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Time-restricted feeding improves insulin resistance and hepatic steatosis in a mouse model of postmenopausal obesity

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Abstract

Background—Menopause is associated with significant hormonal changes that result in increased total body fat and abdominal fat, amplifying the risk for metabolic syndrome and diseases such as diabetes, cardiovascular disease and cancer in postmenopausal women. Intermittent fasting regimens hold significant health benefit promise for obese humans, however, regimens that include extreme daytime calorie restriction or daytime fasting are generally associated with hunger and irritability, hampering long-term compliance and adoption in the clinical setting. Time-restricted feeding (TRF), a regimen allowing eating only during a specific period in the normal circadian feeding cycle, without calorie restriction, may increase compliance and provide a more clinically viable method for reducing the detrimental metabolic consequences associated with obesity.

Methods—We tested TRF as an intervention in a mouse model of postmenopausal obesity. Metabolic parameters were measured using Clinical Laboratory Animal Monitoring System (CLAMS) and we carried out glucose tolerance tests. We also stained liver sections with oil red O to examine steatosis and measured gene expression related to gluconeogenesis.

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Author contributions
LGE, DDS, RP and NW designed the research; HC, WC and LGE conducted the study; LGE, HC, DDS and NW analyzed and formatted the data; LGE, NW and DDS wrote the manuscript and LGE had primary responsibility for the final content; all authors critically reviewed and approved the final manuscript.

Conflicts of interest
None.
Results—Preexisting metabolic disease was significantly attenuated during 7 wk of TRF. Despite having access to the same high fat diet (HFD) as ad libitum fed (ALF) mice, TRF mice experienced rapid weight loss followed by a delayed improvement in insulin resistance and a reduced severity of hepatic steatosis by having access to the HFD for only 8 h during their normal nocturnal feeding period. The lower respiratory exchange ratio in the TRF group compared with the ALF group early in the dark phase suggested that fat was the predominant fuel source in the TRF group and correlated with gene expression analyses that suggested a switch from gluconeogenesis to ketogenesis. In addition, TRF mice were more physically active than ALF fed mice.

Conclusions—Our data support further analysis of TRF as a clinically viable form of intermittent fasting to improve metabolic health due to obesity.

Keywords
Time-restricted feeding; obesity; intermittent fasting; postmenopausal; insulin resistance; hepatosteatosis; mice

1. Introduction

Obesity is a strong risk factor for type 2 diabetes and several types of cancer including breast, colon, liver, and prostate cancer. Just prior to and after menopause, women experience increased adiposity (% fat mass) that contributes to the increased incidence of obesity and the metabolic syndrome with time after menopause [1-3]. Nonalcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), which are manifestations of the metabolic syndrome and precursors to cirrhosis of the liver, also increase following menopause [4, 5]. Furthermore, inflammation and insulin resistance that are associated with obesity are known risk factors for breast cancer especially in postmenopausal women [6]. Older women consume disproportionately more health care than other segments of the population and published data document statistically significant gender differences in chronic disease risk factors, the associations of risk factors with disease, and chronic disease rates [7-10]. Considering the increased risk that obesity poses for human disease, dietary obesity prevention and intervention strategies may be effective for mitigating obesity’s harmful sequelae and reducing medical and emotional burden on society. Indeed, dieting (restricting food intake to lose weight) caloric restriction (CR: energy restriction without incurring malnutrition) and intermittent fasting (IF; cycling between fasting and non-fasting periods) can reverse many of the detrimental effects of obesity [1, 11-13]. One of the most common IF protocols is alternate day fasting (ADF) consisting of 24 h of ad libitum feeding followed by 24 h of fasting or severe CR (75-90% restriction of energy needs on 1-2 days per week) that reduces circulating cholesterol and triglycerides, and decreases blood pressure, fat mass and insulin resistance [14].

At the mechanistic level, CR reduces growth factor activation of Akt and mTORC1 and induces AMPK and the sirtuins, and CR mimetics such as metformin or everolimus are being tested in a number of cancer-related clinical trials [11, 15]. ADF reduces oxidative stress and cancer incidence in rodents [16] but has not been shown to provide any advantages over CR for weight loss in humans [17]. Despite promising data in animals [18,
these dietary interventions have not been adopted in the clinic, possibly reflecting difficulties patients face when trying to incorporate CR or ADF into their daily routine and/or problems with long-term compliance as these regimens are associated with hunger and irritability and the benefits can take weeks or months to materialize [20, 21]. Indeed, a recent IF study reported that while compliance was strong during the study period, the majority of participants found the fasting days made daily living more difficult and only 18% would adhere to the regimen if prescribed by a physician [22]. A nutritional intervention that has the same benefits but is easier to maintain would greatly increase long-term compliance.

