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The Comparison of Long-term Effect Between Intermittent Fasting and Calorie Restriction on Neurological Parameters of Mice

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Abstract

Intermittent fasting (IF) and calorie restriction (CR) were purported to have health benefits. This research aimed to determine the long-term effect of IF and CR on selected neurological parameters in mice. Swiss Webster male mice were divided into 3 groups: *ad libitum* feeding (AL), IF, and CR. Mice in each group received the treatment for 16 weeks. They were then tested for anhedonia, depression, aggressiveness, and social approach. They were also subjected to contextual fear conditioning tests to model PTSD. Compared to AL, sucrose intake in the IF group was lower, while the CR group showed higher intake ($p<0.01$). This anhedonia characteristic shown in the IF group was confirmed not related to depression, as shown by significantly lower immobility time in the forced swimming test compared to AL ($p<0.05$). In the resident-intruder test, attack numbers in the IF group were fewer than in the AL group ($p<0.05$). As demonstrated by the results of the three-chamber test, the reduced aggressiveness in IF mice was unrelated to a deficit in sociability. In the fear extinction test (PTSD model), mice in the IF group showed lower freezing compared to those in AL ($p<0.001$). Although both IF and CR caused a reduction in total food intake, in the mice model tested, IF was shown to have favorable impacts on neurological parameters.

Keywords: Anhedonia; Calorie Restriction; Depression; Intermittent Fasting; Post Traumatic Stress Disorder.

INTRODUCTION

Humans have practiced fasting for cultural or religious purposes for a long time. Recently, fasting was practiced widely for health purposes, and it becomes a lifestyle. There are many ways and designs of cutting out diet intake. However, generally, in a research setting, fasting can be classified into three types, namely calorie restriction (CR), alternate-day fasting (ADF) or intermittent fasting (IF), and dietary restriction (DR) (Trepanowski & Bloomer, 2010). Calorie restriction (CR) is the reduction of food intake by a certain percentage (measured in kcal) of *ad libitum* consumption. CR is usually set at a 20-40% reduction from normal intake (Cherif et al., 2016). Intermittent fasting is a practice that consists of alternating periods between "the feast period", in which the subject may consume food ad libitum, and "the fast period," where fasters are prohibited from consuming any food except water. The duration of the feast period may be varied, but it is usually 16 hours or 24 hours (Welton et al., n.d.).

In many research literatures, fasting or food reduction increased longevity in a diverse group of species, including the bacteria *Escherichia coli*, yeast *Saccharomyces cerevisiae*, nematode *Caenorhabditis elegans*, fruit fly (*Drosophila Melanogaster*), and mouse (Gonidakis et al., 2010; Hwangbo et al., 2020). The possible mechanisms behind the benefit of fasting were proposed in three scenarios: attenuation of oxidative damage, modulation of glycemia and insulinemia; and hormesis, the beneficial action resulting from the response of an organism to a low-intensity stressor (Pinches et al., 2022).

In the hormesis mechanism, IF and CR are considered low-intensity metabolic stressors that induce the release of stress hormones, adrenocorticotropin hormone (ACTH), and corticosterone (Kim et al., 2021). This low-intensity stressor is a kind of exercise to face a higher-intensity stressor. The response of mice to higher stressors after being treated with long-term IF and CR was studied in this research by modeling post-traumatic stress disorder (PTSD) in mice.

Abnormality of HPA (hypothalamic-pituitary-adrenal system) may underlie stress-related psychiatric disorders such as melancholic depression (Juruena et al., 2018). It was found that the practice of fasting enhanced mood in patients with chronic pain syndrome (Michalsen, 2010). Ramadan fasting was found to increase plasma levels of serotonin, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and serotonin plasma levels. Serotonin is one of neurotransmitter involved in mood regulation (Bastani et al., 2017). In another study,

underfeeding patients for 16 days caused decreasing dopamine, a neurotransmitter in the brain that plays a role in reward-motivated behavior (Göhler et al., 2000). Therefore, this study aimed to evaluate the effect of long-term IF and CR on mood-related behavior, particularly depression and anhedonia. Since anhedonia is a core characteristic of major depressive disorder (MDD), this test should be confirmed with a depression test. This study also assessed the effect of IF and CR on aggressiveness and social interaction as one of the behaviors closely related to depression.

MATERIALS AND METHODS

Animals

Twenty-four male Swiss Webster mice aged 7 weeks old were kept for 2 weeks at a constant room temperature of 25ºC with 12 h light and 12 h dark cycle for acclimatization. During these 2 weeks, a continuous supply of food and water was provided, and food intake was measured daily. The average daily food intake during acclimatization was determined as a 100% calorie provision requirement.

