenance. Typically, mice are switched from AIN-93G to AIN-93M at ~10–12 weeks of age. In particular, AIN-93-based diets have been used to investigate the anti-obesity effects of food composition and additives, on the basis that unadulterated forms of these diets can be used as control diets.

Recent research has shown that circadian rhythms might influence diet-induced obesity, and many animal studies have now provided evidence that the timing of food consumption affects the development of obesity and obesity-related metabolic disturbances [14-15]. To investigate this phenomenon further, we have studied the effects of the timing of food consumption and the consumption of functional food ingredients on diet-induced obesity using the AIN-93M (4% fat) diet.

When mice consumed a 4% fat diet twice daily at ZT23:00–01:00 and ZT11:00–13:00, their body mass gain was less than that of the equivalent *ad libitum* group (Figure 1 and Table 2), similar to previously published findings [22]. AIN-93M (4% fat) provides 9.5% kcal from fat, 75.8% kcal from carbohydrate, and 14.7% kcal from protein, as shown in Table 1. According to the Dietary Reference Intakes (DRIs) in Japan, the recommended sources of energy for young adult men are 20%–30% kcal from fat, 50%–70% kcal from carbohydrate, and 20% kcal from protein, in order to maintain and promote health and prevent lifestyle-related diseases [24]. The recommendations in North America and Europe for fat intake by adults are similar (15–35% kcal) [25]. This implies the fat content of the AIN-93M diet might be insufficient for the maintenance of body mass gain during TRF in mice. Therefore, we modified the AIN-93M diet to contain 10% fat, such that it contributed 21.9% kcal, while 64.5% kcal were contributed by carbohydrate and 13.6% kcal by protein (Table 1).

When mice consumed the 10% fat diet *ad libitum*, their body mass gain was similar to that of mice consuming the 4% fat diet *ad libitum* over a 5-week period (Figure 1), indicating that this 6% difference in fat content does not have a significant impact under *ad libitum* feeding conditions. In addition, the body mass gain measured just after the period of food availability during TRF using the 10% fat diet was similar to that of the *ad libitum* group, but the gain by mice consuming the TRF-4% diet was much lower than that of the equivalent *ad libitum* group (Figure 1 and Table 3). However, serum triglyceride, total cholesterol, and phospholipid concentrations did not differ between the TRF-4% and TRF-10% fat groups (Figure 2).

CONCLUSION

The present findings indicate that a diet containing 10% fat is suitable for the maintenance of body mass gain in mice undergoing TRF experiments for evaluation about timing of meals. The diet containing 10% fat provides 21.9% kcal from fat, and this proportion is similar value of recommendation for human (15-35%). In addition to the amounts of fat, its origin is important for the nutritional value of the diet. Therefore, evaluation of the effects of fat origin, for example lard, vegetable oils, and fish oil on physiologic parameters, including body mass gain, is planned for the future. Furthermore, we are continuing study of the effects of the timing of consumption of food ingredients on diet-induced obesity, using 10% fat diet as the most appropriate control.

List of Abbreviations: TRF, time-restricted feeding; ZT, Zeitgeber time.

Competing Interests: There are no conflicts of interest to declare.

Authors' contributions: H.S., S.M. and S.N. designed the research. H.M., W.T. and D.Y. conducted the research. M.S. performed statistical analyses. H.S., T.S. and T.Y. wrote the manuscript. H.S. had primary responsibility for the final content. All authors have read and approved the final version of the manuscript.

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