There is increasing evidence that time-restricted feeding (TRF), the practice of restricting the time of calorie intake, but not the amount of calorie intake, to an 8-12 hour window that corresponds with daily circadian rhythms (e.g., eating during the night or “dark phase” for nocturnal mice), is an alternative approach for metabolic disease and cancer prevention that might be easier to implement in terms of compliance [13]. Food signals entrain peripheral clock rhythms and amplitudes. In fact, synchronizing feeding-fasting with normal circadian rhythm appears to improve oscillations in circadian clock gene expression, enhance energy metabolism, and reduce inflammation, while loss of circadian clock genes dysregulates metabolism and inflammatory responses [23, 24]. For example, loss of the core clock component protein cryptochrome (Cry) leads to constitutive elevation of proinflammatory cytokines in a cell-autonomous manner in mice [25] and loss of the clock gene period circadian clock 2 (Per2) causes systemic and liver-specific perturbations in glucose metabolism and altered food intake behavior [26].

TRF during the dark phase fully protects male mice from obesity, hyperinsulinemia, hepatic steatosis, and inflammation, despite their consuming the equivalent amount of calories as ad libitum-fed (ALF) mice [23, 27]. In female *Drosophila melanogaster*, TRF attenuates cardiac aging and prevents body weight gain compared with ALF, without reducing calorie intake [28]. We have found that restricting access to a pro-inflammatory, western-style high-fat diet, without restricting calorie intake, ameliorates hepatic steatosis and insulin resistance in ovariectomized (OVX) female mice, a postmenopausal model.

### 2. Materials and methods

#### 2.1 Animals and diets

All animal experiments were carried out in accordance with the guidelines of the NIH and were approved by the University of California, San Diego, Institutional Animal Care and Use Committee. Mice were maintained in a facility with a 12 h light/12 h dark cycle with water and food ad libitum. Forty-five female C57BL/6N mice (Charles River, Wilmington, MA) aged 7-8 weeks were ovariectomized (OVX) and body weights measured weekly thereafter. At 10 weeks of age 15 mice were continued on normal chow (NC; 12% kcal from fat; 3.02 kcal/g; Purina 5001, LabDiet, St. Louis, MO) and the remaining 30 mice introduced to a high fat diet (HFD; 60% kcal from fat; 5.24 kcal/g) D12492, Research Diets, New Brunswick, NJ). Both diets in pellet form were initially weighed and placed into standard wire food racks. Remaining pellets were weighed the next day to calculate the amount of
food consumed before additional pellets were added and the new total amount of food weighed.

At 19 weeks of age when the HFD mice reached an average of 40g (9 weeks of HFD feeding), they were divided into ALF and TRF groups of 15 mice each. The TRF group had access to the HFD for 8 h per day during the dark cycle from Zeitgeber time (ZT) 16 (10pm) to ZT time 0 (6am). ZT 0 is lights-on (6 am) and ZT 12 is lights-off (6 pm). The ambient room temperature for group housed mice was 22 ± 1°C. At 6am, the TRF group was moved to clean boxes for the fasting period to prevent foraging and coprophagia, after which time the mice were placed in their home boxes. Mice in all groups were handled daily at the same time to control for any handling stress and minimize experimental variation between groups [29]. Mice were euthanized 7 weeks after commencing the TRF intervention, the bilateral #3 and #4 mammary fat pads were removed and weighed and the liver frozen for further analysis.

2.2 Metabolic cage assessments

Four mice per group (normal chow, ALF, TRF), matched for body weight by group where possible (TRF and ALF), were individually housed in a 12-chamber Clinical Laboratory Animal Monitoring System (CLAMS) with controlled temperature (22 ± 0.5°C), light and feeding (Columbus Instruments, Columbus, OH). CLAMS provides automated access to food and monitors the cumulative amount of food eaten as well as the amount eaten in each bout of feeding with the use of a Mettler Toledo balance with a resolution of 0.01g. Feeders are designed to account for spillage of food and to prevent foraging. Oxygen uptake, carbon dioxide output, respiratory exchange ratio (RER), horizontal and vertical ambulatory movement, feeding and drinking were measured over a 5-day period. Highly variable data from day 1 during acclimatization were excluded from further analyses.

