DYSLIPIDAEMIC, OXIDATIVE STRESS AND IMMUNOINFLAMMATORY ALTERATIONS IN A RAT MODEL OF LATE-NIGHT EATING

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Background: Modern-humans have adapted a 24/7 active and feeding lifestyles with consequential eating at odds with the circadian system that threatens to pose a pandemic of metabolic diseases. Since nocturnally restricted feeding promotes growth and metabolic fitness and that *adlibitum* feeding disturbs diurnal rhythms and metabolic health in rodents, the use of *adlibitum* controls in metabolic researches can have doubtful extrapolative conclusions. Here, we simulated human late-night eating using feed restricted controls with the primary aim of exploring possible dyslipidaemic, oxidative stress and immunoinflammatory alterations of Late-night eating in Wistar rats.

Methods: Sixteen (16) male Wistar rats (aged 8-10 weeks) were randomly assigned into control or late-night eating group (n=8). Fasting weight and blood glucose were obtained and Lipids were analyzed using their respective Randox kits. Malondialdehyde, catalase and superoxide dismutase activities were assayed while Full blood counts and CD 4^+ T-cells were determined using automated analyzers. Data were analyzed using SPSS $V_{20.0}$, compared using Student's t-test and significance set at $p \leq 0.05$.

Results: Our findings have demonstrated that late-night eating is associated with an overall significant decrease in total feeds intake, Fasting blood glucose, High density lipoprotein, catalase, and CD4+ cell counts. On the other hand, Cardiac risk ratio and Atherogenic coefficient are marginally raised, while Platelet lymphocyte ratio, Monocyte lymphocyte ratio and Monocyte High density lipoprotein ratio are insignificantly higher among the late-night eating rats than in controls.

Conclusion: Although our finding could not demonstrate an elevated risk of obesity or diabetes, it has uncovered the dyslipidaemic, oxidative stress and immunoinflammatory alterations associated with late-night eating.

Keywords: CD4 lymphocyte count, Complete blood count, Late-night eating, Lipid ratios, Oxidative stress.

INTRODUCTION

Food availability can act as a prevailing timing cue for circadian synchronization, even in conditions of a non-functional suprachiasmatic nucleus (SCN) (García-Gaytán *et al.,* 2020). The SCN is a (light-entrainable) master clock of the circadian system that orchestrates daily rhythms of physiology and behavior (Ni *et al.,* 2019), including food intake. These rhythms are present even at the base of the food chain, where photosynthetic chemical energy exhibits a predictable diurnal rhythm of 12hours light and 12 hours darkness (12L:12D) conferring an ability to acquire food during the day and store a portion for utilization during the rest/dark period of the day (Longo and Panda, 2016). To enable further cycle of energy scouting, the rest/fasting period serve as a standby for repairs, stress resistance and fitness (Longo and Panda, 2016).

The increased industrialization recorded during the last century, have made humans to adopt a 24/7 active lifestyle with ad Libitum access to food (Wilkinson *et al.,* 2020) and consequential consumption of calorie-dense food later in the day (Longo and Panda, 2016; Zhang *et al.,* 2019; Wilkinson *et al.,* 2020).

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When food intake is confined in synchrony with the circadian rhythm, it ensures best fuel utilization, circadian synchrony and metabolic well-being (Sutton *et al.,* 2018; Jamshed *et al.,* 2019; Zhang *et al.,* 2019; Wilkinson *et al.,* 2020). However, eating during the biological rest/fasting period forces the body to process nutrients at odds with the circadian clocks (Shimizu *et al.,* 2018), imposes circadian desynchrony (Wehrens *et al.,* 2017; Ni *et al.,* 2019), reverses metabolic rhythm (Delahaye, *et al.,* 2018; De-Goede *et al.,* 2019) and contributes to weight gain (Delahaye, *et al.,* 2018; Goel *et al.,* 2019; Ni *et al.,* 2019), obesity (Chaix *et al.,* 2014; Ni *et al.,* 2019) and metabolic dysfunctions (Wehrens *et al.,* 2017; Sakai *et al.,* 2018; Shimizu *et al.,* 2018; Goel *et al.,* 2019), regardless of how much calorie is being consumed (Goel *et al.,* 2019; Ni *et al.,* 2019). On the other hand, restricting access to food exclusively to the night (active phase) in rodents, increases longevity, delays the onset of age-related morbidities (Gat□Yablonski *et al.,* 2016) and reduces the risk of cardiometabolic disorders (Olsen *et al.,* 2017; Jamshed *et al.,* 2019; Bhoumik *et al.,* 2020).

