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TITLE:

Assessment of the Metabolic Effects of Isocaloric 2:1 Intermittent Fasting in Mice

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SUMMARY:

The current article describes a detailed protocol for isocaloric 2:1 intermittent fasting to protect and treat against obesity and impaired glucose metabolism in wild-type and *ob/ob* mice.

ABSTRACT:

Intermittent fasting (IF), a dietary intervention involving periodic energy restriction, has been considered to provide numerous benefits and counteract metabolic abnormalities. So far, different types of IF models with varying durations of fasting and feeding periods have been documented. However, interpreting the outcomes is challenging, as many of these models involve multifactorial contributions from both time- and calorie-restriction strategies. For example, the alternate day fasting model, often used as a rodent IF regimen, can result in underfeeding, suggesting that health benefits from this intervention are likely mediated via both caloric restriction and fasting-refeeding cycles. Recently, it has been successfully demonstrated that 2:1 IF, comprising 1 day of fasting followed by 2 days of feeding, can provide protection against diet-induced obesity and metabolic improvements without a reduction in overall caloric intake. Presented here is a protocol of this isocaloric 2:1 IF intervention in mice.

Also described is a pair-feeding (PF) protocol required to examine a mouse model with altered eating behaviors, such as hyperphagia. Using the 2:1 IF regimen, it is demonstrated that isocaloric IF leads to reduced body weight gain, improved glucose homeostasis, and elevated energy expenditure. Thus, this regimen may be useful to investigate the health impacts of IF on various disease conditions.

INTRODUCTION:

Modern lifestyle is associated with longer daily food intake time and shorter fasting periods[1]. This contributes to the current global obesity epidemic, with metabolic disadvantages seen in humans. Fasting has been practiced throughout human history, and its diverse health benefits include prolonged lifespan, reduced oxidative damage, and optimized energy homeostasis[2,3]. Among several ways to practice fasting, periodic energy deprivation, termed intermittent fasting (IF), is a popular dietary method that is widely practiced by the general population due to its easy and simple regimen. Recent studies in preclinical and clinical models have demonstrated that IF can provide health benefits comparable to prolonged fasting and caloric restriction, suggesting that IF can be a potential therapeutic strategy for obesity and metabolic diseases[2-5].

IF regimens vary in terms of fasting duration and frequency. Alternate day fasting (i.e., 1 day feeding/1 day fasting; 1:1 IF) has been the most commonly used IF regimen in rodents to study its beneficial health impacts on obesity, cardiovascular diseases, neurodegenerative diseases, etc.[2,3]. However, as shown in previous studies[6,7], and further mechanistically confirmed in our energy intake analysis[8], 1:1 IF results in underfeeding (~80%) due to the lack of sufficient feeding time to compensate for energy loss. This makes it unclear whether the health benefits conferred by 1:1 IF are mediated by calorie restriction or modification of eating patterns. Therefore, a new IF regimen has been developed and is shown here, comprising of a 2 day feeding/1 day fasting (2:1 IF) pattern, which provides mice with sufficient time to compensate for food intake (~99%) and body weight. These mice are then compared to an *ad libitum* (AL) group. This regimen enables examination of the effects of isocaloric IF in the absence of caloric reduction in wild-type mice.

In contrast, in a mouse model that exhibits altered feeding behavior, AL feeding may not be a proper control condition to compare and examine the effects of 2:1 IF. For example, since *ob/ob* mice (a commonly used genetic obese model) exhibit hyperphagia due to the lack of leptin regulating appetite and satiety, those with 2:1 IF exhibit ~20% reduced caloric intake compared to *ob/ob* mice with AL feeding. Thus, to properly examine and compare the effects of IF in *ob/ob* mice, a pair-feeding group as a suitable control needs to be employed.

Overall, a comprehensive protocol is provided to perform isocaloric 2:1 IF, including use of a pair-feeding control. It is further demonstrated that isocaloric 2:1 IF protects mice from high fat diet-induced obesity and/or metabolic dysfunction in both wild-type and *ob/ob* mice. This protocol can be used to examine the beneficial health impacts of 2:1 IF on various pathological conditions including neurological disorders, cardiovascular diseases, and cancer.

PROTOCOL:

All methods and protocols here have been approved by Animal Care Committees in The Animal Care and Veterinary Service (ACVS) of the University of Ottawa and The Centre for Phenogenomics (TCP) and conformed to the standards of the Canadian Council on Animal Care. It should be noted that all procedures described here should be performed under institutional and governmental approval as well as by staff who are technically proficient. All mice were housed in standard vented cages in temperature- and humidity-controlled rooms with 12 h/12 h light/dark cycles (21–22 °C, 30%–60% humidity for normal housing) and free access to water. Male C57BL/6J and *ob/ob* mice were obtained from the Jackson Laboratory.

1. 2:1 isocaloric IF regimen

1.1. For lean and diet-induced obesity mouse models, prepare either a normal diet (17% fat, ND) or high fat diet (45% fat, HFD).

NOTE: 60% HFD can be used to induce severe diet-induced obesity; yet, due to the softness of the food pellet, it is relatively difficult to accurately measure daily food intake. An automated continuous measurement system can improve versatility for multiple types of diets.

1.2. Measure baseline body weight and body composition of each mouse at 7 weeks of age using a scale and EchoMRI, respectively.

NOTE: Refer to section 3 for body composition measurement.

1.3. Based on body weight and body composition results, randomly and equally divide 7 week-old male C57BL/6J mice into two groups: *ad libitum* (AL) and intermittent fasting (IF) groups.

1.4. Place two to three mice per cage and ensure free access to drinking water.

NOTE: The number of mice per cage can affect food intake behavior. It is recommended to maintain an equal number of mice per cage in all groups during the study.

1.5. Provide 1 week of acclimation to the new cage environment and diet before starting the IF regimen.

1.6. Fasting period: move mice to a clean cage with fresh bedding at 12:00 PM. Do not add food for the IF group, while providing a weighed amount of food to the AL group.

NOTE: For each fasting cycle, it is important to change cages for both AL and IF groups to ensure that both groups are exposed to the same amount of handling time.

1.7. After 24 h, measure the weights of mice in both groups and leftover food in AL cages.

NOTE: Make sure to include the weight of food crumbs on the food hopper and bottom of the cage, especially when using HFD, as mice often remove small pellets or fragments of food from the hopper and keep them near nest sites. The average energy intake per mouse at the end of each 2:1 cycle (3 days) is around 35 kcal, equivalent to ~10 g for a normal diet (3.3 kcal/g) and ~7 g for HFD (4.73 kcal/g).

1.8. Feeding period: provide a weighed amount of food at 12:00 PM for both AL and IF groups.

1.9. After 48 h of providing the food, measure the weight of leftover food and mice.

1.10. Repeat steps 1.6–1.10 for the duration of the study (e.g., 16 weeks).

2. Pair-feeding (PF) control group

NOTE: For an IF experiment in which altered feeding behavior is observed in a mouse model (e.g., hyperphagia in *ob/ob* mice), it is necessary to have a pair-feeding group as a control for proper calorie-independent comparison to IF.

2.1. For the PF control group, stagger the experiment schedule such that the same amount of food consumed by IF group is offered to the PF group (**Figure 2**).

2.2. Measure the amount of food consumed by the IF group over 2 days of refeeding period.

2.3. Divide this amount of consumed food in the IF group evenly into three proportions and provide it daily to the PF group at 12:00 PM.