2.3 Glucoregulatory assessments

Glucose tolerance test (GTT). At 25 weeks of age, 7 mice per group were fasted for 6 h prior to the GTT. Fasting commenced at ZT 0, when the feeding period ends for the TRF group and the dark phase ends for all of the mouse groups. Thus, all mice were in similar postprandial states during fasting assessments. Blood was collected via the tail vein at 0 min and then 1 g/Kg glucose injected intraperitoneally. Blood glucose was monitored using a hand held glucometer (One Touch Ultra, LifeScan, Milpitas, CA) at intervals up to 120 min. Terminal fasting plasma insulin was measured on 10 mice per group by ELISA; terminal fasting plasma glucose was measured by the glucose oxidase method (YSI, Model 2950, Yellow Springs, Ohio). Homeostatic model of insulin resistance (HOMA-IR) was calculated as follows: (fasting plasma insulin concentration (mU/ml)) × (fasting blood glucose levels (mg/dl))/(405) [30].

2.4 Cryosectioning and oil red O tissue staining

Liver tissue was harvested at ZT 6 following fasting from ZT 0 on the final day of the experiment. Tissue was embedded in optimal cutting temperature compound (OCT), sectioned on a cryostat at 7 μm thickness, air-dried and fixed in 10% neutral buffered formalin (Thermoscientific, Malaga, WA). Oil red O (Sigma-Aldrich, San Louis, MO; 0.5%
in isopropanol) was diluted 3:2 in water and sections stained for 30 min, washed in water and counterstained with methylene blue (Sigma-Aldrich). Samples were examined by light microscopy and imaged using a SPOT Idea digital camera (Diagnostic Instruments, Sterling Heights, MI). The area of oil red O lipid staining from 4 representative images in 3 mice per group was measured using Image J software [31].

2.5 mRNA isolation and semi-quantitative real time PCR (RT-PCR)
RNA was prepared using Trizol (Life Technologies, Grand Island, NY) and cDNA synthesized. RT-PCR was carried out using a StepOne Plus machine (Life Technologies). Primers are listed in Supplemental Table 1.

2.6 Statistics
Data were analyzed by one-way or two-way ANOVA as indicated in the figure legends followed by multiple comparisons corrected using Tukey's method. RT-PCR data were log2 transformed and analyzed by one-way ANOVA followed by Holm-Sidak's multiple comparisons test as indicated in the figure legends. All data were analyzed using Graphpad Prism software (Graphpad software, La Jolla, CA) and the level of significance was set at p<0.05.

3. Results
3.1 Obese female OVX mice lose weight with TRF
Age-matched female OVX mice were fed either normal “low fat” chow (NC) or HFD (60% kcal from fat) ad libitum. Nine weeks after initial HFD-feeding and associated body weight accumulation (average >40g total body weight), the HFD-fed mice were split to ALF and TRF HFD-fed groups. The ALF group of mice continued to steadily gain weight over this time (15% increase from 9 wk to end of study, p < 0.0005; Fig 1A). The TRF group of mice lost 17% of their body weight (p < 0.0001) within 3 wk of commencing the TRF program and then their weight stabilized thereafter (Fig 1A). While the body weight of the TRF group after 6 weeks remained significantly higher than the NC group (1.34-fold increase, p<0.0001), it was significantly lower than the ALF group (1.85-fold increase compared with NC, p<0.0001; Fig. 1A & B). A similar pattern of weight difference in the mammary fat pads emerged at the end of the study (Fig. 1C), but the magnitude of weight difference between the NC group and the ALF (6.5-fold increase, p<0.0001) and TRF (4.5-fold increase compared with NC, p<0.0001) groups was more pronounced.

Food and water intake was measured at 13 min intervals by the CLAMS apparatus over a four day period in four mice per group. Daily intake is the average of the cumulative intake from all the intervals on a particular day. After accounting for differences in the energy density of the NC and HFD diets, daily caloric intake was lower in the ALF and TRF groups versus the NC group (Fig. 1D). Although there was a trend towards lower daily calories consumed in the TRF group compared with the ALF group, this difference was not statistically significant. It should be noted that food intake can be different in mice housed individually in metabolic cages compared with group-housed mice and this may account for some discrepancies compared with previous studies which find no difference in calories.
consumed per day between NC and ALF groups [23, 32]. Daily water intake was lower in the ALF and TRF groups compared with the NC group (Fig. 1E), most likely due to moisture and texture differences in the diets, the NC diet being much drier than the HFD.