Consequently, the increasingly varied lifestyles and eating patterns of our current society with eating window of 16-18 hours (Ni *et al.,* 2019; Zhang *et al.,* 2019) would and continue to pose a global threat of adverse metabolic diseases up and until research and public health efforts are effectively mobilized in that regards. Interestingly, lifestyle changes have been advocated as interventions for the prevention and/or treatment of metabolic disruptions (Olsen *et al.,* 2017; Jamshed *et al.,* 2019). These include fasting and time restricted eating in which feeding window is reduced and aligned to the natural feeding rhythms (Gat□Yablonski *et al.,* 2016; Olsen *et al.,* 2017; Jamshed *et al.,* 2019; Zhang *et al.,* 2019; Bhoumik *et al.,* 2020).

Although research interests have recently been focused in that regard, to the best of our knowledge and literature consultations, studies using time restricted protocols have consistently adopted an *adlibitum* feeding regimen for their control animals. This begs the question of what the normal should be, and whether outcomes from such researches would be compounded by mistimed and/or increased energy intake of the *adlibitum* imposed controls. This is because, *adlibitum* feeding in nocturnal animals has been critiqued as not being a perfect protocol (Kasanen *et al.,* 2018) and that it should not be viewed as being a *bona fide* physiological state (Gat□Yablonski *et al.,* 2016). Similarly, *adlibitum* feeding in nocturnal rodents has been reported to disturb the diurnal rhythms of physiology and behavior, increase the risk of metabolic diseases, spontaneous tumours and reduces their life span (Gat□Yablonski *et al.,* 2016; Kasanen *et al.,* 2018). Interestingly, it has been noted that a daily feeding window of 12 hours improves metabolic fitness of rodents (Chaix *et al.,* 2014) and that optimal growth promotion and development can be achieved in mice and adult male Wistar rats that are confined, respectively, to only 10 and 5 hours of food access in a day (Hatori *et al.,* 2012; García-Gaytán *et al.,* 2020). Therefore, limiting feed access only to the active phase (night) of rodents should be regarded as a physiological state (Sun *et al.,* 2018; Zhang *et al.,* 2019), and thus, be considered as the control references for metabolic related researches (Gat□Yablonski *et al.,* 2016).

Consequently, while many metabolic related researches have been conducted aiming to explore the beneficial effect of time restricted feeding; their *adlibitum* control protocols could hinder extrapolative conclusions. To ensure scientific quality and relevance, the design of timed/caloric restriction in rodents would thus require the achievements of an undisturbed diurnal rhythm (Kasanen *et al.,* 2018) at least, in the controls. Consistent with this notion, we focused at simulating a modern-day human lifestyle of extended eating window amidst delayed night sleep and termed it a rat model of late-night eating in humans.

In that regard, we aimed at exploring the hematological, metabolic and oxidative stress biomarker changes associated with that abnormal feeding pattern.

MATERIALS AND METHODS

Experimental animals, animal groupings and research protocol: A total of 16 male Wistar rats aged between 8-10 weeks, weighing 100g±12g were purchased from the animal house of the Department of Human Physiology, Bayero University, Kano, and were housed in metallic cages measuring 38cm×46cm×24cm with saw dust beddings with room temperature of 22^{0C}-25^{oC}. They were randomly divided into 2 equal groups; group 1 (controls) and group 2 (late-night eating models) and allowed an acclimation period of two weeks during which they were maintained under the natural 12L/12D condition and allowed feeding during the dark periods of the day only. For the 6 weeks of the intervention period, feeds and tap water were made available throughout the dark portion of the day for the controls rats while the models were in addition, allowed to feeds during the first five hours of photophase (from 6:30 am to 11:30 am), at which time, the two groups were subjected to a gentle handling sleep restriction protocol as described elsewhere (Dissi *et al.,* 2021). The research protocol was reviewed and approved by the animal use and care committee of Ahmadu Bello University, Zaria, Nigeria (ABUCAUC/2020/64) and the ethical committee's guidelines as well as the National Institutes of Health guidelines for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) were adhered to.

Determination of body weight changes and feeds intake: A weighing scale (American weigh triple beam scale, model: TB-2610. Readable load of 610 g with readability and sensitivity of 0.1g) was used to obtain the fasting weight of the experimental animals on days 0, 15, 22, 29, 36, 43, 50 and 56 of the experiment. Measurements were done between 6:00 pm to 6:30 pm of the respective days accordingly. Weekly weight changes were deduced by subtracting weight of a particular week from the weight of a previous week (i.e w_2- w_1, w_3-w_2 e.t.c). On the other hand, Feeds were pelletized and oven dried for maximum particular cohesion and the weight of feeds intake per day was obtained by subtracting the weight of leftover pellets from the weight of provided feeds. Thereafter, cumulative total weekly feeds intake was obtained by adding daily intakes.