NOTE: Providing equal amount of food daily is critical. In the case of mice with hyperphagia, if the pair-fed mice are provided with an amount of food less than their voluntary consumption at once, they will likely consume all provided food and become effectively fasted. This may then prevent proper comparison to IF-treated mice and confound the result.

2.4. Repeat steps 2.1–2.3 for the duration of the study.

3. Body composition analysis

NOTE: Since long-term IF affects body weight in mice, body composition can be measured at appropriate cycles (e.g., every 3 or 4 cycles) using a body composition analyzer to quantify fat and lean mass in live, non-anesthetized mice.

3.1. Turn on the body composition analyzer.

NOTE: Before starting the program, leave the machine on for at least 2–3 h to warm up.

3.2. Run a system test on the body composition analyzer to test its measurement accuracy. If

necessary, calibrate the system using canola oil and water samples.

3.3. Measure the body weight of each mouse.

3.4. Place the mouse in a small animal cylindric holder.

3.5. Place the holder into the body composition analyzer and insert a delimiter to constrain physical movement of the mouse during the measurement.

3.6. Run the scanning program.

NOTE: It takes approximately 90–120 s to analyze.

3.7. After measurement, remove the holder from the equipment and bring the mouse back to the cage.

NOTE: A more detailed protocol can be found in a previous publication[9].

4. Glucose and insulin tolerance tests

4.1. For glucose tolerance test (GTT), measure body weight and body composition of each mouse before subjecting to fasting and mark the tail with a permanent marker for easy and rapid indexing.

4.2. Place mice in new cages without food at 7:00 PM for overnight fasting.

NOTE: Overnight fasting is the standard protocol, yet due to mouse physiology (e.g., increased glucose utilization after prolonged fasting[10,11]), shorter fasting (~6 h) can be used as described for ITT.

4.3. After fasting 14–16 h (9:00 AM in the following morning), measure body weight and body composition of each mouse and calculate the amount of glucose dosage based on body weight.

NOTE: To avoid overestimation of glucose intolerance in obese mice, lean mass obtained from the body composition analysis can be used to calculate glucose dosage[12,13].

4.4. For each mouse, cut the tip of the tail (0.5–1.0 mm) using clean surgical scissors. After wiping off the first drop of blood, draw a fresh drop of blood from the tail and measure baseline fasting blood glucose level with the glucometer.

4.5. Subject mice to an intraperitoneal injection of glucose (1 mg/g of body weight).

NOTE: Based on the objective of an experiment (e.g., examining incretin effects), oral administration of glucose can be performed by oral gavage. The protocol for oral GTT (OGTT)

can be found in another study[14].

4.6. Measure blood glucose from the tail at 0, 5, 15, 30, 60, and 120 min post-glucose injection.

4.7. After finishing the GTT, provide a sufficient amount of food.

4.8. For the insulin tolerance test (ITT), remove food at 9:00 AM.

NOTE: Since both GTT and ITT are stress-inducing experiences for mice that can elevate blood glucose levels and change physiology, it is recommended to perform ITT after providing at least 2–3 days of recovery after GTT experiment.

4.9. After fasting for 6 h (3:00 PM), measure baseline blood glucose from the tail as described in step 4.4.

4.10. Subject mice to i.p. injection of insulin (0.65 mU/g of body weight).

4.11. Measure blood glucose from the tail at 0, 15, 30, 60, 90, and 120 min post-insulin injection.

4.12. After finishing ITT, provide a sufficient amount of food.

5. Indirect calorimetry

NOTE: Energy metabolism of IF-treated mice can be further evaluated through indirect calorimetry over a single cycle of IF. This will measure oxygen consumption (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER), and heat (kcal/h).

5.1. Turn on the power of the indirect calorimeter system at least 2 h before running the experiment.

NOTE: This system warm-up is important for accurate measurement.

5.2. Prepare cages with clean bedding, fill water bottles, and add the pre-weighed amount of chow to the food hoppers.

5.3. Check the condition of the Drierite and lime soda. If a color indicator of the Drierite appears pink, which indicates that the Drierite has absorbed a high amount of moisture, it is necessary to replace or top with fresh Drierite.

5.4. Calibrate the system using a gas with the specific composition (0.5% CO_2, 20.5% O_2).

5.5. Measure body weight and body composition of each mouse, which will be used to normalize VO_2 and VCO_2 data.

5.6. Gently place one mouse per cage.

5.7. Assemble metabolic cages, place them in the temperature-controlled environment chamber, and connect to gas lines and activity sensor cable.

5.8. After setting up the experiment profile by adding appropriate experimental parameters using the software, run the program for measurement. The purpose of the first day's measurement is to provide a period of acclimatization and measure a baseline energy metabolism.

5.9. At 12:00 PM the following day, subject mice to 24 h of fasting by removing food and crumbs from the hopper and bottom of the cage. If necessary, replace with clean bedding.

5.10. After 24 h, add the pre-weighed amount of chow to the food hopper for the refeeding period.

5.11. Continue to measure for the next 48 h. Check regularly whether the system is running without hardware or software interruption.

5.12. After completing measurement, terminate the program and bring mice back to their original cages. Measure the amount of leftover food to examine food intake.

5.13. The detailed protocol for indirectly calorimetry can be found in a previous study[9].

REPRESENTATIVE RESULTS:

Figure 1 shows the feeding analyses after 24 h fasting and the comparison between 1:1 and 2:1 intermittent fasting. A 24 h fasting period resulted in a ~10% reduction in body weight, which was fully recovered after 2 days of refeeding (**Figure 1A**). A 24 h fasting period induced hyperphagia during the subsequent 2 days of refeeding (**Figure 1B**). Nevertheless, the comparison of energy intake between 1:1 alternative day fasting and 2:1 intermittent fasting revealed that the 1 day of the refeeding period in 1:1 IF was not sufficient (~80%) to compensate for the caloric loss by fasting, compared to the AL condition (**Figure 1C**). On the other hand, 99% of energy intake was fully compensated during 2 days of refeeding in 2:1 IF. This regimen enables examination of the effects of isocaloric IF that are independent of caloric intake difference.

Figure 2 illustrates a schematic timeline for the isocaloric 2:1 IF and PF regimens. To minimize the differences in caloric intake, an observation made in alternate day fasting[6,7], this protocol established a new IF regimen comprising of 2 day feeding and 1 day fasting periods (2:1 IF)[8], which enabled the examination of the health effects of isocaloric IF in wild-type mice. However, in *ob/ob* mice, which exhibited hyperphagic behavior, 2:1 IF-treated *ob/ob* mice showed a 21% caloric intake reduction, compared to *ob/ob* AL mice[15]. Since this prevents a proper caloric-

independent comparison, a PF control group that maintained the same caloric intake as IF-treated *ob/ob* mice was used. Briefly, the total amount of food consumed during 2 days of feeding in 2:1 IF mice were divided equally into three daily amounts, then provided to the PF group.

