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Does Time restriction feeding reverse the High fat diet induced obesity in *Drosophila melanogaster*?

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ABSTRACT:
Obesity and its co-morbidity are the most challenging tasks for the current health care system. It is a multifactorial disorder either due to genetic predisposition, high calorie-dense diet, circadian disruption, etc. Circadian disruption is due to people's altered lifestyle choices have resulted in increased obesity that can be measured in terms of lipid profiles, overweight, and endogenous antioxidant level. As consequence of this increased diabetes and cardiovascular problems among the current generation. The present study is an attempt to understand the importance of disciplined lifestyle and eating at the right time. Many studies in this regard have revealed time-restriction feeding and its benefits. The current study aims to investigate the adverse effects of a high-fat diet on odd-time eating and high-fat diet with that of time-restriction feeding (TRF). To address this problem, we choose *Drosophila melanogaster* flies that were fed with different food regime as high-fat diet (HFD), a normal diet (ND) as a control, HFD+TRF and ND+TRF. The biochemical assays were used to detect obesity, the stress and TRF normalizes the triglyceride level to reduce endogenous antioxidants in the HFD group was observed and TRF has an effective response to obesity by reducing body adiposity caused by a high-fat diet.

Keywords: *Drosophila melanogaster*,TRF, HFD, SOD, catalase

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INTRODUCTION

Circadian rhythm disruption (CRD) is an emerging risk factor for obesity and metabolic diseases, which can be caused by prolonged exposure to light, shiftwork, or a shiftwork-like lifestyle (Morris et al., 2016; Stone et al., 2016). In the current world, obesity has economic burdens and is a worldwide problem. Two out of every three world populations are overweight or obese, with related complications and the primary cause of death within the adult population (Diop, Birse, & Bodmer, 2017). Obesity increases the risk factors for many conditions like diabetes, dyslipidemia, hypertension, stroke, certain cancers, heart disease, arthritis and muscle

dysfunction disorganization (Villanueva et al., 2019).

It has been shown that the circadian clock genes modulate the temporal expression and function of metabolic regulations by optimizing nutrient metabolism according to daily cycles in food availability and energy expenditure (Barber et al., 2016; Diop, Birse, & Bodmer, 2017). Due to reciprocal interaction between metabolism and circadian rhythm, this daily eating-fasting rhythm acts synchronically with the molecular clock to drive daily rhythms in anabolic and catabolic metabolism and dependent physiology. (Turek et al, 2005; Sundaram & Yan 2016). It has been reported that metabolic imbalance caused by oxidative stress could leads to metabolic syndrome (Villanueva et al., 2019).

There's a long-time connection between a high-fat diet and oxidative stress (Che et al., 2021). It has been recommended that long-term consumption of a high-fat diet acts as an oxidative stress inducer (You et al., 2005), since it suggestively weakens the hepatic enzyme antioxidant system by damaging and upsurging the lipid peroxidation level (LPO) in the liver and plasma (Chung et al., 2018). A high-fat diet (HFD) is a diet containing at least 35% of total calories expended from both unsaturated and saturated fats (Chaix & Zarrinpar, 2015) which is the root cause of molecular and cellular level damages, and it activates an oxidative stress progression (Chandrashekara et al., 2014)

Studies have shown that oxidative stress produces different responses like activation of heat shock proteins and mitogen-activated protein kinase (MAPK) signaling pathways to protect cells against oxidative damage, and production of lipid peroxidation, alteration of proteins, and insulin resistance. Furthermore, an HFD that endorses an enhanced supply of fatty acids and triglycerides results in an increase in the oxidation of fatty acids in order to produce energy (Trindade de Paula et al., 2016). Conversely, putting sleep/wake and feed/fast cycles on a timer or in tune with environmental entrainment while reducing calorie intake promotes daily rhythm in gene expression and normalizes function (Villanueva et al. 2019). Several studies involving model organisms and volunteers emphasized the opposite of obesity and metabolic disorders (Sutton et al. 2018; Gabel et al. 2018). Further, the ectopic fat accumulation in the indirect high muscle of *Drosophila* may lead to the production of reactive oxygen species shows reduction in lifespan, mitochondrial degradation, sarcomere disorganization and disturbed the sleep/wake cycle (Bondia-pons et al. 2012).