Animal sacrifice, samples collection and biochemical analysis: The research protocol lasted for 6 weeks, after which the two groups were allowed to resume their acclimation protocols for 24 hours before being anaesthetized using an intraperitoneal injection of a cocktail of diazepam (2mg/kg) and ketamine (20mg/kg). Blood samples were then taken via cardiac puncture and were put in two separate containers; one, containing ethylene diamine tetra-acetic acid (EDTA) (at the ratio of 1–2 mg of anticoagulant to 1ml of blood) and the other, plain container without EDTA. The blood samples containing EDTA were used to determine full blood counts (FBC) and $CD4^{+}T$ cells, while blood samples in the plain containers were allowed to stand at room temperature for 30 minutes before being centrifuged at 2000G for 15 minutes at room temperature using a bench top centrifuge. Using a Pasteur's pipette, the serum layers were aspirated and transferred into smaller, sterile, labeled, blank tubes and stored in a refrigerator at $0°C$ for subsequent analysis of the metabolic, oxidative stress and other biochemical parameters.

Biochemical analysis: Biochemical analysis of samples was done at the laboratory units of Haematology Department, Aminu Kano Teaching Hospital and Human Physiology Department of Yusuf Maitama Sule University, Kano.

Determination of fasting blood glucose: This was done using a digital Glucometer and strips (Accu-Check Active® Roche Diagnostics, GMBH 68298; Germany) on days 0, 14 and 56 between 5:30 pm to 6:00 pm accordingly.

Determination of lipid profile and ratios: Total cholesterol (T.Chol.), serum triglyceride (Trigs) and Serum High-density lipoprotein (HDL) were quantified using their respective Randox kits and chemistry Autoanalyser (mindry Ba-88a). In addition, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were computed as LDL = TC - (HDL+ Trigs/5) and VLDL =Trigs/5 whereas total lipids was calculated as the sum total of all the components of the lipid profile. Similarly, TyG index, Cardiac Risk Ratio (CRR), Atherogenic Index of Plasma (AIP), Atherogenic Coefficient (AC) and Castelli's Risk Index-II (CRI-II) were determined as previously reported (Dissi *et al.,* 2021).

Determination of oxidative stress biomarkers: Lipid peroxidation was estimated calorimetrically by measuring malondialdehyde (MDA) using the method of Albro *et al*. (1986) and Das *et al*. (1990). Catalase (CAT) activity was measured spectrophotometrically using Abebi's method (1974) while Superoxide dismutase (SOD) was determined by the methods described by Fridovich (1989).

Determination of haematological parameters and ratios: Full blood count was done using an Automated Hematology analyzer (Mindray BC-10) while CD 4^+ T-cells were estimated by impedence-based flow cytometry using an automated Cyflow counter 1 (Partec, Germany, 2017). Monocyte to high-density lipoprotein cholesterol ratio (MHR), monocyte-lymphocyte ratio (MLR) and platelet-lymphocyte ratio (PLR) were obtained by dividing monocyte with HDL and lymphocyte counts as well as dividing platelets by lymphocytes respectively.

Statistical analysis: Data was analyzed using the Statistical Package for Social Sciences (IBM SPSS version 20.0). Student's t-test was used to compare difference between groups and data were summarized as Mean±Standard error of means (SEM). In all cases, $p \leq 0.05$ was considered as statistically significant.

RESULTS

From the results, we noted the average weekly feeds intake of the late-night eating (LNE) models to be significantly lower from the first through the fourth intervention weeks. Thereafter the difference in the amount of consumed feeds begins to narrow and remain statistically insignificant up to the last intervention week. However, due to the larger initial differences, the overall total difference in feeds intake remain statistically lower in the late-night eating model rats (table 1).

On the other hand, it could be noted that at no point during the intervention weeks, has weight gain appeared significant. But it could be observed that the margin of initial weight gain adopts an increasing trend up to the third intervention week, before trending towards attenuation at the beginning of fourth week (table 2). Interestingly, despite the overall significant difference in feeds consumption between the groups, body weight gain remains statistically similar at the end of the research period, with percentage difference in feeds intake and body weight gain of 11.4% 16% respectively.

We have also demonstrated that fasting blood glucose is significantly lower in the late-night eating model group but TyG index appeared to be similar between the groups (table 3). On the other hand, while Trigs and VLDL levels are not different between the groups, HDL cholesterol level is significantly lower, whereas, CRR and AC are noted to be marginally higher in the abnormal feeding rats than in controls (table 3).