For a comprehensive overview on the metabolic outcomes of 2:1 IF, we compared the effects of AL, IF, and PF in body weight, food intake and body composition in wild-type and *ob/o*b mice under normal diet (ND) and HFD. Compared to AL, IF treatment led to lower body weight increase in ND-fed and HFD-fed WT mice without significant differences in food intake (**Figure 3A,B**). Body composition analysis revealed that IF specifically reduced fat mass without changes in lean mass in wild-type mice (**Figure 3C**). It is possible that a slightly, albeit not significantly, lower accumulated energy intake over 16 weeks of the IF program could result in reduced body weight gain of IF animals. However, IF experiment with the pair-feeding regimen confirmed that the decreased body weight gain by IF was not due to altered energy intake (**Figure 3D,E**). Unlike wild-type animals, body weight of *ob/ob* mice subjected to IF (Ob-IF) was lower than that of Ob-AL mice (**Figure 3G**). This is due to hyperphagia (excessive eating) of *ob/ob* mice, leading to mildly higher (21%) food intake in AL mice, compared to IF-treated animals (**Figure 3H**). Therefore, to specifically examine the metabolic effect of IF in a caloric-independent manner, a pair-feeding control group was employed. However, unlike wild-type mice[8], Ob-PF mice were indistinguishable compared to Ob-IF mice in body weights and body composition[15] (**Figure 3I**). These results suggest that leptin is likely implicated in isocaloric IF-mediated body weight reduction in mice.

The major metabolic benefit conferred by isocaloric IF is the improved glucose homeostasis. As shown in **Figure 4A,B,C,D**, HFD-IF mice exhibited a significant improvement in glucose homeostasis. GTT showed that blood glucose is more rapidly cleared in IF-treated mice, while ITT revealed higher insulin sensitivity in HFD-IF mice, compared to HFD-AL or HFD-PF mice. Unexpectedly, despite the failures in IF-mediated weight reduction, Ob-IF animals exhibited significantly improved glucose handling with smaller glucose excursions in GTT, compared to Ob-PF mice (**Figure 4E**), whereas insulin sensitivity was indistinguishable between Ob-IF and Ob-PF mice (**Figure 4F**). This improved glucose homeostasis in Ob-IF mice is likely mediated by increases in plasma level of glucagon-like peptide-1 (GLP-1) and glucose-stimulated insulin secretion (data not shown)[15]. Overall, by using this 2:1 IF protocol and proper caloric-independent PF control, we showed the metabolic benefits of isocaloric IF in wild-type and *ob/ob* mice.

One of the metabolic effects of IF in wild-type mice is higher total O_2 consumption, used to estimate the energy expenditure (**Figure 5A,B**). This elevation in O_2 consumption was found only during feeding period in IF mice, but not fasting period, compared to AL mice. The increased energy expenditure was largely mediated by adipose thermogenesis, such as browning of white adipose tissues and activation of brown adipose tissue (data not shown)[8,16]. IF-mediated adipose thermogenesis would presumably explain how wild-type mice subjected to IF exhibited the reduced body weight gain with no difference in food intake, compared to AL mice. On the other hand, IF failed to increase O_2 consumption in *ob/ob* mice (**Figure 5C-D**), and

even led to a reduction in energy expenditure during fasting period. Consistently, IF-induced adipose thermogenesis was completely abolished in *ob/ob* mice (data not shown). These data suggest a possible limitation of IF as it may work differently for individuals with different genetic and environmental backgrounds.

FIGURE AND TABLE LEGENDS:

Figure 1: Feeding analyses after 24 h fasting and comparison between 1:1 and 2:1 IF. (**A**) Daily body weight changes of mice before and after 24 h fasting (n = 10). (**B**) Daily energy intake before and after 24 h fasting (n = 5 cages; 2 mice per cage). (**C**) Comparison of energy intake between alternate day fasting (i.e., 1 day feeding/1 day fasting, 1:1 IF) and 2:1 intermittent fasting (i.e., 2 day feeding/1 day fasting). In the 1:1 IF regime, only ~80% of food intake was compensated during the subsequent 1 day of refeeding compared to food intake over 2 days of feeding. On the other hand, 99% of energy intake was achieved when 2 days of refeeding was given, compared to that over 3 days of feeding. Data are expressed as mean ± SEM. This figure was reproduced with permission from Kim et al.[8].

Figure 2: Schematic illustration of the isocaloric 2:1 IF regimen. For PF control, the amount of food consumed during the 2 days of feeding by IF-treated mice is divided into three equal portions, which is then provided daily to PF mice during the next cycle. AL = *ad libitum*; PF = pair-feeding. Part of this figure was reproduced with permission from Kim et al.[8].

Figure 3: Comparison of AL, IF, and PF effects on body weight, food intake, and body composition between wild-type and *ob/ob* mice. (**A,B,C**) Body weight, food intake, and body composition in AL or IF-treated wild-type mice under normal diet (ND) or high fat diet (HFD) during 16 weeks of IF regimen. Data are expressed as mean ± SEM. (ND-AL: n = 7; ND-IF: n = 8; HFD-AL: n = 7; and HFD-IF: n = 8); one- or two-way ANOVA with Student-Newman-Keuls post-hoc analysis; **p < 0.01 *vs*. HFD-AL. (**D,E,F**) Body weight, food intake, and body composition in PF *vs.* IF mice fed with high fat diet (HFD) during 12 weeks of IF regimen. (PF: n = 6 and IF: n = 6); two-tailed unpaired Student's *t*-test; *p < 0.05 *vs*. HFD-PF; NS = not significant. (**G,H,I**) Body weight, food intake, and body composition in AL, PF, or IF-treated *ob/ob* mice fed with normal chow (Ob-AL: n = 4; Ob-PF: n = 7; Ob-IF: n = 6); Ob-AL *vs*. Ob-PF: *p < 0.05; Ob-AL *vs*. Ob-IF: *p < 0.05; Ob-PF *vs*. Ob-IF. Panels A–F were reproduced with permission from Kim et al.[8]. Panels G–I were reproduced with permission from Kim et al.[15].

Figure 4: Improved glucose homeostasis by IF in both wild-type and *ob/ob* mice. (**A,B**) Intraperitoneal GTT and ITT in HFD-AL and HFD-IF wild-type mice after 16 weeks of IF regimen. The inset shows area under curve (AUC); *p < 0.05 *vs.* HFD-AL. (**C,D**) GTT and ITT in HFD-PF compared to HFD-IF wild-type mice after 12 weeks of IF regimen. The inset shows AUC; *p < 0.05 *vs.* HFD-PF. (**E,F**) GTT and ITT in Ob-IF compared to Ob-PF mice after 16 weeks of IF regimen. The inset shows AUC (*p < 0.05 *vs*. Ob-PF). Panels A–D were reproduced with permission from Kim et al.[8]. Panels E and F were reproduced with permission from Kim et al.[15].

Figure 5: Energy expenditure analysis in IF-treated wild-type and *ob/ob* mice. (**A**) Traces of O_2

consumption during one cycle of 2:1 IF in wild-type mice (i.e., 1 day fasting followed by 2 days of feeding). (**B**) Average of O_2 consumption per hour during fasting, feeding, and one cycle of 2:1 IF. Data are expressed as mean ± SEM (HFD-AL: n = 6; and HFD-IF: n = 12); *p < 0.05 *vs*. HFD-AL. (**C**) O_2 consumption traces of *ob/ob* mice during one cycle of 2:1 IF. (**D**) Average of O_2 consumption per hour during fasting, feeding, and one cycle of 2:1 IF (Ob-PF: n = 7; Ob-IF: n = 6); *p < 0.05 *vs*. Ob-PF. Panel B was reproduced with permission from Kim et al.[8]. Panels C and D were reproduced with permission from Kim et al.[15].