Variable metabolic pathways, physiological conditions, growth and development of *Drosophila melanogaster* have homology with mammals. *Drosophila melanogaster* has comparable growth, replica, and senescence stages as humans and conjointly shows deterioration in exercise and cognitive behavior throughout ageing (Jafari, 2010). Recent proof suggests that feeding of high-fat diets redoubled the accretion of lipid hydroperoxides, speed up the ageing method, and evoked higher mortality in *Drosophila melanogaster.* Furthermore, feeding a high-fat diet activates the JAK-STAT signaling pathway, which reduces insulin sensitivity and shortens the lifespan of *Drosophila melanogaster* (Villanueva et al., 2019). Further, changes in the circadian cycle could impair the other systems of the body, like reproductive function, and muscle physiology, by causing an impact on nutrient metabolism (Patke, Young, & Axelrod, 2020). Pathological obesity is characterized by ectopic lipid production in non-adipose tissue and impairment of glucose metabolism (Jung & Choi 2014). Disruption in circadian rhythms causes dysmetabolism and obesity-related co-morbidities with feeding-fasting behaviour and time limitation, i.e., feeding individuals at specific times to sync with their natural pattern (Ruan, Yuan, & Eltzschig, 2021).

Time-restricted feeding (TRF), where the intake of food is restricted to certain hours of the day, but not restricted to total dietary intake. In *Drosophila melanogaster*, TRF has been discovered to attenuate age-related cardiac decline, as well as increase sleep and muscle function (Cabrera, Young, & Axelrod, 2020). A few clinical studies confirmed that time-restriction feeding has potential as a health-promoting intervention and improve a variety of metabolic markers such as weight, body fat, pressure level, energy intake, and endurance, specifically in people at risk for metabolic syndrome (Cabrera et al., 2020).

Drosophila fat bodies are considered equivalent to vertebrate adipose tissue, both metabolically and in their endocrine role (Trinh and Boulianne 2013). Both models of diet-induced and genetically induced obesity models exhibited myofibril disorganisation and mitochondrial abnormalities and attenuated TFR compared with age-matched ALF 3 week old female flies. The TRF suppressed the disruption of the M-line and Z disc of myofibrils. There is a need for a model for obesity-related metabolic syndrome in laboratory animals to check the efficacy of the present day lifestyle and its consequences. The present study is an attempt to understand the adverse effects of a high-fat diet and the ameliorating effects of time-restricted feeding as measured using the *Drosophila melanogaster* model.

MATERIALS AND METHODS

Fly culture and diet preparation

The *Drosophila melanogaster* Canton-s strain was obtained from DOS in Zoology, University of Mysore. The experimental stocks were first established initially on corn wheat media, and four groups of five-day-old flies were placed in four different diet regimes. The first group received the normal balanced diet (normal diet) and the second group had ND+ time restricted (TRF). The HFD diet for the third group consists of 5% lard and 1% emulsified (tween). The fourth group was given HFD+TRF (time-restricted feeding), which meant that food was only offered once a day (12 hours after photophase). All four groups of flies were kept in an environmental chamber with a 12 h dark/12 h light cycle, 75% humidity, and a temperature of 25°C were maintained. All adult flies were transferred to the Drosophila raring chamber (population chamber) (n = 30) for the time-restricted feeding and cultured for five generations with the same diets, which were then used for further analysis.

Body weight analysis

Bodyweight analysis using fifth-generation flies aged ten days were anesthetized and transferred to empty vials, which allowed them to wake up after 30 minutes. The bodyweight of all 30 flies was measured. Similarly, a 10th, 20th, and 30th-day fly of different feeding regimes was assessed (method of Jumbo- Lucioni et al., 2020).