Diet Treatment

Animals were divided randomly into 3 groups, which were *ad libitum* feeding (AL), intermittent fasting (IF), and calorie restriction (CR). Each group consisted of 8 mice, housed two per cage. Mice in the AL group were provided with a continuous supply of food. In contrast, those in the IF group were fed every other day (a 24-hour fasting period was followed by a 24-hour feeding period, in which food was available ad libitum). Mice in the CR group were fed 65% of the calorie requirement, which was 4.5 g/mice/day. All mice were provided with a continuous supply of drinking water throughout the study. Diet treatment was initiated on 9-week-old mice, which is roughly equivalent to 18-year-old humans. The treatment was implemented for 16 weeks. At the end of the treatment, mice were 25 weeks old, equivalent to 40 years old human (Dutta & Sengupta, 2016). Food intake in each cage was measured daily, and the body weight of each mouse was weighed weekly. After the completion of diet treatment was ended, the mice were tested for their behavior.

Sucrose Preference Test

In each cage, mice were provided with two drinking bottles; bottle A contained drinking water, and bottle B contained 15% sucrose solution. The position of each bottle was shifted every 24 hours to avoid bias in place preference. This treatment was performed for 4 days: the first two days were for acclimatization, and the following two days were for data measurement. Acclimatization was aimed to avoid neophobia since the mice were never exposed to sucrose drinking. Each bottle was weighed before and after the test. Sucrose preference was calculated with the formula:

$$\frac{sucrose\ intake}{sucrose + water\ intake} x100\%$$

Forced Swim Test

A mouse was placed in a tank filled with water for 6 minutes. The duration of immobility time was measured during the last four minutes. The mouse was considered immobile if it was floated and only made the necessary movements to keep its nostrils above the water's surface.

Resident-Intruder Test

This test was aimed at measuring aggressiveness. The bedding was not changed 2 weeks before the test in order to enhance olfactory stimulus as a territorial cue within the cages. The test was performed by introducing a stranger mouse of the same sex to the cage of a test mouse mice for 5 minutes. Any sign of aggressiveness was recorded. In case of an attack, the latency as well as incidence was calculated.

Three-Chamber Test

The three-chamber test apparatus consists of three inter-connected compartments. One side chamber was assigned as a stimulus chamber (Chamber B) in which the mouse could be exposed to an olfactory stimulus through a hole. An empty cage was placed in the other side chamber as the control chamber (Chamber C). A middle chamber (Chamber B) was sandwiched between the side chambers. One day before the test, a mouse was allowed to explore the apparatus for 10 minutes. Mouse was observed if there was any sign of chamber preference. On the testing day, before the test, the mouse was habituated for 5 minutes by placing it in the middle chamber. During the test session, a stimulus mouse was placed into a cage in chamber A, an empty cage was placed in chamber C, and the test mouse was replaced in chamber B. The percentage of time spent in each chamber during the habituation session and test session was calculated.

Contextual Fear Conditioning and Fear Extinction

Fear conditioning is performed by pairing the conditioned stimulus (CS), the environment of the fear conditioning box; with the unconditioned stimulus (US), electrical footshock. The mouse will learn to associate CS with the US. This method is commonly used for PTSD modeling in animals.

On the first day, a mouse was habituated in a fear conditioning box for 3 minutes. During this phase, the duration of freezing was measured and recorded as the freezing baseline. In the following 6 minutes, electric foot shocks (1.2 mA, duration 1 sec) were delivered repeatedly in 19 sec intervals. The behavior of the mouse was recorded using a digital video camera mounted on

the ceiling of the outer box. At 24 h later, the mouse was re-exposed to the fear conditioning box for 6 minutes without exposure to electric foot shock. This phase was called extinction training. At 48 hours following extinction training, the mouse was re-exposed to the fear conditioning box without exposure to the foot shock and tested for its memory retention to extinction training. This phase was called the extinction test. The duration of freezing in each stage (habituation phase, fear conditioning phase, fear extinction training phase, and fear extinction test phase) was measured and compared between the groups. The freezing response was defined as complete immobilization of the mouse, except that it was needed for respiration (Shoji et al., 2014).

Data Analysis

Data analysis was performed using one-way ANOVA followed by the post hoc LSD for comparing parametric data between-group comparisons. The percentage of attack in the aggressiveness test was analyzed using the Fisher exact test. A value of $p < 0.05$ was considered significant. Data in the text was presented as means ± SD.