DISCUSSION:

It has been well-documented that IF provides beneficial health effects on various diseases in both humans and animals[8,15-19]. Its underlying mechanisms, such as autophagy and gut microbiome, have recently been elucidated. The presented protocol describes an isocaloric 2:1 IF regimen in mice for investigating calorie-independent metabolic benefits of IF against diet-induced obesity and associated metabolic dysfunction. Unlike the alternate day fasting (1:1 IF) protocol that results in a reduction in overall caloric intake[6,7], providing 1 more day of refeeding in the 2:1 IF regimen enables maintenance of an isocaloric condition in wild-type mice.

Additionally, compared to 1:1 IF, the 2:1 IF regimen may reduce possible fasting-mediated stress or torpor in mice[20] and is also comparable to a popular dietary method, the 5:2 diet[2]. Although its effects have not been tested, the regimen can be modified by providing additional days of refeeding (e.g., 3:1 or 4:1 IF). Moreover, this protocol presented can be easily adjusted to an hourly-scale called time-restricted feeding (TRF), in which access to food is limited to 8 h per day during the active phase[21], which is known to achieve an isocaloric diet regimen and provide metabolic benefits against HFD -induced obesity and diabetes[19,21,22].

As shown in the feeding analysis (**Figure 1B**), hyperphagic behavior immediately after 24 h of fasting decreases gradually in wild-type mice, which enables isocaloric IF. However, this isocaloric condition cannot be attained in *ob/ob* mice, as they lack leptin signaling-mediated satiety and energy metabolism, leading to a continuous hyperphagic phenotype[23,24]. Therefore, before performing an IF experiment, it is recommended to examine feeding behavior of the mouse model of interest. To examine the effects of IF using a hyperphagic mouse model (e.g., *ob/ob*, *db/db*, *Sim1*$^{+/-}$, *MC4R*$^{-/-}$)[24-26], as described in this protocol, employment of a pair-feeding group as an isocaloric experimental control is important for making proper comparisons. It also requires careful planning when testing a mouse model with a hypophagic phenotype (e.g., melanin-containing hormone KO mice)[27].

An important factor to consider for IF studies is housing temperature, which affects various physiological and behavioral parameters in mice. Particularly, cold exposure (4–6 °C) significantly increases energy intake to maintain core body temperature[28]. In contrast, in thermoneutral conditions (30 °C) under which heat gain is balanced by heat loss, reductions in food consumption is markedly reduced[8]. With respect to metabolic outcomes, cold exposure induces adipose thermogenesis, which is hampered by thermoneutral condition. Therefore, it is expected that housing temperature influences the metabolic phenotypes of IF and

appropriate feeding:fasting ratio to achieve isocaloric IF.

Indeed, it has been previously demonstrated that isocaloric 2:1 IF can be achieved in thermoneutral conditions, leading to improved metabolic health in diet-induced obesity and metabolic dysfunction without differences in food intake between IF and AL groups[8]. However, isocaloric IF may not be achievable with 2:1 ratio at cold temperatures because mice under cold exposure will show a hyperphagic phenotype, which leads to underfeeding in the IF group. Since cold exposure and IF display comparable metabolic outcomes and mechanisms (i.e., adipose thermogenesis and improved glucose homeostasis) that help fight obesity, there is interest in combining these two interventions to maximize metabolic impact. Therefore, to properly test this, performing the feeding analysis before running an IF experiment and utilizing a pair-feeding control group under cold exposure are recommended.

Other factors that may potentially affect the outcomes of IF studies include housing density. Similar to the previous study, which showed reduced food consumption in more densely housed mice[29], mice from a cage of five consumed significantly less food than those from a cage of two (unpublished results). In addition, it has been demonstrated that housing density significantly affects ambient temperature, as the temperature inside cages that house five mice is 1–2 °C higher than the ambient temperature[30]. Although this study concluded that housing density did not significantly affect food intake (examined for 5 weeks), in an IF study lasting 12–16 weeks, temperature inside the cage affected by housing density may still influence food intake and energy metabolism. Together, it is important to keep the same number of mice housed in a cage and minimize changing the number per cage over the course of a study.

In summary, this report shows a simple and reproducible protocol for testing isocaloric 2:1 IF in mice. Although the current study is focused on metabolic benefits of IF in diet-induced obesity and metabolic dysfunction, it can be easily adapted to investigate the protective and therapeutic effects of isocaloric IF against other conditions, such as cardiovascular and neurological diseases.

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The authors have nothing to disclose.

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Figure 1

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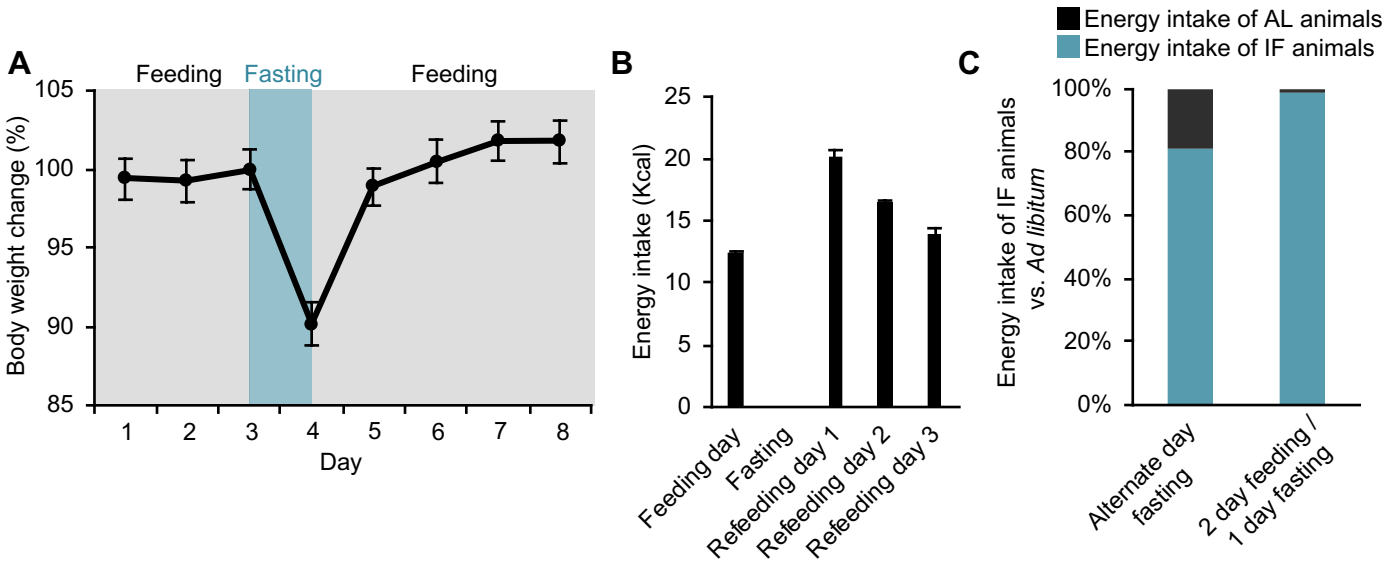