Lipid Assay

Males were placed in groups of ten in clean dry vials, dried for 36 hours at 70°C, and weighed to the nearest microgram. The lipid content of the flies was determined by deflating them in tubes containing 1.3 mL ether. The lipid was extracted over 36 hours at 25.2°C using a gel-rocker set to 2000 rpm and gentle agitation. Ether was changed every 12 hours. After 36 hours in the ether, the flies were rinsed with 1 mL fresh ether, dried for 2 hours at 70°C, and weighed to determine lipid- free dry weights. The difference in dry weight "before" and "after" ether extraction was used to calculate the total lipid content. Five vials were set up for each therapy and population (Zwaan et al., 1995).

Biochemical level

Estimation of Glycogen

Glycogen levels were measured according to the method described by Palanker et al. (2009). Rapid immersion in > 95°C water killed flies without causing any behavioural changes. The 30th day age group flies were stored at -70 °C overnight before being homogenised in ice cold extraction buffer (20mM HEPES, 0.10M Sodium azide, 0.2mM PMSF, and 1mM EDTA, pH 7.0), centrifuged at 17000 rpm for 3 minutes at 4°C, and 25 μl of supernatant was aliquoted and incubated with 100 μl of glucose reagent (Sigma) at 37°C for a standard curve. Each sample was examined three times, with statistical analysis based on the average mean of the three results.

Triglyceride measurements

The content of triglycerides was determined using the Palanker et al. protocol (2009). Ten flies of 30th day were freeze killed and homogenised in 100 μl of PBS containing 0.5 percent Tween 20 before being incubated for 5 minutes at 70°C. The heat-treated homogenate was centrifuged, and the supernatant was incubated for 30 minutes at 30°C with Triglyceride Reagent (Sigma). After 5 minutes at 37°C incubation with Free Glycerol Reagent (Sigma), absorbance was measured at 525 nm with a Beckman DU 640 spectrophotometer. Protein content was used to normalize triglyceride levels. The assay was performed three times, with the mean calculated were used for statistical analysis.

Protein preparation

The protein extraction from the flies was done according to Clark and Keith's standard protocol (1988). From each treatment, ten flies (10 males) of 30th day treated aged group were collected, freeze killed, and homogenised in 100 μl of protein extraction buffer (0.05M Tris-HCl, 150mM Nacl, 0.1 percent Triton X-100, 1 % Deoxycholate, 0.1 percent SDS, 1mM EDTA, pH 7.4). At 4°C, the homogenates were centrifuged for 10 minutes at 14000rpm. In 1.5 ml of Bradford reagent, a small amount of each sample (25μl) was added (Sigma). Optical density was measured at 600 nm. Using bovine serum albumin (BSA) as a standard, a standard curve was created. The protein estimation was repeated three times.

Extract Preparation for Enzyme Activity

Flies of 30th treatment group from the holding vials were sexed under light CO_2 anaesthesia and stored at −20°C before being used. Three replicates of 25 flies per sex per treatment per population were homogenised in ice-cold protein extraction buffer (20 mM Tris–HCl, pH 8.0; 1mM EDTA; 1 mM phenyl methane sulfonylfloride; and 0.1 percent Triton-X 100), then centrifuged for 30 minutes at 15,079g at 4°C. For activity testing, the supernatant was used as a crude enzyme extract. The BCA-Protein Assay Kit (Sigma–Aldrich) was used to determine protein concentration according to the manufacturer's instructions.

Antioxidant Enzyme Assays/In vivo antioxidant activity

All spectrophotometric measurements were taken on a double-beam UV Visible spectrophotometer (UV-1800; Shimadzu, Japan).