On the other hand, comparative analysis of oxidative stress and systemic inflammatory markers revealed a statistically similar serum MDA levels across the groups. On the other hand, serum catalase is found to be significantly lower among the late-night eating model rats (table 4). In addition, both MHR and PLR values are slightly higher in the LNE rats than in controls (table 4).

Total WBC count, although appears non significantly lower with relative monocytosis, $CD4^{+}$ T cells appeared significantly lower among the late-night eating models. Similarly, total and percentage lymphocytes were slightly reduced by the abnormal feeding protocol (table 5).

We have also observed that RBC count, HCT and HGB levels are nonsignificantly lower, whereas, platelet cell count appears insignificantly higher in the late-night eating model rats. On the other hand, other erythrothrombotic parameters and indices of the two groups remain essentially similar (table 6).

Table 1: Weekly and Total Weight of Feeds Consumed during the intervention period

Variables	**Controls**	**LNE models**	**t-value**	**P-value**
week 1	191.99±8.80	144.44±8.92	3.794	0,002*
week 2	181.51±6.20	167.60±4.12	1.871	0.082
week 3	210.00±8.53	188.25±4.70	2.337	0.035*
week 4	205.25±7.04	179.00±9.18	2.269	0.040*
week 5	178.25±11.56	163.50±9.29	0.994	0.337
week 6	146.88±11.23	132.25±12.90	0.855	0.407
Total	1519.44±15.95	1347.04±27.17	5.472	0.001*

All values are expressed in grams and summarized as mean±SEM; LNE=late-night eating; *=statistically significant difference

Table 2: Weight and weight change profile of the animals over the Research period

Variables	**Controls**	**LNE models**	**t-value**	**P-value**
post acclimation weight	121.63±9.42	134.25±8.92	-0.973	**0.347**
Final weight in grams	174.63±8.56	171.25±8.35	0.282	**0.782**
Weight change in week 1	12.75±5.00	8.63±11.96	0.318	**0.755**
Weight change in week 2	11.25±2.49	5.63±12.71	0.434	**0.671**
Weight change in week 3	7.63±1.43	3.75±1.56	1.836	**0.088**
Weight change in week 4	7.75±1.93	7.00±2.05	0.267	**0.794**
Weight change in week 5	10.38±1.05	9.63±1.96	0.337	**0.741**
Weight change in week 6	3.25±2.00	2.38±1.45	0.354	**0.728**
Total weight change	53.00±7.58	37.00±5.32	1.728	**0.106**

All Values are presented in grams and summarized as mean±SEM; LNE=late-night eating.

Table 3: Comparing fasting blood glucose, lipid profile and lipid ratios between the groups

Variables	**Controls**	**LNE models**	**t-value**	**p-value**
Initial FBG (mg/dl)	96.25±2.30	100.75±3.64	-1.045	0.314
Final FBG (mg/dl)	126.25±4.14	111.00±3.64	2.859	0.013*
FBG Changes (mg/dl)	30.50±5.14	10.20±2.21	2.684	0.018*
TyG index	6.22±0.17	6.15±0.13	0.359	0.725
Trigs (mg/dl)	8.66±1.08	8.66±1.10	0.004	0.997
HDL (mg/dl)	2.81±0.17	2.17±0.15	2.793	0.014*
LDL (mg/dl)	17.25±2.18	16.94±1.99	0.105	0.918
VLDL (mg/dl)	1.73±0.22	1.73±0.22	0.004	0.997
T.Chol. (mg/dl)	21.79±2.26	20.84±2.10	0.306	0.764
T.Lipids (mg/dl)	52.23±4.57	50.34±4.34	0.301	0.768
CRR	7.73±0.61	9.57±0.69	-2.012	0.064
CRI	6.11±0.64	7.75±0.69	-1.741	0.104
AC	6.73±0.61	8.57±0.69	-2.012	0.064
AIP	0.459±0.068	0.582±0.068	-1.282	0.221

All values are in mg/dl and presented as mean±SEM; FBG= fasting blood glucose; TyG index=triglyceride-glucose index; Trigs=triglyceride; HDL = high density lipoprotein; LDL=low density lipoprotein; VLDL=very low density lipoprotein; T.Chol=total cholesterol; T.Lipids=total lipids; CRR= cardiac risk ratio; CRI= castelli risk index; AC= atherogenic coefficient; AIP= atherogenic index of plasma; LNE=late-night eating; *=statistically significant difference