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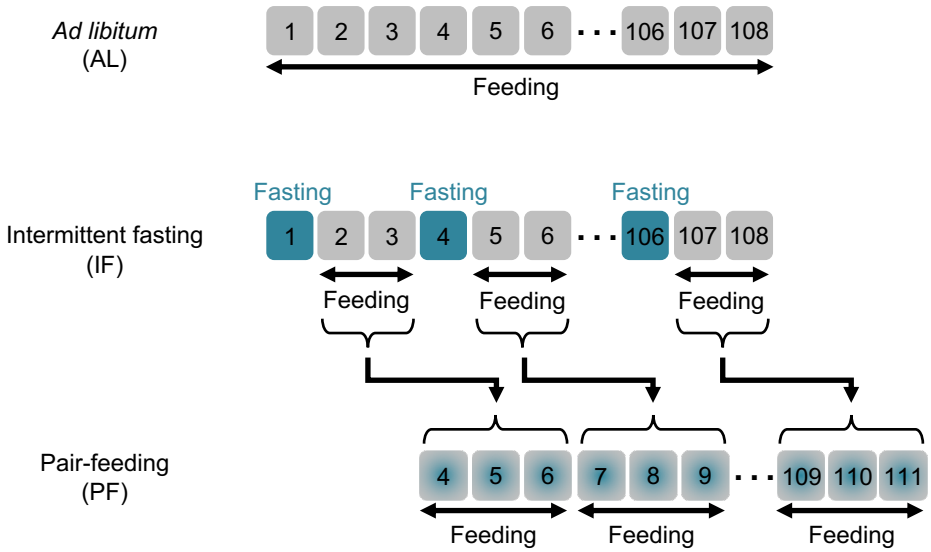

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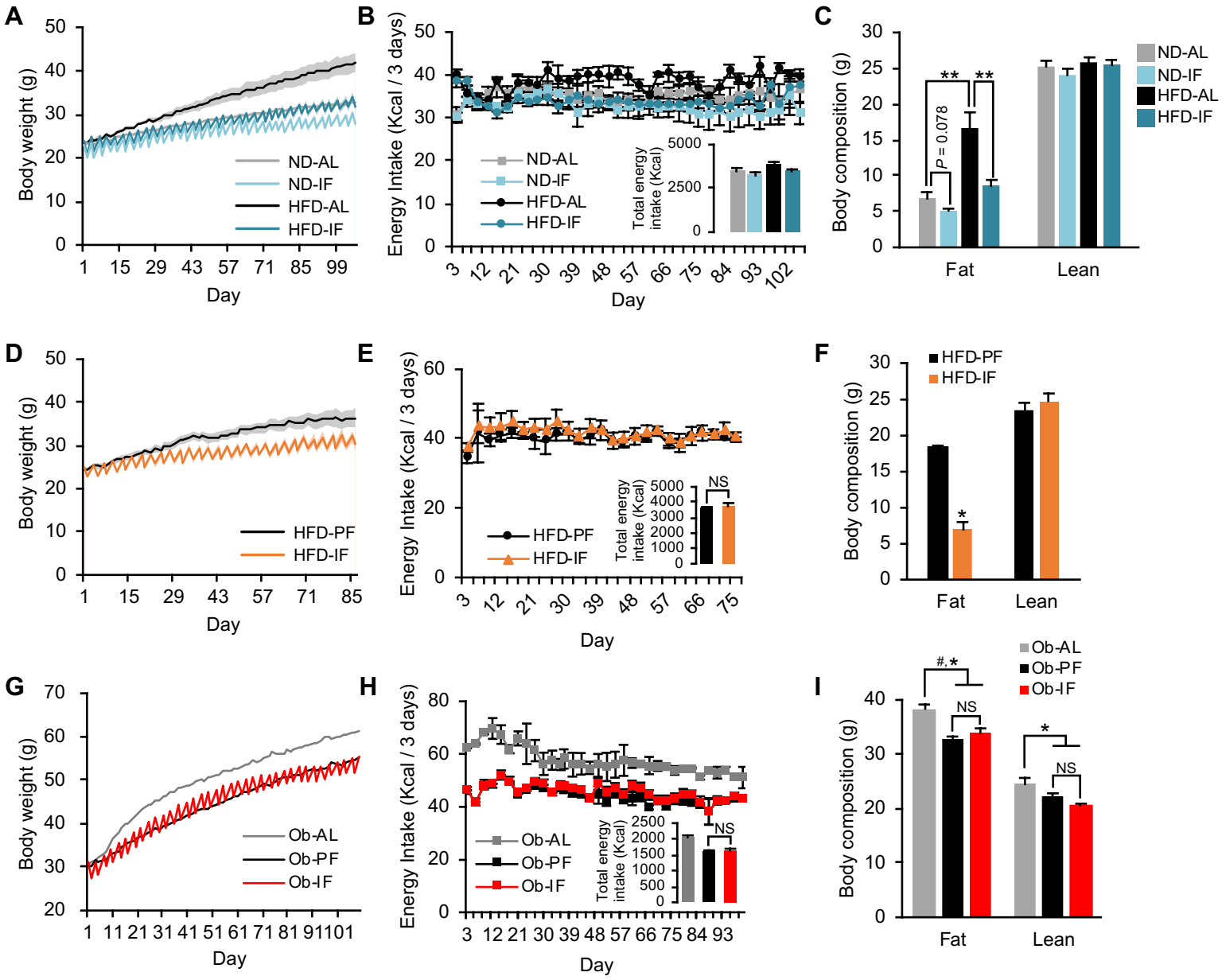

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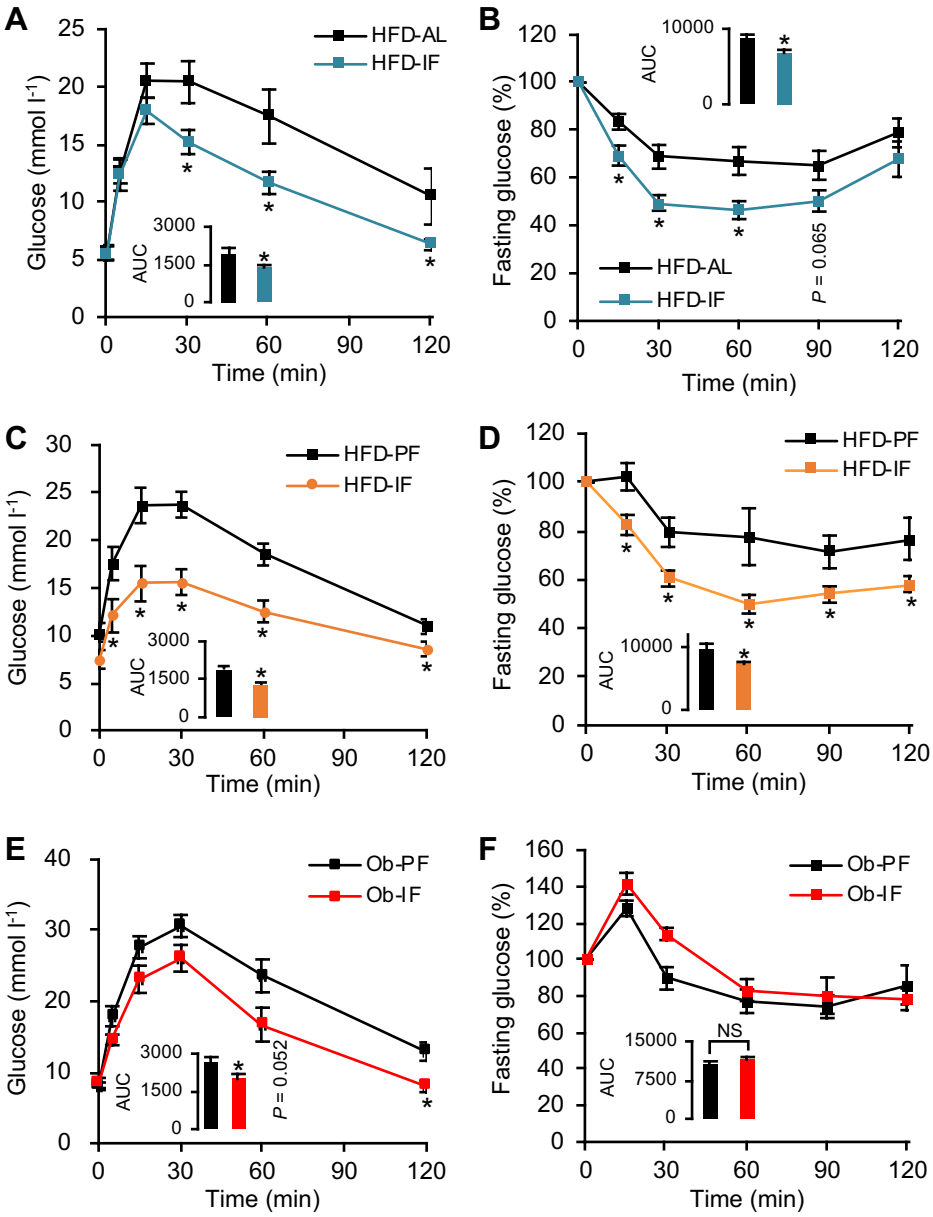