Catalase Assay

Catalase activity (EC 1.11.1.6) was measured by using protocol of Aebi. In a total volume of 1 mL, the activity buffer contained 100 mM KPO_4 buffer, pH 7.0; 20 mM H_2O_2; and 50 mL enzyme extract. In a cuvette, phosphate buffer and enzyme extracts were added, and the reaction was started with H_2O_2. Using the auto-rate assay mode on the spectrophotometer, the decrease in H_2O_2 was monitored by measuring absorbance at 240 nm continuously for 2 minutes with intervals of 30 seconds. One unit of enzyme is equal to the amount of enzyme needed to convert 1 mol of H_2O_2 to product in one second. The activity of enzymes was measured in units per milligramme of protein.

Superoxide Dismutase (SOD) Assay

Activity of SOD (EC 1.15.1.1) was determined by following the method of Beauchamp and Fridovich with minor modifications. In a total volume of 3 mL, the activity buffer contained 100 mM KPO_4 buffer, pH 7.8, 0.05 mM EDTA, 45 mM l-methionine, 65 mM nitro-blue tetrazolium, and 35 mL protein extract. In the end, riboflavin (2mM) was added and mixed. For 30 minutes, the tubes were kept under a light source (20 W). As a control, assay activity buffer (containing Riboflavin) was used. At 560 nm, the absorbance was measured. Under specified conditions, one unit of SOD activity (U) is defined as the amount of enzyme required to cause a 50% inhibition in the rate of reduction of nitro-blue tetrazolium. Units of activity or units per milligram of protein were used to express the findings.

MDA (Malondialdehyde) levels

To estimate MDA levels in the samples, flies were homogenised in a bead-based PowerLyser® homogenate with 150 μL of 0.5 $MHClO_4$and 150 μL of distilled water, and the homogenate (10,000 g for 10 minutes at 4°C). The supernatant was injected into the HPLC as described by Karatas et al. (2002).

Statistical Analyses

In all cases except survival function analysis, the population means were used as the units of analysis. The significance of the difference between the means was assessed using a one-way analysis of variance. The difference among treatments was compared by the Tukey–Kramer Minimum Significant Difference (MSDa0.05) Test. The significance of the difference between adult survival curves was analyzed using the Kaplan–Meier log-rank test.

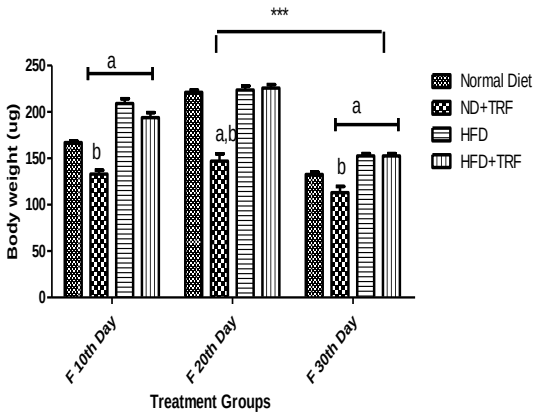
Body weight and lipid content data were expressed in Mean ± SEM (n=30), data were analyzed by two-way ANOVA, Bonferroni's test. $p>0.05$. aP compared to normal diet, bP compared to HFD and abP compared to TRF. Biochemical content data to ANOVA followed by Tukey's post hoc test wasdone .MDA level, Catalase and Superoxide dismutase content of the Drosophila treated with different diet and TRF diet in both male and female flies. All the values are expressed in Mean ± SD, n=3. Data

were analyzed by two-way ANOVA using Bonferroni test.

RESULTS

1. Body weight analysis

1A)



1B)