### 3.2 Metabolic assessment following TRF

To investigate the physiological alterations that might explain TRF-induced changes, mice were subjected to a CLAMS analysis to assess activity, respiration, feeding and drinking during the last week of the study. After the first 24 h period of acclimatization, mice on the TRF regimen showed a spike in food and water intake when food first became available during the dark phase of each 24 h cycle (Fig. 2A,C) and this contributed to a significant increase in calories consumed by these mice during the 8 h TRF feeding period compared with the NC and ALF mice (Fig. 2B). NC mice ate and drank predominantly during the dark phase, while feeding in the ALF group was spread more evenly over the whole 24 h cycle (Fig. 2B,D). Calculations of the food energy consumed showed that the NC mice consumed more total calories than the ALF mice. The NC-fed mice showed greater physical activity during the dark phase than ALF mice; TRF mice exhibited the same level of physical activity as the NC mice during the 8 h TRF period (Fig. 2E-H). As expected the respiratory exchange ratio (RER) for the ALF mice was approximately 0.75 and did not vary, indicating that the ALF mice were in an oxidative state for the duration of the study consistent with the composition of the diet and the constant food intake (Fig. 3A,B). The RER for the NC mice varied between 0.95 (reflecting primarily carbohydrate oxidation) during the dark eating phase to 0.85 during the light phase, as the mice switched to a combination of carbohydrate and fat oxidation. The mice on NC eat less during the light phase but never show a fasting switch to oxidative metabolism. The TRF mice fasted for 16 h per day as reflected in RER values of 0.7 (reflecting primarily fat oxidation) from 6am to 10pm. The RER increased in the TRF mice during the 8 h TRF period from 10pm to 6am due to their consumption of carbohydrate and protein in the HFD during this time. Consistent with the higher RER, the NC-fed mice exhibit greater O2 usage (Fig. 3C,D) and CO2 generation (Fig. 3E,F), and less heat generation (Fig. 3G,H) compared to the ALF and TRF mice. This results in lower cage temperatures (Fig. 3I,J), since diurnal changes in cage temperature are indicative of physical activity. ALF-fed mice generate more heat due to greater mitochondrial fatty acid oxidation and hence have slightly higher cage temperatures than NC-fed mice. The TRF mice generate the same heat as the ALF animals during the feeding phase but less during the fasting phase (Fig. 3G,H).

### 3.3 TRF improves glucose tolerance and insulin resistance

Our interventional TRF approach resulted in glucose tolerance that was only slightly improved after 4 weeks, despite this being the period of greatest weight loss during the study. After 2 additional weeks of the TRF regimen, a significant improvement in glucose tolerance was observed in the TRF group compared with the ALF group, suggesting that improvements in insulin resistance continued to occur in the TRF group in the absence of significant weight loss (Fig. 4A-C). Mice in the ALF and TRF groups were matched for body weight (~40g) for the GTT, since body weight differences alone can affect the GTT. Terminal fasting plasma glucose was elevated in ALF mice compared to NC mice, but fasting plasma glucose in TRF mice had normalized to levels observed in NC mice (Fig. 4A-C).
4D). HOMA-IR was significantly reduced by the TRF regimen (Fig. 4E), indicative of improved insulin sensitivity.

3.4 Amelioration of hepatosteatosis with TRF

Hepatic steatosis develops in OVX mice fed a HFD and has been used to model NAFLD [33]. In our study, lipid content in the liver was visualized using oil red O staining of cryosections (Fig. 5A). The ALF mouse group showed a 6-fold increase in the amount of fat in the liver (% area) compared to the NC group, while the TRF group showed only a 3.5-fold increase (Fig. 5B). We postulate that this partly explains our observation of better glucose tolerance in the TRF mice compared to ALF mice (Fig. 4A, B) as hepatic steatosis is associated with liver insulin-resistance and glucose intolerance.