Table 4: Biomarkers of oxidative stress and systemic inflammation of the groups

Variable	Controls	LNE models	t-value	p-value
MDA (µmol/L)	6.54±2.60	5.47±0.60	0.401	0.695
CAT (U/L)	0.130±0.023	0.042±0.008	3.567	0.003*
SODa (U/min)	1.89±0.09	1.96±0.01	-0.813	0.430
SODi (%)	94.38±4.51	98.05±0.25	-0.813	0.430
MHR	0.36±0.05	0.46±0.08	-1.093	0.293
MLR	0.097±0.007	0.113±0.014	-1.008	0.330
PLR	43.89±3.97	57.81±7.34	-1.667	0.118

All values are presented as mean±SEM; MLR=monocyte to lymphocyte ratio; MHR=monocyte to HDL ratio; GLR=Granulocyte to lymphocytes ratio; MLR=Monocyte to lymphocytes ratio; LGR=Lymphocytes to granulocytes ratio; LNE=late-night eating; *=statistically significant difference

Table 5: Leucocytic parameters of the groups

Variables	Controls	LNE models	t-value	p-value
WBC (x10^3/µL)	12.33±1.13	10.60±1.47	0.929	0.368
LYM (x10^3/µL)	10.04±0.99	8.31±1.24	1.085	0.296
MON (x10^3/µL)	0.96±0.11	0.94±0.13	0.144	0.888
GRA (x10^3/µL)	1.31±0.06	1.35±0.22	-0.164	0.872
LYM (%)	81.05±0.83	78.84±2.47	0.850	0.409
MON (%)	7.85±0.56	8.71±0.59	-1.059	0.307
GRA (%)	11.10±0.67	12.45±1.99	-0.644	0.530
CD4 (cells/µL)	37.00±5.46	14.50±4.67	3.132	0.007*

All values are presented as mean±SEM; WBC=white blood cells; LYM=lymphocytes; MON=monocytes; GRA=granulocytes; LNE=late-night eating; *=statistically significant difference.

Table 6: Haemato-thrombotic cell counts and indices of the groups

Variables	Controls	LNE models	t-value	p-value
RBC	7.08±0.19	6.06±0.76	1.301	0.214
HGB	13.75±0.28	11.98±1.50	1.162	0.265
HCT	35.54±0.64	30.84±3.74	1.237	0.236
MCV	50.36±0.67	51.26±0.86	-0.828	0.421
MCH	19.43±0.22	19.60±0.32	-0.459	0.654
MCHC	38.680.18	38.30±0.70	0.520	0.611
RDWC	17.39±0.39	17.00±0.27	0.824	0.424
RDWS	28.73±0.67	30.11±0.59	-1.547	0.144
PLTC	417.38±16.89	464.25±63.10	-0.718	0.485
MPV (fL)	7.41±0.14	7.40±0.11	0.072	0.944
PCT	0.311±0.016	0.347±0.048	-0.719	0.848
PDW (fL)	18.80±0.83	16.86±1.16	1.356	0.197
PLCR (%)	9.76±0.67	9.34±0.52	0.503	0.623

All values are presented as mean±SEM; RBC=red blood cells; HGB= hemoglobin; HCT= haematocrit; MCV= mean corpuscular volume; MCH=mean corpuscular hemoglobin; MCHC= mean corpuscular hemoglobin concentration; RDWC= red cell distribution width coefficient of variation; RDWS= red cell distribution width standard deviation; PLTC=platelets count; MPV=mean platelet volume; PCT=plateletcrit; PDW=platelet distribution width;; PLCR=platelet large cell ratio; LNE=late-night eating.