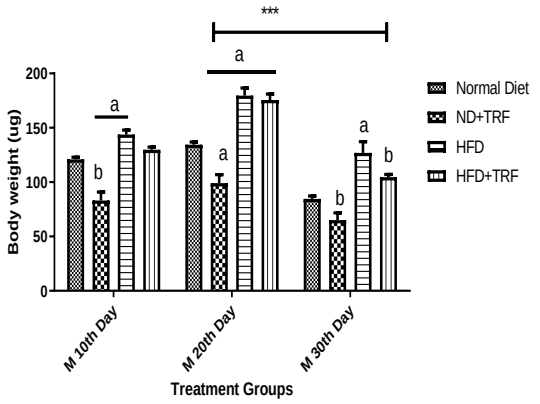
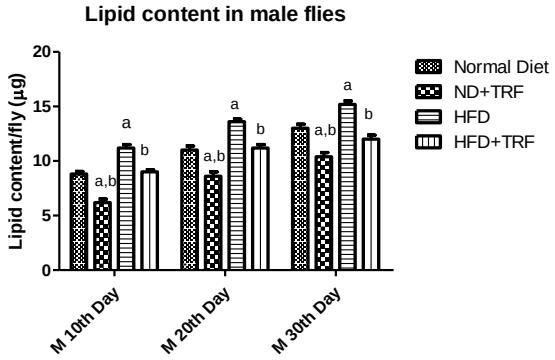


Figure 1: Bodyweight analysis in normal and high fat diet fed groups at different days of TRF. 1A) Body weight of female flies, 1B) Body weight of male flies.

Figures 1A and B show the Male and Female flies body weight of 10th, 20th, and 30th days flies under different nutrition regimes. Bodyweight of the 30th-day flies showed significant reduced size when compared to the other group. The female and male flies of 10th and 30th generation fed with the Normal diet +TRF, HFD and HFD group having TRF showed body weight loss. The results showed weight reduction in ND+TRF in all three generation. Overall there was significant changes were seen between 20th to 30th day. Two way ANOVA revealed there is a significant difference between different nutrition groups with age group F=221.8, df=2, p<0.0001 for females and F=81.79, df=2, P<0.0001 for males. The 10th day groups were similar in both male and female with all diet, whereas flies showed significant with ND+TRF, HFD+TRF and HFD. Interestingly HFD+TRF AND HFD both group are non-significant.

2. Lipid content

2A)



2B)

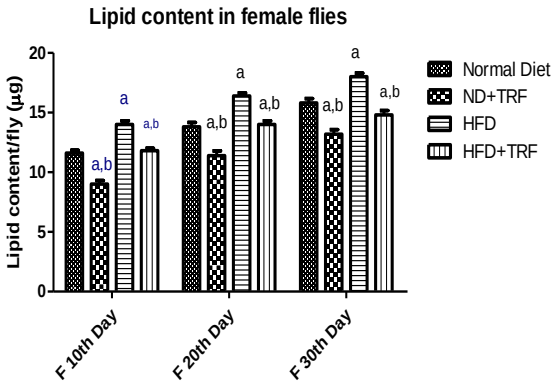


Figure 2: Lipid Content analysis of normal and high fat diet fed groups at different days of TRF 2A) Lipid content of male flies, 2B) Lipid content of female flies.

The lipid content of the different groups, namely ND, ND+TRF, HFD and HFD+TRF is depicted in figures 2A and B. Male and female flies showed the same type of results. There is a significant increase between the different groups of HFD, HFD+TRF, ND+TRF treatments. The lipid accumulation in the HFD group is significantly higher compared to all other groups, both in males and females. Surprisingly, in the HFD+TRF group, the lipid content is equivalent to that of the control group. ANOVA F=114, df=3, P<0.0001 for females and F=109, df=3, P<0.0001 for male

flies. Lipid content is increased and significantly high in different age groups of flies compared to the 10th and 20th days. The 30th day exhibited the highest fat content; lipid in the HFD group is comparatively slight reduced with that of the HFD+TRF group flies. Lipid content was measured after treating with high fat diet (HFD) and Time restricted feeding (TRF) in comparison with flies fed with normal diet (ND). There is a significant change in lipid level after HFD and recovered after TRF.