RESULTS AND DISCUSSION

Food Intake

The average basal food intake was 7.04 g/mice/day. Food intake in the CR group was set from this basal value. Therefore, mice in the CR group were fed 4.5 g/mice/day. Throughout this study, the average food intake in the AL group tended to decrease over time, from 8.05 g/mouse/day at the beginning of treatment to 5.26 g/mouse/day. At the beginning of treatment, the average food intake in the IF group was 6.4 g/mouse/day, lower than AL. After adapting to a new diet, starting from week four until week sixteen, mice in the IF group always consumed more food than the AL group, to as much as twice the quantity. This is similar to the observation reported earlier (Anson et al., 2003). However, since the IF group received the feed every other day, the average daily intake in the IF group was lower than in the AL group (Figure 1A).

Throughout observation, the average body weight of mice in all groups tend to increase, as shown in Figure 1B. Although total intake in the IF group was 32% lower than that of the AL group, no significant difference in body weight was observed between the two groups. This might be explained by metabolic shifting to the ketogenic pathway, resulting in ketone bodies (Carteri et al., 2021). The average body weight of mice in the CR group, however, was significantly lower compared to AL and IF (p-value <0.05). At the end of the treatment, the average body weight in the AL, IF, and CR groups were 51.66 ± 3.82 g, 51.34 ± 2.61 g, and 47.14 ± 4.24 g, respectively.

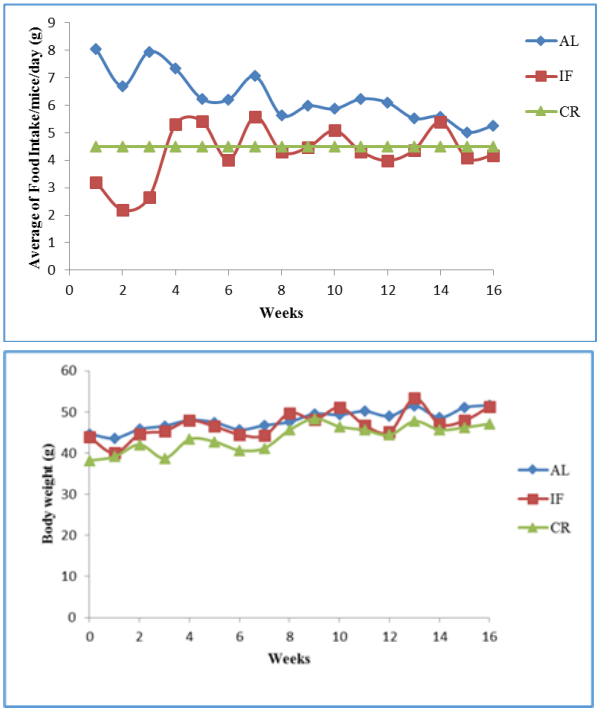

Figure 1. Profiles of average food intake **(A)** and body weight **(B)** during diet treatment for 16 weeks. AL: *ad libitum* feeding, IF: intermittent fasting, CR: calorie restriction.

Sucrose Preference Test and Forced Swim Test

Anhedonia refers to the reduced ability to experience a pleasure. In this study, this characteristic was tested using a sucrose preference test. Mice in the IF group showed no preference between water and sucrose, unlike the other group, which prefers sucrose. Compared to those in the AL group, mice in the IF group showed a significantly lower preference for sucrose ($p < 0.01$), indicating a symptom of anhedonia (Table 1). However, the average sucrose consumption in the CR group was higher compared to that in the AL group, indicating hedonic behavior ($p < 0.01$). Even though mice in CR and IF groups showed decreasing food intake, they showed the opposite results in terms of sucrose preference. In this study, chronic intermittent fasting, but not diet restriction, induced anhedonia characteristics in mice tested with a sucrose preference test. A decrease in dopamine, a neurotransmitter responsible for reward-seeking behavior might explain this behavior. A study conducted by Elesawy et al. (2021) showed that IF caused a decrease in dopamine while increasing serotonin.

Table 1. Sucrose Preference Test and Forced Swim Test Result in Mice after 16 weeks Diet Treatment.