DISCUSSION

The primary aim of the current study is to explore the hematological, metabolic and oxidative stress biomarker changes associated with late-night eating in a rat model. Our observation shows a significant decrease in total feeds consumption among the late-night eating models beginning from the first through the 4th week before assuming a trend towards attenuation, is consistent with the finding of decreased appetite in light-fed rats subjected to a 3 weeks study protocol (García-Gaytán *et al.,* 2020). It is also in tandem with the observation that externally imposed feeding restrictions would require about 4 weeks of adaptation to reach a relative stablity (Acosta-Rodrıguez *et al.,* 2017; Zhang *et al.,* 2019) perhaps, because late-night eating model rats exhibits an unstable activity levels during the first 4 intervention weeks (Ni *et al.,* 2019). More so, our finding of better food-efficiency among the late-night eating models is in consonant with recent late-night eating models in mice (Acosta-Rodriguez *et al.,* 2017; Delahaye, *et al.,* 2018) and rats (Shimizu *et al.,* 2018; Ni *et al.,* 2019) which also revealed an obesogenic tendency without necessarily increasing feeds consumption (Acosta-Rodriguez *et al.,* 2017; Delahaye, *et al.,* 2018; Shimizu *et al.,* 2018) due to better fuel efficiency (Opperhuizen, 2016; Acosta-Rodriguez *et al.,* 2017; Delahaye, *et al.,* 2018; Shimizu *et al.,* 2018), reduced energy expenditure (Olsen *et al.,* 2017) and perhaps, circadian dysruption (Shimizu *et al.,* 2018). In contrast, although some studies had observed a significant reduction in body weight gain and lower or unaltered feeds intake among day-fed mice (Zhang *et al.,* 2019) and rats (de Almeida *et al.,* 2016), others have reported no differences in body weight gain (Opperhuizen, 2016; Cisse *et al.,* 2018; De-Goede *et al.,* 2019), except for increased fat deposition (Opperhuizen, 2016), or caloric intake (Cisse *et al.,* 2018; De-Goede *et al.,* 2019). These differences could be due to inconsistencies in time and timing of feeds, duration of intervention, specie or sex differences. For example, while de Almeida *et al*. (2016) provided chow between ZT24 and ZT12 for over 6 weeks using 10 weeks old female Wistar rats, De-Goede *et al*. (2019) used adult male Wistar rats and allowed access to chow pellets for only 10 h in the middle of the light phase using a 4 weeks intervention protocol. On the other hand, Zhang *et al.* (2019) employed a 30% calorie restriction in a 4 week protocol using 8 weeks old male C57BL/6J mice. Notwithstanding, human (Sakai *et al.,* 2018; Gabel *et al.,* 2019; Goel *et al.,* 2019; Wilkinson *et al.,* 2020) as well as rodent (Bhoumik *et al.,* 2020) studies restricting food to the early active phase has translated into lower body weight gain and reduced adiposity even when feeds intake is unaltered (Olsen *et al.,* 2017; Sun *et al.,* 2018; Gabel *et al.,* 2019; Hu *et al.,* 2019; Aouichat *et al.,* 2020) demonstrating a protective effect against obesity. In this regards therefore, our finding of better fuel efficiency among the late-night eating model rats constitutes a long-term potential risk for overweight and obesity.

Studies have shown that timing of fasting and feeding behavior in line with regular sleep and wake periods enhances optimal glucose metabolism, such that eating late-at-night or during the light phase (in case of rats) can adversely affect glucose metabolism in rodents (de Almeida *et al.,* 2016; De-Goede *et al.,* 2019; Hu *et al.,* 2019) as well as humans (Sakai *et al.,* 2018; Wilkinson *et al.,* 2020). In agreement with these studies, we hypothesized that late-night eating model rats would display hyperglycaemia and worsened insulin sensitivity; however, our result demonstrates the contrary. We observed a significant reduction in the fasting blood glucose of the late-night eating models with a similar TyG index compared to the controls. As heat shock proteins protect cells from oxidative stress (Thanan *et al.,* 2015) and play a critical role in insulin signaling (Butterfield *et al.,* 2014), their increased expression by

Ad libitum feeding (Gat□Yablonski *et al.,* 2016) would demonstrate the possible mechanism through which late-night eating could cause the development of insulin resistance (Butterfield *et al.,* 2014) via increased oxidative stress (Butterfield *et al.,* 2014; Thanan *et al.,* 2015; Gat□Yablonski *et al.,* 2016). Interestingly, our observed similar MDA and SOD but lower catalase activity could have only been a trend toward oxidative stress, hence, our statistical similarity in TyG index among the groups. This unexpected finding has similarly been observed in dark fed mice who exhibited lower fasting glucose compared to *adlibitum* fed mice and in day-fed mice compared to night-fed (Zhang *et al.,* 2019). Similar observations were also made in humans following late meals under controlled laboratory conditions (Wehrens *et al.,* 2017). In similar findings, neither plasma insulin (Opperhuizen, 2016; Acosta-Rodriguez *et al.,* 2017), nor glucose concentrations (Sun *et al.,* 2018; Ni *et al.,* 2019) were seen to be affected by timing of feed intakes in rodents. Circadian synchronized feeding has been reported to reprogram hepatic metabolic flux, decrease lipogenesis and redirects cholesterol to bile acid production (Longo and Panda, 2016). Our finding, although have not demonstrated difference in the serum levels of triglycerides and VLDL, but has revealed a significantly lower HDL cholesterol level and marginally higher CRR and AC values in the late-night eating model rats, thus, pointing to an increased risk of adverse cardiovascular events. In agreement to our finding, a recent 16 weeks long, cafeteria diet, protocol confining feed access to 8 hours between zeitgeber time13 to 21 reported a significant reduction in serum triglycerides, total cholesterol, LDL, AIP, AC and CRI with significant increase in serum HDL compared to rats fed *adlibitum* (Aouichat *et al.,* 2020). In similar temporal, but 4 weeks long protocol using normal chow, *adlibitum* fed mice exhibited significantly higher triglycerides and LDL levels with no significant difference in total cholesterol or HDL when compared to the nocturnally restricted mice (Hu *et al.,* 2019). In another 4 weeks long, high sucrose diet protocol, *adlibitum* fed rats were found to have increased triglycerides and total cholesterol levels compared to those confined to a feeding regimen between zeitgeber time12 to 24 (Sun *et al.,* 2018). Since a sixteen (16) weeks long and cafeteria provided protocol was able to demonstrate a bigger effect size compared to our findings, the subtle differences between these findings and ours could be duration and/or diet dependent. In consonant with our general finding, late-night eating have repeatedly demonstrated a tendency towards elevated atherogenic and decreased cardio protective lipids in both rodent (Ni *et al.,* 2019) and human (Sakai *et al.,* 2018; Goel *et al.,* 2019; Wilkinson *et al.,* 2020) models.