3.5 Modulation of hepatic gene expression reflects beneficial effects of TRF

Mice were sacrificed at ZT 6 after a 6 h fast and RNA extracted from the liver for assessment of the expression of genes involved in lipid and glucose metabolism. As expected from the reduced adiposity, the lipid biosynthetic genes Acc2 and Fasn, and the lipid transport genes Cd36 and Slc27a1 were still elevated in the ALF mice compared to NC mice despite being fasted for 6 h (Fig. 6A). These genes were not elevated in the TRF group however, indicating less lipogenesis in the liver. The lipid storage genes Cidea and Figf2l were elevated by the HFD in both the ALF and TRF groups, but surprisingly the Cidec gene was only elevated in the ALF group and not the TRF group, consistent with the reduced steatosis (Fig. 6B). Other lipid storage genes such as Fitm2, Lipin1, Plin1, and Plin2 were unchanged (Suppl Fig. 1). The lipid oxidation genes Acadl, Acaa1a, and Acadvl were elevated in the ALF group as were the peroxisomal genes Pex11a and Crot (Fig. 6C). Interestingly unlike the other genes Crot expression was much higher in the TRF group suggesting increased lipid transport into peroxisomes for oxidation (Fig. 6C). Similarly, Cideb expression was higher in the TRF group. The first enzyme in the β-oxidation pathway Acox1 was expressed at a slightly lower level in the TRF group (Fig. 6C) but other genes involved in peroxisomal oxidation were unchanged (Supp Fig. 1). Pyruvate carboxylase (Pcx), pyruvate dehydrogenase kinase 4 (Pdk4) and pyruvate kinase (Pklr) were increased in the ALF and TRF groups indicating that pyruvate is being utilized for oxaloacetate production to maintain mitochondrial β-oxidation (Fig. 6D). Glucose-6-phosphatase (G6pc) is markedly elevated in the TRF group but PEPC (Pck1) is slightly reduced (Fig. 6D) suggesting increased gluconeogenesis. The livers of ALF mice show mild elevation of inflammatory genes such as Arg1 and Emr1 (F4/80) and TRF mice show higher expression of the Kupffer cell marker Clec4f (Fig. 6E), but cytokine levels are unchanged by the ALF or TRF regimes (Suppl Fig. 1). Hence hepatic inflammation was not a feature of NAFLD in our model and was not significantly altered by TRF.

4. Discussion

Efforts to address the obesity epidemic have focused on dieting because reducing calorie intake overcomes the deleterious effects of obesity by ameliorating insulin resistance and impaired glucose tolerance in both rodents and humans [34, 35]. Unfortunately restricting caloric intake induces hunger and irritability in humans and these fasting regimes may
require active intervention by nutritionists or clinical researchers to ensure compliance [20].
While these interventions may work in the short-term to correct metabolic dysfunction, they
are not suitable for long-term health improvements due to low compliance in the obese
population. Recent studies show that a prolonged daily fast (> 12 h) occurring
predominantly during the inactive or sleeping phase, without calorie restriction is sufficient
to prevent obesity and insulin resistance in male mice [23, 36]. Such a fasting regimen
implemented on weekdays only is sufficient to maintain the metabolic improvements,
suggesting that TRF has the potential to be an adoptable, feasible lifestyle modification for
humans [36].

Sex hormones influence adipose tissue deposition and function, and females differ in their
distribution of adipose tissues pre and post menopause. After menopause, fat deposition
shifts from the subcutaneous depot to the visceral depot. Obesity causes insulin resistance
and tissue inflammation in male mice, but is much less detrimental in intact, estrogenized
female mice [37-39]. The protective effect of estrogen is lost in whole body ERα knockout
mice [40] or OVX mice lacking ovarian steroids, that respond to obesity similar to male
mice [39]. As OVX mice are often used as a model for postmenopausal hormone changes,
we wanted to ask whether TRF would be effective in improving the metabolic profile of
obese OVX female mice. The current studies show that in female obese OVX mice, TRF is
effective as an interventional program to reduce obesity and improve metabolic profiles.