Group	Sucrose intake (%)	Immobility time (s)
AL	67.98 ± 4.7	194.89 ± 19.12
IF	50.29 ± 4.06**	153.43* ± 40.15
CR	87.86 ± 10.08**	157.725* ± 35.94

Note: Data presented in mean ± SD. *) $p < 0.05$ compared to the AL group. **) $p < 0.01$ compared to the AL group

Anhedonia is a core feature of Major Depressive Episodes, according to DSM-5. It is considered a key diagnostic criterion for the depressive subtype melancholia (DSM-5TM, 2013). To confirm whether anhedonia shown in the IF group was related to depression or not, mice were tested using a forced swim test as a representation of "learned helplessness". The demonstration of decreased immobility of mice in both groups compared to the AL group led to a suggestion that anhedonia in the IF group was unrelated to depression (Table 1). Both long-term IF and CR in mice had an "antidepressant-like effect" property. Depression is often associated with abnormality of serotonergic signaling pathways, mainly due to low activity (Cowen & Browning, 2015). It is supported by the evidence that Selective Serotonin Reuptake Inhibitors (SSRIs) improve depression symptoms. Several studies found that food deprivation increased serotonin release and turnover (Bastani et al., 2017; Elesawy et al., 2021; Mohamed Shawky et al., 2015). Acute starving can cause increasing serotonin turnover and 5-hydroxyindoleacetic acid levels as a metabolite of 5-HT. Meanwhile, the long-term starving (14 days) downregulates the density of the 5-HT transporter in the frontal cortex (Huether et al., 1997). Both these mechanisms ultimately cause an increase in serotonin signaling output. Enhancement of serotonergic signaling is also caused by an increase in Brain-Derived Neurotrophic Factor (BDNF), which was induced by long-term intermittent fasting (Elesawy et al., 2021). The difference between the long-term effect of IF and CR mechanisms in improving mood may be related to serotonergic and dopaminergic signaling. While both IF and CR may similarly affect serotonergic signaling, they affect dopaminergic signaling oppositely.

Resident-Intruder Test and Three-Chamber Test

The number of mice which attacked the intruder was the fewest in the IF group. Of eight mice in each group, the number of mice that exhibited attack to the intruder was seven in the AL group, three in the IF group, and six in the CR group. However, when analyzed using the Fisher exact test, this percentage was not significantly different ($p > 0.05$) since the sample size was too small to detect the difference (Table 2).

Table 2. Aggressiveness of Mice Tested with Resident-Intruder Test.

Group	Parameter of Aggressiveness		
	% Attack	Attack Count	Latency of Attack (s)
AL	87.5	8.57 ± 2.51	53.14 ± 67.94
IF	37.5	4.33 ± 1.53*	57.00 ± 49.11
CR	62.5	10.00 ± 3.39	126 ± 80.10

*) $p < 0.05$ compared to the AL group

The average latency of the attack was not significantly different between groups. Meanwhile, the attack count of the IF group was significantly lower than the AL group ($p<0.05$). The lack of aggressiveness showed by mice in the IF group might indicate a deficit of sociability. To confirm this possibility, mice were checked on social interaction using a three-chamber test. During the habituation session, mice did not exhibit a chamber preference. During the test session, mice spent more time in the stimulus chamber. Compared to the AL group, mice in the IF group spent more time in the stimulus chamber ($p < 0.05$), demonstrating no deficit in sociability (Table 3). Several test mice in the IF group showed pro-social behavior, such as the effort to overthrow the cage to free the stimulus mouse.

Table 3. The Percentage of Time Spent in Each Chamber During Three-Chamber Test.

Group	Time spent (%)		
	Stimulus Chamber	Middle Chamber	Non-Stimulus Chamber
	Habituation Session		
AL	35.24	30.47	34.29
IF	32.46	30.37	37.17
CR	33.79	32.1	34.11
	Test Session		
AL	38.79	28.04	33.17
IF	54.68*	19.15	26.17
CR	36.82	28.13	35.05

*) $p < 0.05$ compared to the AL group

Besides affecting mood, serotonergic signaling also has a role in mediating aggressiveness. The 5-HT system dampened aggression in mice and humans. A high level of aggressiveness may be related to a low concentration of 5-HT. It can be reduced by the administration of pharmacological agents that strengthen serotonergic, such as 5-HT precursor, 5-HT reuptake inhibitor, and 5-HT receptor agonist (Randy J. Nelson & Silvana Chiavegatto, 2001). Reduced aggressiveness in IF mice might be related to the enhancement of serotonergic signaling, which also causes an "antidepressant-like effect" characteristic.