3. Estimation of TRF on biomolecules level.

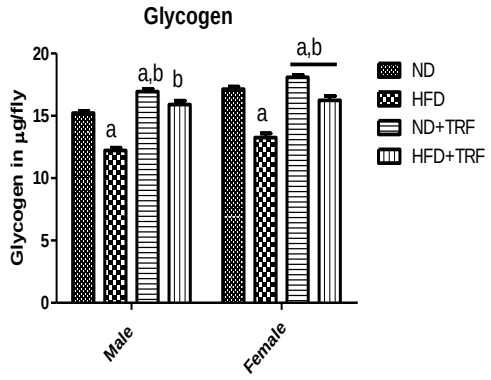


Figure 3A: The Glycogen content

The glycogen content of both males and females in 30 days fed flies. The glycogen content is significantly different in ND+TRF than in all other groups. The HFD+TRF has not shown any significant level with that of HFD flies in both males and females, when compared ND+TRF groups had no change. HFD flies have enhanced glycogen content both in males and females. ANOVA followed by Tukey's post hoc test revealed a significant difference in the different groups F=10.6, df=3, P<0.022.

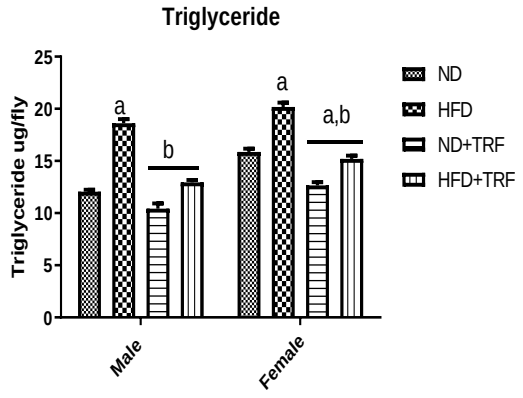


Figure 3B: The triglyceride content

Both male and female flies of the HFD group showed a highly significant increase in triglycerides compared to other groups. Interestingly, the ND+TRF and the HFD+TRF groups showed reduced triglycerides compared to the ND group. Interestingly, Highly reduced triglyceride is observed in ND+TRF followed by HFD+TRF, which means the time-restricted feeding group has reduced triglyceride content compared to the other groups. ANOVA as F=6.53, df=3 (between the treatment column) and p<0.05.

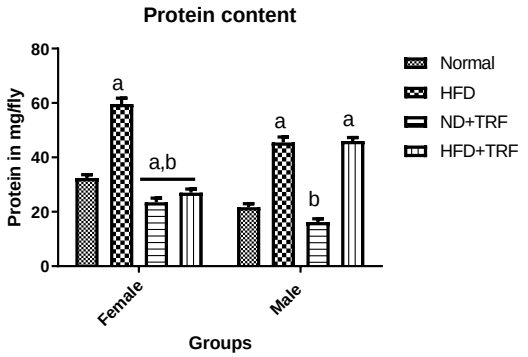


Figure 3C: Protein content

A total protein content analysis of different feeding groups flies of females and males had significant lowed in HFD feed flies compared to other groups but had increased level in HFD+TRF. When compared to ND flies, ND+TRF and HFD+TRF showed significant differences. The total protein content in male flies was significant decreased protein content of both male and female in ND+TRF and HFD+TRF, whereas HFD+TRF had increased protein content in the flies. The ND+TRF group showed decreased protein content. F=7.154, df=3, P<0.05.

4. Estimation of endogenous antioxidant level in the *Drosophila*

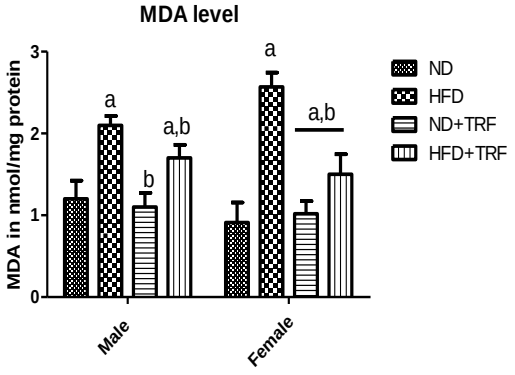


Figure 4A: MDA levels (malondialdehyde)

Depicts the MDA level, an endogenous antioxidant enzyme levels were higher in the HFD group, interestingly ND+TRF showed very little change in MDA. TRF+HFD and ND+TRF groups showed slight decrease, the HFD+TRF group had reduced levels in males and females of MDA was observed F=59.7, df=3, P<0.0001.