Figure 5

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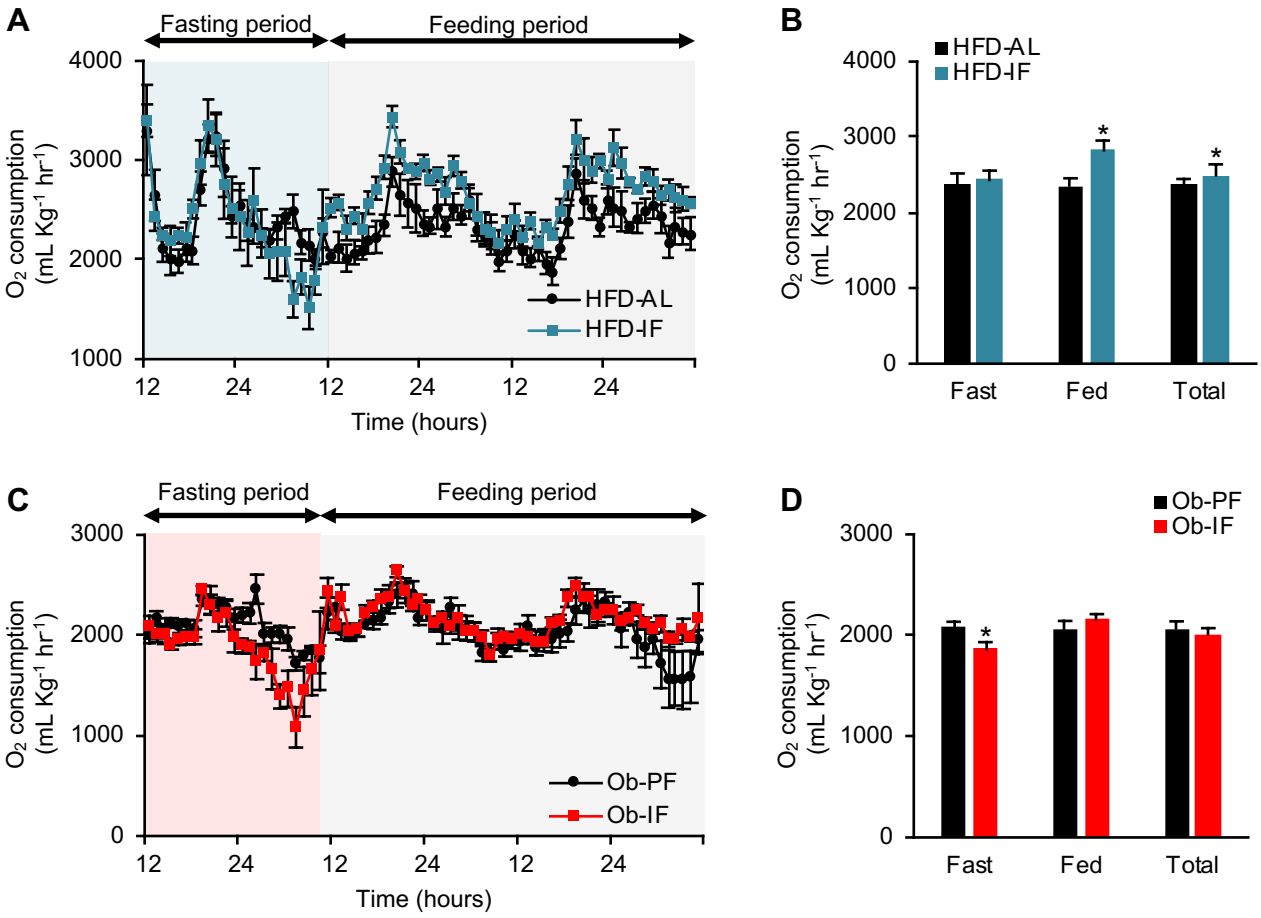

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Comprehensive Lab Animal Monitoring System (CLAMS)	Columbus Instruments		Indirect calorimeter
D-(+)-Glucose solution	Sigma-Aldrich	G8769	For GTT
EchoMRI 3-in-1	EchoMRI	EchoMRI 3-in-1	Body composition analysis
Glucometer and strips	Bayer	Contour NEXT	These are for GTT and ITT experiments
High Fat Diet (45% Kcal% fat)	Research Diets Inc.	#D12451	3.3 Kcal/g
High Fat Diet (60% Kcal% fat)	Research Diets Inc.	#D12452	4.73 Kcal/g
Insulin	El Lilly	Humulin R	For ITT
Mouse Strain: B6.Cg-Lepob/J	The Jackson Laboratory	#000632	Ob/Ob mouse
Mouse Strain: C57BL/6J	The Jackson Laboratory	#000664	
Normal chow (17% Kcal% fat)	Harlan	#2918	
Scale	Mettler Toledo		Body weight and food intake measurement

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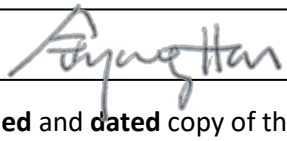
Signature:	
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We thank the editor and two reviewers for the helpful comments to improve our manuscript. As described in the detailed point-by-point rebuttal below, we have carefully addressed all the concerns and suggestions raised by the reviewers in the revised manuscript.

We wish that you and the reviewers would be satisfied with our revision. Should you need additional information, please let us know.

Thank you again for your kind consideration of our manuscript.

Sincerely,

Kyoung-Han Kim and Hoon-Ki Sung

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you. We carefully went through the manuscript for grammatical and spelling errors during this revision.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Thank you. We have formatted our revised manuscript based on the JoVE guidelines.

3. Please revise lines 223-230 and 237-241 to avoid overlap with previous publications.

Thank you for the comment and sorry the overlapping issue with our previous work. To address this, we have re-written the indicated lines to avoid the overlapping as follows:

PREVIOUS VERSION (lines 223-230 of the original manuscript): Body composition analysis revealed that IF specifically reduced fat mass without changes in lean mass in wild-type mice (**Figure 3C**). We speculated that the lower body weight of IF animals might be attributed to the slight decrease in accumulated energy intake over 16 weeks of the IF program. However, IF experiment with the pair-feeding regimen revealed that IF-mediated decrease in body weight is not attributed to an energy intake difference (**Figure 3D-E**). Unlike wild-type animals, *ob/ob* mice subjected to IF exhibited lower body weight than Ob-AL mice (**Figure 3G**). This is due to hyperphagic behaviors of *ob/ob* mice, leading to mild (21%) increase in total food intake in AL mice, compared to IF-treated animals (**Figure 3H**).