Contextual Fear Conditioning and Fear Extinction

The results of fear conditioning and extinction tests are presented in Figure 2. During the habituation session, mice in all groups actively explored the fear conditioning box, a normal behavior for a mouse exposed to a new environment. The baseline of freezing was not significantly different between groups. Then, mice were exposed to a fear conditioning session, in which electrical shocks were delivered. During this session, all groups exhibited similar freezing duration, so the differences in diet treatment did not seem to affect this response. At 24 hours after the conditioning session, mice were trained for extinction. Initially, mice exhibited freezing remarkably since the association between box and electrical foot shock was formed previously. Over time, however, they started to explore the box since their anticipation of electrical foot shock was countered. Therefore, in this stage, the duration of freezing time was

reduced compared to the previous session. The duration of freezing time was not significantly different between the groups in this session. At 48 hours after extinction training, mice were tested for the memory retention of extinction training. In this stage, the duration of freezing in all mice was shorter compared to that during extinction training. In mice treated with intermittent fasting, the duration of freezing was 30.72 ± 25.42 s, significantly different ($p < 0.001$) from that of the AL, 116.77 ± 53.27 s. This result suggests that long-term intermittent fasting facilitates extinction learning in PTSD-modeled mice.

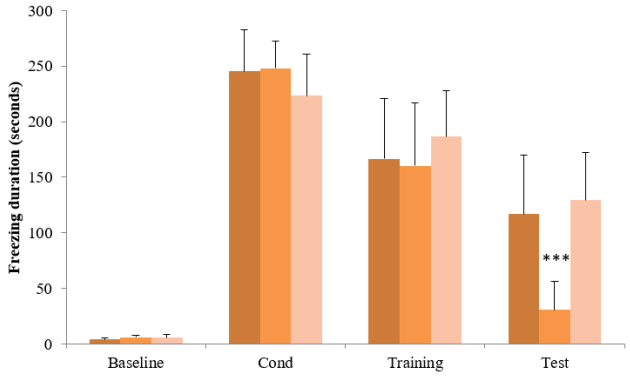

Figure 2. Frequency of freezing in fear conditioning and fear extinction. (Darkest to lightest brown: AL, IF, CR, respectively)

Fear conditioning was widely used for Post Traumatic Stress Disorder modeling. In this method, the previously neutral conditioned stimulus (CS) was paired with an unpleased unconditioned stimulus (US). In this study, CS was a closed box where an unconditioned stimulus, which was an electrical foot shock, was delivered. It was expected that anytime the mouse was put in the same box, it would demonstrate a species-specific defensive response (SSDR), freezing (Maren, 2008). This process is called conditioning. This response to fear is analog to humans having PTSD. After the association of CS to the US was formed, the mouse was exposed to the same box but without exposure to followed electrical foot shock. During this extinction training, the mouse is learning new associations that the box is not dangerous anymore. Several days later, the test animal is tested for retention of memory during extinction.

In this study, it was found that mice treated with long-term intermittent fasting were more resistant to PTSD compared to the AL group when exposed to stressful events. Instead of intervening in the fear conditioning process, IF might facilitate the fear extinction process. In response to stress, the HPA (hypothalamic-pituitary-adrenal) axis will be activated and induce glucocorticoid release from the adrenal cortex. In proper doses, it is important to prepare the body to fight the threat. However, in high concentrations, it can endanger neurons in the hippocampus and cerebral cortex such that they become more vulnerable to excitotoxic, metabolic, and oxidative injury (Dekkers et al., 2022). Two types of receptors of glucocorticoids that have been elucidated are glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). GR may mediate the excitotoxic mechanism of glucocorticoid, while MR has a protective effect, such as maintaining the expression of the antiapoptotic gene Bcl-2 in pyramidal cell populations. Intermittent fasting has been reported to increase corticosterone (belonging to glucocorticoid) concentration in rats, decrease GR level, and not affect MR level (Sabatino et al., 1991). Thus, the probability of glucocorticoid binding to MR will be increased compared to GR. It may explain the mechanism behind the neuroprotective effect of IF, including resistance to PTSD. Other studies showed that IF increases corticosterone in mice but prevents corticosterone surge when mice are exposed to distress, thus facilitating fear extinction (Shojaie et al., 2017). Fear extinction may also be facilitated by BDNF, which increases due to intermittent fasting (Elesawy et al., 2021). A study conducted by Peters et al. (2010) showed that the administration of BDNF directly into the Prefrontal cortex (PFC) can facilitate extinction in mice.

CONCLUSIONS

Even though both intermittent fasting and calorie restriction caused a reduction in total food intake, their beneficial effects on tested behaviors were different. Long-term IF resulted in reduced sensitivity of the brain reward system, which was not related to depression. In addition, long-term IF had an "antidepressant-like effect". The level of aggressiveness in mice treated with intermittent fasting was lower than that in the AL group, and it was shown that a deficit in sociability did not cause it. Intermittent fasting is regarded as a chronic mild stressor such that mice were more resistant to PTSD. Long-term calorie restriction resulted in increasing sensitivity of the brain reward system but lowered depression.

Competing Interests: The authors declare that there are no competing interests.

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