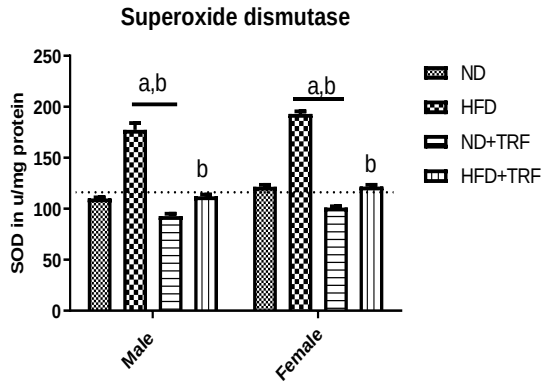


Figure 4B: SOD Level

Depicts the superoxide dismutase, an endogenous antioxidant enzyme. Similar to MDA, the TRF group depicted reduced levels of SOD compared to the HFD group, indicating the increased oxygen levels in the HFD group. Whereas the HFD+TRF group and ND+TRF showed reduced levels of SOD as equivalent to the ND group, the comparison between the groups is significant F=88.82, df=3, P<0.0001.

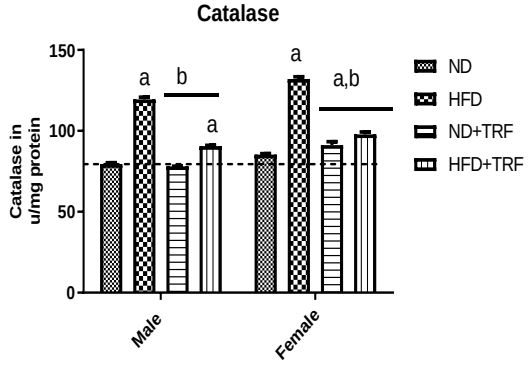


Figure 4C: Catalase level

Depicts the catalase activity of the flies fed with HFD, HFD+TRF, ND, and ND+TRF groups. Both male and female flies showed maximum catalase activity in the HFD group compared to all other groups, ND+TRF and HFD+TRF also showed significant increase in activity. The comparison between groups is significant at F=173, df=3, P<0.0001.

DISCUSSION

It is imperative for the obesity model and metabolic diseases in laboratory animals to test the interference of lifestyle to alleviate these disorders. Time-restriction feeding, or intermittent fasting, has gained attention in recent years for its health benefits in humans. In this study, we demonstrated the effect of TRF on obesity and biochemical parameters. First, we modeled obesity in both male and female *Drosophila* by feeding them a high-fat diet in control groups. For the TRF group, flies were fed a high-fat diet for 12 hours and then restricted to 12 hours of food for 30 days. Normal flies were fed with a normal diet, and normal flies were also kept for TRF for comparison with the high-fat diet TRF group (Gill, Melkani & Panda 2015).

The high-fat diet-fed flies successfully gained weight after 30 days. There was a significant weight loss found in the HFD+TRF group when compared with HFD alone. A recent study reported that 8-h TRF caused 2.6% weight loss after 12 weeks. Similarly, 10-h TRF resulted in a 3.6% weight loss over 16 weeks and a 3.0% weight decrease after 12 weeks (Che et al., 2021). While TRF may be a well-known strategy to slenderize, improvements in insulin sensitivity, blood pressure, and oxidative stress have been seen even without weight loss in a few human studies (Cienfuegos et al., 2020). Several randomized controlled studies have reported that weight loss decreases the mortality rate in individuals (Allison et al., 1999).