OVX mice in the ALF and TRF groups showed 6.5 and 4.5-fold increases in the weight of
the subcutaneous mammary fat pads, respectively, compared with mice eating NC.
Parametrial adipose tissue showed similar changes to the mammary fat pads with significant
increases in both the ALF and TRF groups (data not shown), however the data were not
included due to a concern that the ovariectomy surgery which disrupts the parametrial
adipose tissue, would confound the interpretation. TRF led to a 17% reduction in the body
weight of the OVX mice by 4 weeks and prevented the further increase seen for the ALF
mice, but did not reverse the obesity. This difference in adiposity could be partially
explained by a trend towards a lower food intake in the TRF group compared with the ALF
group. While this is in contrast to young male mice that show no difference in food intake
on a TRF regimen [23], middle aged male mice (12 months of age) on a similar TRF
regimen have been shown to have reduced caloric intake and gain significantly less weight
than age and sex-matched ALF mice [41]. Other potential contributing factors include an
initial reduction in food intake leading to a loss of fat mass in the TRF group, a longer
fasting duration leading to enhanced use of fatty acids as fuel and depletion of fat stores,
and increased locomotor activity leading to increased calorie expenditure and depletion of fat
stores. The TRF group showed increased physical activity during the nocturnal feeding
period but similar activity during other times. This increased activity may contribute to the
early weight stabilization but does not explain the metabolism, which continued to improve
over the next 6 weeks in the face of constant body weight.

The liver is a central organ in lipogenesis, gluconeogenesis and cholesterol metabolism. The
availability of Western diets is increasing the prevalence of obesity and metabolic syndrome
and promoting pathophysiological changes that result in NAFLD, the most common liver
disorder in developed countries [42]. Insulin resistance in the liver can cause glucose

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intolerance, failure to suppress gluconeogenesis, and steatosis [43] and HFD-induced obesity causes hepatic insulin resistance. We found that TRF reduced the severity of hepatic steatosis and improved insulin resistance, despite the consumption of similar quantities of HFD. One potential explanation is that the 16 h fast forces the mouse to switch to oxidative metabolism of lipid as an energy source causing the liver to halt lipogenesis and increase lipid oxidation, effectively clearing and preventing steatosis. Chylomicrons deliver lipids to the adipose tissue during the 8 h consumption of the HFD and to the liver by the exogenous pathway via chylomicron remnants, but the cessation of feeding stops the flow of lipids from the gut so lipids transferred to the liver by chylomicron remnants are used as an energy source rather than stored. Support for this model comes from gene expression in the liver following TRF.

Expression of genes involved in lipid synthesis and storage are normalized to NC levels despite the consumption of the HFD, in particular. Cidec, an indicator of lipid storage, was reduced 8-fold from ALF levels with TRF, and is suppressed during fasting. Fgf21 is induced by both ALF and TRF regimes. This may seem paradoxical, but one of the roles of FGF21 is to stimulate β-oxidation that is critical during fasting, but is also needed when HFD is the major source of energy. In contrast to the changes in lipid metabolism, genes regulating glucose metabolism did not show consistent alterations in expression upon TRF.

The expression data was generated from mice following a 6 h fast, however that may mask changes in glucoregulatory genes, so it will be interesting to measure expression on livers from mice sacrificed at different times during the 24 day.

In summary, we have shown that obese OVX female mice on a TRF regimen show metabolic improvements that appear to be a combination of those found in middle-aged male mice and younger male mice. A limitation of the study was the inability to accurately measure changes in the parametrial fat of the OVX females due to surgical interference and future studies with intact females will address this shortcoming.

This TRF study, as well as published mouse and human studies, support the translational potential of TRF regimens in modulating obesity-related disease risk [13, 23, 44, 45]. In fact, we have recently shown that restricting the majority of food intake to <11 hours per day was prospectively associated with reduced incidence of breast cancer recurrence [46], the first report of a clinical outcome associated with TRF. Evidence from mouse and human population studies supports testing the TRF regimen in a randomized controlled clinical trial. As an intervention, TRF is simple and feasible and therefore could be implemented in multiple contexts, at scale, with sustainability. TRF could be effective in lower socioeconomic populations that (1) have the greatest risk of metabolic dysregulation, obesity, and type 2 diabetes and (2) may struggle with access and compliance to multifaceted, expensive, or time-consuming dietary interventions such as commercial weight loss programs. If randomized controlled clinical trials show that habitual TRF improves metabolic health in humans, this would be an important public health discovery to reduce the risk of type 2 diabetes and other chronic diseases.
5. Conclusions