→ REVISED VERSION (lines 317-325 of the revised manuscript): Body composition analysis revealed that IF specifically reduced fat mass without changes in lean mass in wild-type mice (**Figure 3C**). It is possible that a slightly, albeit not significantly, lower accumulated energy intake over 16 weeks of the IF program could result in reduced body weight gain of IF animals. However, IF experiment with the pair-feeding regimen confirmed that the decreased body weight gain by IF was not due to altered energy intake (**Figure 3D-E**). Unlike wild-type animals, body weight of *ob/ob* mice subjected to IF (Ob-IF) was lower than that of Ob-AL mice (**Figure 3G**). This is due to hyperphagia (excessive eating) of *ob/ob* mice, leading to mildly higher (21%) food intake in AL mice, compared to IF-treated animals (**Figure 3H**).

PREVIOUS VERSION (lines 237-241 of the original manuscript): As shown in **Figure 4A-D**, HFD-IF mice showed improved glucose homeostasis with smaller glucose excursion in GTT, increased insulin sensitivity in ITT, compared to HFD-AL or HFD-PF mice.

→ REVISED VERSION (lines 331-335 of the revised manuscript): As shown in **Figure 4A-D**, HFD-IF mice exhibited a significant improvement in glucose homeostasis. GTT showed that blood glucose is more rapidly cleared in IF-treated mice, while ITT revealed higher insulin sensitivity in HFD-IF mice, compared to HFD-AL or HFD-PF mice.

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For example: Harlan, Research Diets, Echo-MRI

Thank you. We have removed commercial language, including Harlan and Research Diets and replaced them with generic terms.

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Thank you for the comment. We have revised our manuscript to address this comment.

Figures:
1. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, or .pdf file (4 in total). Please remove ‘Figure 1’ etc. from the figures themselves.

We have made individual PDF files for each figure. ‘Figure X ’ labelling of each figure has been removed.

2. Figure 1: Please define the error bars. Also, please use ‘day 1/2/3’ instead of ‘day1/2/3’ (include a space).

Thank you for the comments. The definition of the error bars for Figure 1 has been added and the X-axis title, Day 1/2/3, has been modified.

References:
1. Please do not abbreviate journal titles.

We have revised references with non-abbreviated journal titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Thank you. We have added all materials and equipment used in the protocol to the Tagble of Materials.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This manuscript provides a valuable perspective on the appropriate methodology to apply to intermittent fasting as a nutritional intervention, particularly to ensure that the fasting effects can be isolated from additionally inadvertently underfeeding. The authors provide evidence describing the importance of applying a 2-day feeding to 1-day fasting interval, relative to a 1-day feeding:1-day fasting, to ensure that the protocol provides sufficient energy in the refeeding period to compensate for the energy deficit accrued in the fasting day. The authors also provide evidence for the application of pair-feeding in particular animal models or experimental approaches expected to alter feeding behavior. The authors provide a comprehensive description of the 2-day feeding:1-day fasting isocaloric intermittent fasting feeding regimen, the application of a Pair-feeding control group within this context, a description of the body composition analysis and description of the glucose and insulin tolerance tests and provide data representing the application of these feeding approaches.

Major Concerns:
This manuscript nicely describes the application of the intermittent fasting feeding regimen on body composition and glucose homeostasis and ensuring that energy intake is carefully matched. However, energy balance and therefore body composition depends not only on energy intake but also energy expenditure. While the described protocol very nicely controls for the energy intake, it would be worth providing representative data on how such an intervention can alter energy expenditure to provide readers further perspective on the phenotype that might be manifested under this type of regimen and its influence on energy balance. For example, the HFD-IF show a reduced increase in body weight relative to their HFD-PF, clearly showing that the fasting itself is eliciting an effect on body weight. Since energy intake is matched, presumably this difference in body weight gain is due to an increase in whole-body energy expenditure in the HFD-IF. In contrast, the Ob-PF show no difference in body weight relative to the Ob-IF, yet oxygen consumption is lower only in the fasted state in the Ob-IF (2nd attached manuscript). These are very interesting findings resulting from the fasting itself.

We thank the reviewer for the positive comments and in particular, very constructive suggestions about energy expenditure data.

We cannot agree more that one of main mechanisms of IF-mediated metabolic benefits is adipose thermogenesis thereby increasing energy expenditure without affecting food intake. Particularly, we have previously shown that energy expenditure was increased during refeeding period after fasting and it is largely mediated by adipose thermogenesis, including browning of white adipose tissue and activation of brown adipose tissue [Kim et al, Cell Res, 2017]. On the other hand, in our follow-up study, we have demonstrated that this elevated energy expenditure with increased adipose browning was not evident in IF-treated *ob/ob* mice, suggesting that the effect of IF on energy expenditure can be changed in different types of obesity [Kim et al., Sci Rep, 2019]. As the reviewer commented, data above would presumably explain why wild-type mice subjected to IF exhibited the reduced body weight gain without a difference in food intake, compared to AL mice. However, we respectively disagree that the body weight reduction effect by IF is mediated by IF because we have shown that adipose-specific VEGF KO (Ad-VEGF-KO) mice, which exhibited impaired IF-induced adipose thermogenesis, still showed reduced body weight gain compared to AL-treated Ad-VEGF-KO mice. This suggests the absence of effect in body weight gain observed in IF-treated *ob/ob* mice is not simply due to inhibited thermogenesis, but likely caused by lack of leptin's metabolic functions. Nevertheless, we agree with the reviewer that the addition of energy expenditure data is beneficial to potential readers who would use this protocol.

To address the Reviewer's comment, thus, we have added new representative results of energy expenditure in IF-treated wild-type and *ob/ob* mice as Figure 5, and have demonstrated that as follows:

(lines 343-354 of the revised manuscript)
One of the metabolic effects of IF in wild-type mice is higher total O_2 consumption, used to estimate the energy expenditure (**Figure 5A-B**). This elevation in O_2 consumption was found only during feeding period in IF mice, but not fasting period, compared to AL mice. The increased energy expenditure was largely mediated by adipose thermogenesis, such as browning of white adipose tissues and activation of brown adipose tissue (data not shown)[8,16]. IF-mediated adipose thermogenesis would presumably explain how wild-type mice subjected to IF exhibited the reduced body weight gain with no difference in food intake, compared to AL mice. On the other hand, IF failed to increase O_2 consumption in *ob/ob* mice (**Figure 5C-D**), and even led to a reduction in energy expenditure during fasting period. Consistently, IF-induced adipose thermogenesis was completely abolished in *ob/ob* mice (data not shown). These data suggest a possible limitation of IF as it may work differently for individuals with different genetic and environmental backgrounds.

Minor Concerns:
The Discussion describes two topics that are not particularly discussed in the Introduction or addressed in the Methods (housing density and temperature). I wonder whether it is worth describing how housing temperature can influence the phenotype and/or the fasting:feeding ratio - is it worth having more refeeding days if exposed to a colder environment? Can you run such a protocol in a cold environment? If so, how cold? Some animals struggle to survive in the cold when fasted for a prolonged period. This might be worth discussing as many groups are interested in combining these two metabolic stimuli.