The body weight analysis of HFD groups is highly significant compared to other diet groups. Interestingly, the HFD+TRF group has shown reduced body weight compared to the HFD group. Further, the data indicated that 30 days of time restriction period feeding normalise the bodyweight of the *Drosophila*. On the 10th day of flies, all the groups were significant with a normal diet, but only ND+TRF showed significant results with HFD-treated groups on the 10th day. Correspondingly, the lipid content of both males and females increased in the HFD group compared to other feeding groups. Similarly,

the HFD+TRF group has shown the same lipid content as that of the normal diet. The flies in the 30th day age group had more lipid content when compared with the 10th and 20th age groups.

The biochemical assay includes glycogen, triglyceride and protein content showed significant activity in both male and female flies, HFD-treated flies showed the lowest glycogen content when compared to the normal and TRF groups. However, the flies in ND and TRF showed a much larger increase than those in HFD, indicating that there was a significant interaction between diet and hypoxia when the regulation of glucose levels was considered (Heinrichsen & Haddad, 2012). Flies exposed to HFD showed a significant difference in glucose levels between diets, although there was a slight increase in glucose levels between the normal diet and ND+TRF in flies. The triglyceride levels of the HFD group, both in males and females, showed increased TG levels when compared to other groups. TG levels are slightly higher in females compared to males. It may play an important role in egg-laying and longevity. There was increased in Protein content level of TRF controlled group when compared to others.

In the present study, SOD and catalase levels are increased in HFD groups compared to other groups. SOD catalyses the dismutation of the superoxide into H_2O_2, whereas CAT detoxifies H_2O_2 and converts the lipide hydroperoxides into nontoxic alcohols. Elevated ROS (represented by high MDA) levels thereby inflict disruption on cellular metabolism. Cellular ROS is controlled by the upregulation of defence mechanisms that have protein antioxidants like glutathione, uric acid, bilirubin, vitamins C and E, and accelerator enzymes (SOD), enzymes (CAT), and peroxidase (GSH-Px) (Patke 2020). The enhanced ROS level is involved in damaging the cellular events. The results also showed that malondialdehyde levels were significantly high in the HFD group and conversely reduced in the HFD + TRF group. Depicting the end product of gut metabolism.

The circadian timing of food intake affects weight gain and that restricted feeding of a high-fat diet during the dark cycle prevents adipogenesis in non-obese wild-type mice (Chaix et al, 2014; Hatori et al., 2012). TRF reduces the negative effects of HFD by entraining the circadian clock and metabolic regulator to fixed feeding timing. The mechanism is still unknown at the molecular level (sundaram & Yan 2016), but ad-lib consumption may cause attenuation (disruption) of the diurnal rhythm of food intake, which in turn corrected by TRF. (sundaram & Yan 2016; Arble et al., 2009; Kohsaka et al., 2007). Further, time-restricted feeding (TRF) is another form of intermittent fasting wherein energy intake is scheduled for specific hours in a day (Varady et al., 2009). Restricted feeding of a high-fat diet during the light cycle for a short time (4 hrs) resulted in lower body weight compared to mice fed a low-fat diet ad libitum with the consumption of the same number of calories. In the present study, the body weight, biochemical assays and endogenous antioxidant levels are high in HFD and have an ameliorating effect in TRF without restricting the calorie. Thus, adverse effects of circadian disruption can be corrected by simple alteration in the eating timings without restricting the calorie.

CONCLUSION

TRF is an effective dietary strategy to promote weight loss and to decrease lipid storage with remarkable changes in blood biomarkers. In the current study, HFD feeding has successfully induced obesity in the *Drosophila melanogaster*. We evaluated the effect of obesity as well as TRF on the blood biomarkers, endogenous antioxidant, and ROS level. TRF significantly reduced the body weight, glycogen, and lipid level when compared to HFD fed flies. TRF treatment reverses the elevated ROS level by enhancing the endogenous antioxidants like SOD and Catalase. Therefore, time restriction feeding is one way to control obesity and its comorbidity.

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ABBREVIATIONS

CRD: Circadian rhythm disruption
HFD: high fat diet

TRF: time restriction feeding
MDA: malondialdehyde
SOD: superoxide dismutase
ND: Normal diet

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