Our results in obese OVX female mice support the findings in obese male mice that TRF is an effective strategy for improving the metabolic health of obese individuals. Whether TRF reverses hepatic steatosis and restores glucose tolerance or simply prevents its progression in our model, remains to be determined. Although further long-term animal studies are required to define the molecular basis for the beneficial effects of TRF on metabolism, animal studies on TRF correlate well with human studies [47, 48] and therefore hold considerable translational potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ADF</td>
<td>alternate day fasting</td>
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<td>ALF</td>
<td>ad libitum feeding</td>
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<td>CLAMS</td>
<td>clinical laboratory animal monitoring system</td>
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<td>OCT</td>
<td>optimal cutting temperature compound</td>
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Metabolism. Author manuscript.
OVX  ovariectomy
Per2  period circadian clock 2
RER  respiratory exchange ratio
RT-PCR  real-time polymerase chain reaction
TRF  time-restricted feeding
ZT  Zeitgeber time

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Figure 1. TRF reduces obesity in HFD-fed OVX mice
A. Body weights of mice on NC, ALF and TRF over time. TRF was initiated when the mice were 19 weeks of age (week 9 of HFD) when average body weights of HFD-fed mice were over 40g. ****p<0.0001 represents difference of TRF group from ALF and NC groups following 2way ANOVA and Tukey's multiple comparisons test. B. Final body weights. C. Thoracic and inguinal mammary fat pad weights. D. Daily food intake of mice during the final week of the study. E. Daily water intake by mice during the last week of the study. Data are means ± SEM for 10 mice per group in A, B and C and 4 mice per group in D and E. Data in B-E were analyzed by one-way ANOVA and Tukey's multiple comparisons test. **p<0.01, ***p<0.001, ****p<0.0001.
Figure 2. TRF increases physical activity
Four mice per group were placed in Comprehensive Lab Animal Monitoring System (CLAMS) housing for a 5-day period to measure metabolic parameters. After 1 day of acclimatization, data were averaged over the 24-hour period for 4 days (left panels) with the black bar representing the dark phase, the open bar representing the light phase and the magenta bars representing the period of feeding for the TRF group in each figure. A. Food intake, C. Water intake, E. X Ambulatory (e.g., walking/running movement in the X-axis plane) and G. Z activity (e.g., reaching/rearing in the Z-axis plane). Measurement averages

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for each time period are presented in bar graphs (right panels). B. Food intake, D. Water intake F. X Ambulatory (e.g., walking/running movement in the X-axis plane), and H. Z activity (e.g., reaching/rearing in the Z-axis plane). Data are means ± SEM for 4 mice per group analyzed by two-way ANOVA and Tukey's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.005 and **** p<0.001.
Figure 3. Fat is the fasting source of fuel for TRF mice
An RER of 0.7 during the fasting period for TRF mice indicated that fat was the major source of fuel, whereas ALF mice burn a mixture of fats and carbohydrates, and NC mice burn mainly carbohydrates. Data for the respiratory exchange ratio (RER) (panels A and B); the volume of oxygen consumed (panels C and D), the volume of carbon dioxide produced panels (E and F), the heat generated (panels G and H), and the cage temperature (panels I and J) were collected and analyzed by two-way ANOVA and Tukey’s multiple comparisons test. *p<0.05, **p<0.01, ***p<0.005 and **** p<0.001.
Figure 4. TRF improves glucose tolerance in HFD-fed OVX mice
A. IP-GTT on mice after 4 weeks of TRF. B & C. IP-GTT after 6 weeks of TRF (panel B) with corresponding area under the curve (AUC) graph of glucose normalized to body weight (BWt). Data are means ± SEM for 7 mice per group **p<0.01, ***p<0.005 vs. ALF. D. Terminal fasting blood glucose. E. HOMA-IR at the end of the study. Data for panels D and E are means ± SEM for 10 mice per group analyzed by one-way ANOVA and Tukey’s multiple comparisons test. *p<0.05, **p<0.01, ***p<0.005.
Figure 5. Hepatosteatosis is reduced in HFD-fed OVX mice after TRF
A. Livers were frozen and cryosections stained with oil red O (red) for lipid. B. The area of oil red O was quantitated using Image J. Data are means ± SEM for 6 mice per group analyzed by one-way ANOVA and Tukey’s multiple comparisons test. ****p<0.0001.
Figure 6. Normalization of metabolic genes following TRF

Genes involved in metabolism in the liver were assayed by real-time PCR. A. Liver synthesis and transport genes. B. Lipid storage genes. C. Lipid oxidation genes. D. Glucose metabolism genes and E. Inflammation genes. Data are means ± SEM of samples from 6 mice per group analyzed by one-way ANOVA followed by Holm-Sidak's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.005 and **** p<0.001.