Upon the emergence of day (light), naive B cells in the payer's patch decreases, whereas they are noted to increase in the bone marrow until the onset of darkness, thus during the feeding period, B-cells decreases in the bone marrow and increases in the payers patch and vice versa (Nagai *et al.,* 2019). In addition, during the active (feeding) phase, noradrenalin-dependent b2-adrenergic stimulation up regulates CCR7 and CXCR4 thereby suppressing cell egress from the lymph nodes to the blood (Suzuki *et al.,* 2016) on the other hand, decreased noradrenalin levels allow for the migratory efflux of lymphocytes back into the circulation during the resting (fasting) phase (Nagai *et al.,* 2019). This circadian variation is noted to influence the bactericidal properties of night-fed mice who were observed to exhibit an enhanced bacteria-killing capacity when challenged prior to, but not the inactive (resting) phase (Cisse *et al.,* 2018). Consequently, when feeding is extended into the resting period, bone marrow to blood egress as well as lymph nodes to blood migration of lymphocytes, and by extension immunological competence, could be adversely affected as has been observed in abnormally fed mice who were unable to mount an LPS-primed bacterial killing during both the active and the inactive phases (Cisse *et al.,* 2018).

This impairment could have been a result of the ability of abnormal feeding time to induce circadian deficits in signaling and synthesis as well as an altered immune cell differentiation and expansion especially in the compliment system and T-helper lineages (Cisse *et al.,* 2018). Therefore, our finding of significantly lower $CD4^+$ T, insignificantly lower total WBC count as well as the slight reduction in total and percentage lymphocytes in the late-night eating model rats signified a trend towards reduced immunological competence.

Moreover, while decreased inflammatory activity could compromise immune response to infection or tissue injury and repair processes, its exaggeration is devastating to metabolic homeostasis and quite implicative in the pathogenesis of chronic and autoimmune diseases (Jordan *et al.,* 2019). Since monocytes are critical in induction and maintenance of inflammation (Jordan *et al.,* 2019), and that fasting reduces the accumulation of pathogenic monocytes (Cignarella *et al.,* 2018) and enhances the up regulation of PPAR-α, with subsequently anti-inflammatory effects (Jordan *et al.,* 2019), it is tempting therefore to relate feeding during the abnormal time with altered inflammatory response. This idea can be strengthened by a recent observation of increased expression of inflammatory cytokines, including TNFα, IL1β, and IFNγ in a late-night eating model rats (Ni *et al.,* 2019). Consequently, our observed relative monocytosis and slightly higher MHR and PLR values in the LNE rats indicates a tendency towards higher systemic inflammatory status.