We appreciate the reviewer's suggestion on housing temperature. It is indeed one of the most influential factors that largely affects feeding behaviour. For example, cold exposure around 4-6°C significantly increases energy intake to maintain core body temperature, whereas thermoneutral condition (30°C), where heat gain is balanced by heat loss, results in marked

reductions in food consumption. To address the reviewer's suggestion and questions, we have added a new paragraph in the Discussion as shown below. In particular, we discussed about a current interest in combining two interventions, IF and cold exposure, to maximize the metabolic benefits. We are hoping this revision improves the quality of our manuscript. We deeply thank the reviewer again for the supports.

(lines 471-488 of the revised manuscript)
An important factor to be considered for IF studies is housing temperature, which affects various physiological and behavioral parameters in mice. Particularly, cold exposure (4-6°C) significantly increases energy intake to maintain core body temperature[28], whereas under thermoneutral condition (30°C), where heat gain is balanced by heat loss, reductions in food consumption is markedly reduced[8]. With respect to metabolic outcomes, cold exposure induces adipose thermogenesis, which is hampered by thermoneutral condition. Therefore, it is expected that housing temperature would influence the metabolic phenotype of IF and the appropriate feeding-fasting ratio to achieve isocaloric IF. Indeed, we have previously demonstrated that isocaloric 2:1 IF can be achieved in thermoneutral condition, leading to improved metabolic health against diet-induced obesity and metabolic dysfunction without a differences in food intake between IF and AL groups[8]. However, isocaloric IF might not be achievable with 2:1 ratio at cold temperature because mice under cold exposure would show a hyperphagic phenotype, which would lead to underfeeding in the IF group. Since cold exposure and IF display comparable metabolic outcomes and mechanisms, such as adipose thermogenesis and improved glucose homeostasis, against obesity, there are interests in combining these two interventions to maximize the metabolic impacts. To properly test this, therefore, performing the feeding analysis before running IF experiment and utilizing a pair-feeding control group under cold exposure are recommended.

Reviewer #2:

Manuscript Summary:
This metods article describes protocols for isocaloric 2:1 intermittent fasting (IF) and pair-feeding that would be useful for studies investigating the effects of IF on other disease conditions. Overall, it is well summarized methods article on the IF using a mouse model and I have a minor point:

Minor Concerns:
- Introduction (the 2nd paragraph): It is recommended that the explanations on Figures 1A and 1B need to be more specifically described. It is not clear that the sentence in lines 64-65 is related with Figure 1C or 1A/1B.

We thank the reviewer for the positive comments. We also apologize if you felt that the paragraph in the Introduction was confusing. Our purpose was providing a brief introduction about 2:1 intermittent fasting (IF), compared to alternate day fasting, rather than specific description, since we provide the detail descriptions on results shown in Figure 1A-C in the sections of the REPRESENTATIVE RESULTS and FIGURE LEGENDS. To minimize a possible confusion, we have removed the indication to Figure 1 and we believe that in that way readers can easily find the detailed description below. We hope this change makes the revised manuscript improved and we thank the reviewer again for the support and comments.

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ORIGINAL ARTICLE

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Intermittent fasting promotes adipose thermogenesis and metabolic homeostasis via VEGF-mediated alternative activation of macrophage

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Intermittent fasting (IF), a periodic energy restriction, has been shown to provide health benefits equivalent to prolonged fasting or caloric restriction. However, our understanding of the underlying mechanisms of IF-mediated metabolic benefits is limited. Here we show that isocaloric IF improves metabolic homeostasis against diet-induced obesity and metabolic dysfunction primarily through adipose thermogenesis in mice. IF-induced metabolic benefits require fasting-mediated increases of vascular endothelial growth factor (VEGF) expression in white adipose tissue (WAT). Furthermore, periodic adipose-VEGF overexpression could recapitulate the metabolic improvement of IF in non-fasted animals. Importantly, fasting and adipose-VEGF induce alternative activation of adipose macrophage, which is critical for thermogenesis. Human adipose gene analysis further revealed a positive correlation of adipose VEGF-M2 macrophage-WAT browning axis. The present study uncovers the molecular mechanism of IF-mediated metabolic benefit and suggests that isocaloric IF can be a preventive and therapeutic approach against obesity and metabolic disorders.

Keywords: intermittent fasting; thermogenesis; vascular endothelial growth factor; adipose macrophage
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Introduction

While fat (white adipose tissue, WAT) is often associated with development of obesity and type 2 diabetes, it is essential for energy homeostasis by storing excess energy and releasing lipids in response to energy deficits [1, 2]. Recent studies have discovered that WAT also contributes to whole-body metabolism by regulating thermogenic activity via the browning of WAT, which increases energy expenditure and improves insulin sensitivity [3]. In this regard, WAT browning has been suggested as a therapeutic approach for obesity and metabolic diseases.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)

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Thermogenesis-independent metabolic benefits conferred by isocaloric intermittent fasting in *ob/ob* mice

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Intermittent fasting (IF) is an effective dietary intervention to counteract obesity-associated metabolic abnormalities. Previously, we and others have highlighted white adipose tissue (WAT) browning as the main underlying mechanism of IF-mediated metabolic benefits. However, whether IF retains its efficacy in different models, such as genetically obese/diabetic animals, is unknown. Here, leptin-deficient *ob/ob* mice were subjected to 16 weeks of isocaloric IF, and comprehensive metabolic phenotyping was conducted to assess the metabolic effects of IF. Unlike our previous study, isocaloric IF-subjected *ob/ob* animals failed to exhibit reduced body weight gain, lower fat mass, or decreased liver lipid accumulation. Moreover, isocaloric IF did not result in increased thermogenesis nor induce WAT browning in *ob/ob* mice. These findings indicate that isocaloric IF may not be an effective approach for regulating body weight in *ob/ob* animals, posing the possible limitations of IF to treat obesity. However, despite the lack of improvement in insulin sensitivity, isocaloric IF-subjected *ob/ob* animals displayed improved glucose tolerance as well as higher postprandial insulin level, with elevated incretin expression, suggesting that isocaloric IF is effective in improving nutrient-stimulated insulin secretion. Together, this study uncovers the insulinotropic effect of isocaloric IF, independent of adipose thermogenesis, which is potentially complementary for the treatment of type 2 diabetes.

Over the past few decades, the prevalence of obesity has dramatically increased across all genders and age groups, reaching a global epidemic level. As obesity is strongly associated with the development of other chronic health conditions, such as type 2 diabetes, hypertension, and non-alcoholic fatty liver disease (NAFLD), development of feasible and practical treatments to counteract obesity is urgently needed. A number of factors contribute to obesity, including genetic determinants, environmental and behavioural traits[1–3]. In particular, polymorphisms in various genes regulating appetite and metabolic rate were identified to predispose individuals to obesity.

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Author Contributions

K.H.K., H.K.S. and J.R.K. designed the project. J.H.L., K.H.K., H.K.S. and Y.H.K. wrote the manuscript. Y.H.K., J.H.L., J.E.S., E.D., Y.J. and J.H.M. performed mouse metabolic experiments. Y.H.K. analysed mouse metabolic data. J.H.L., Y.H.K. and J.L.Y. performed gene expression analysis, with assistance from Y.J. Tissue process and histology staining were conducted and analyzed by J.L.Y., Y.J., H.J. and N.T. R.Y.K. and N.T. performed plasma incretin level analysis. C.C.H., K.O.D. and E.M. provided scientific discussion and technical support. All authors contributed to the discussion and commented on the manuscript.

Additional Information

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