Taken together, our finding of reduced immunological competence and a trend towards higher systemic inflammation has elucidated the tendency of late-night eating to cause an adverse immunoinflammatory alteration. The mechanism(s) through which late-night eating could cause this is, yet, subject to further research, however our finding has corroborated findings from abnormal feeding regimens in rat (Ni *et al.,* 2019; Upadhyay *et al.,* 2019; Bhoumik *et al.,* 2020) and mice (Hatori *et al.,* 2012; Jordan *et al.,* 2019; Zhang *et al.,* 2019) models as well as from calorie restricted and intermittent fasting (Cignarella *et al.,* 2018; Jordan *et al.,* 2019) protocols. While severe caloric restriction increases oxidative stress (Stankovic *et al.,* 2013), mild fasting is known to decreases MDA level, improve antioxidants capacity (Stankovic *et al.,* 2013; Nurmasitoh *et al.,* 2018), cardiovascular (Nurmasitoh *et al.,* 2018) and other health (Stankovic *et al.,* 2013; Martens *et al.,* 2020) outcomes. On the other hand, *adlibitum* feeding increases the expression of heat shock proteins (Gat□Yablonski *et al.,* 2016) as a consequent of increased oxidative stress (Thanan *et al.,* 2015; Gat□Yablonski *et al.,* 2016). It is not surprising therefore, that we found significantly higher Catalase activity among the late-night eating model rats. in consonant to our finding, eight-week old female Wistar rats confined to eat only between 9pm to 6am daily displayed an improvement in oxidative stress capacity over those allowed *adlibitum* access (Upadhyay *et al.,* 2019). Similar beneficial effects have been observed in rodent models (Gat□Yablonski *et al.,* 2016; Nurmasitoh *et al.,* 2018; Bhoumik *et al.,* 2020) as well as in human subjects (Sutton *et al.,* 2018; Martens *et al.,* 2020). Notwithstanding, a finding contrary to ours is noted (Garcia-Gaytan *et al.,* 2020). While it would be expected for a time restricted feeding/fasting to improve oxidative status (Nurmasitoh *et al.,* 2018; Sutton *et al.,* 2018), we noted that the protocol employed by Garcia-Gaytan *et al.,* was restricting rats to a 5 hour/day eating window during the light (resting/fasting) phase. This protocol could have created a double edge sword of severe energy deprivation and circadian disruption. Both severe caloric restriction (Stankovic *et al.,* 2013) and circadian disruption (Lee *et al.,* 2013) are known to increase oxidative stress; therefore, the disposition of an overwhelming oxidative status in the time restricted rats reported by Garcia-Gaytan *et al.,* Could have been the consequence of their timing and time allocated to feeding rather than due to a contrasting outcome.

Since meal timing affect various aspects of the circadian clocks (Cisse *et al.,* 2018; Ni *et al.,* 2019; García-Gaytán *et al.,* 2020) and also oxidative stress response capacity is modulated by the circadian system (Butterfield *et al.,* 2014; Nurmasitoh *et al.,* 2018), it is likely that circadian disruption via mistimed feeding (Wehrens *et al.,* 2017; Ni *et al.,* 2019) could disrupt an oxidative stress handling capacity via circadian-desynchronization induced down regulation of nuclear factor erythroid-2-related factor-2 (Nrf2) expression (Lee *et al.,* 2013). Consequently, our finding of increased oxidative stress potentials could therefore, be a result of significant circadian disruption and the one hour phase advancement observed among the late-night eating model rats (Dissi *et al.,* 2020).

Our observed trend toward lower RBC count and higher platelet cell count in the late-night eating model rats with other erythrothrombotic parameters and indices remaining similar across the groups could be a consequent of our observed circadian desynchrony, oxidative stress and a trend towards systemic inflammation. The circadian system influences haematopoiesis (Hajam and Rai, 2020), daily haematological variations (Makeri *et al.,* 2017), oxidative stress (Habibi*et al.,* 2019) and food intake (Wehrens *et al.,* 2017; Ni *et al.,* 2019). Similarly, feed intake is known to modulate haematopoiesis (Jordan *et al.,* 2019), oxidative stress (Nurmasitoh *et al.,* 2018; Sutton *et al.,* 2018) and circadian system (Shimizu *et al.,* 2018). Since we have observed oxidative stress and circadian dysruption among the late-night eating model rats, the interconnected relationships between them begs for the enquiry whether affecting one could not result in a cumulative toll to the other; therefore our findings, although not statistically significant, but can however be viewed in that regards.

Although there are few studies that focused on blood alterations in late-night eating for comparison, nonetheless, our finding is in agreement with the findings of an 8 hour (Gabel *et al.,* 2019) and 10 hour (Wilkinson *et al.,* 2020) time restricted feeding during the active phase among metabolic syndrome (Wilkinson *et al.,* 2020) and obese (Gabel *et al.,* 2019) participants whom demonstrated no significant change in hemoglobin, haematocrit, platelet counts (Wilkinson *et al.,* 2020) or any of the complete blood count parameters (Gabel *et al.,* 2019), each, after 12 weeks of intervention.

CONCLUSION AND RECOMMENDATION

In conclusion, our findings have uncovered the dyslipidaemic, oxidative stress and immunoinflammatory alterations associated with late-night eating. Consequently, to promote general health, prevent metabolic and other related diseases, we wish to recommend nutritional lifestyle changes such that eating window is reduced to between 10-12 hours, meals temporally distributed and exclusively in synchrony with our circadian phase of eating.

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