ORIGINAL ARTICLE

The Effects of Dawood Fasting on The Morphology of Intestinal Mucosa in Balb/c Mice

Ika Fidianingsih[1], Nurahmi Widyani Ratri[2], Muhammad Wathoni Ikhlas[2], Maftuhah Zahara[2], Reinike Larasati Fajrin[2], Titis Nurmasitoh[3], Irena Agustiningtyas[4]

[1] Department of Histology, Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta,
[2] Student of Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta, Indonesia
[3] Department of Physiology Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta, Indonesia
[4] Department of Microbiology, Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta, Indonesia

ABSTRACT

Introduction: Long-term fasting (>24 hours) can result in mucosal atrophy, reduced number of goblet cells (GCs) and Peyer's patches (PPs), and changes in the gut microbiota. This study aimed to determine whether there is a difference in the morphology of intestinal mucosa between mice treated with Dawood fasting and those fed an ad libitum (AL) diet. **Methods:** An experimental study used ten mice (BALB/c) divided into two groups. One group was given food and drink AL (the AL group). The treatment group (the F group) fasted intermittently in 14-hour (5 p.m. to 7 a.m.) every other day. The study was conducted for 56 days. The faeces from the intestine were diluted 100-fold and cultured for microbiota colony counts. Haematoxylin and eosin staining was performed to observe the villus length (VL) and the area of PPs, and periodic acid-Schiff staining was used to examine the number of GCs. **Results:** There were no significant differences in the VL, GCs, PPs and the number of microbiota between the F group and the AL group with the respective p = 0.26 (369.54 ± 48.41 vs 307.16 ± 61.16) μm, p = 0.33 (10.42 ± 1.27 vs 9.15 ± 2.44), p = 0.8 (0.164 ± 0.069 vs 0.159 ± 0.089) mm^2, and p = 0.64 (1.85 ± 0.97 vs 2.22 ± 1.43) CFU/ml. **Conclusion:** Dawood fasting has no effect on the histopathological condition of the intestinal mucosa, including the VL, GCs count, area of PPs and the number of microbiota in mice.
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Keywords: Intermittent fasting, intestinal villi, goblet cell, Peyer's patches, intestinal microbiota.

Corresponding Author:
Ika Fidianingsih, MSc
Email: ika_fidianingsih@uii.ac.id
Tel: +6287839556572

INTRODUCTION

The presence or absence of food intake within a certain period can influence the structure of intestinal mucosa. This condition can affect the intestine's physiological function, leading to absorption disorders and other gastrointestinal symptoms (1). The fasting performed by Muslims remains a matter of concern for some individuals (2). In Islam, fasting means abstinence from eating and drinking (and other activities that invalidate it) from dawn to dusk for no more than 24 hours, or between 12 and 20 hours depending on the region, preceded by a pre-dawn meal and ending with breaking the fast. Fasting is observed every day during the month of Ramadan (Ramadan fasting) or every other day (Dawood fasting) in the other months (3). The concern is that fasting can lead to such gastrointestinal symptoms as nausea, vomiting, diarrhoea and constipation (4–6). Fasting can also aggravate acute inflammatory bowel disease (IBD), making the practice inadvisable in individuals with such conditions (7). However, a study showed that fasting in IBD patients at the remission stage will not have a severe adverse effect (8). Fasting can reduce seven out of ten IBS symptoms, including abdominal pain, abdominal distension, diarrhoea, anorexia, nausea, anxiety and increase overall quality of life (9).

The understanding of gastrointestinal disorders is associated with previous studies' findings that indicate intestinal mucosal atrophy in fasting experimental animals. Wistar rats that fast for 24, 48, 60 and 72 hours suffer from jejunal mucosal atrophy, reduced proliferation and enhanced apoptosis of epithelial cells (10). Long-term fasting (>24 hours) can reduce the number of goblet cells (GCs) and mucus production, thereby facilitating bacterial adhesion on the surface of epithelial cells and increasing intestinal permeability. Such a condition will increase the likelihood of colitis, IBD, cystic fibrosis and mucinous adenocarcinoma (11). Fasting for 36 hours changes the mucosal immune system with the reduction in size of and number of cells in Peyer's patches (PPs), fewer intraepithelial lymphocytes and less lamina propria (12) due to apoptosis. Mice fasting for 36 hours

show decreased bacteria (*Lactobacillus murinus*) in the small intestine and colon (13). In a study by Sonoyama et al. (2009), Syrian hamsters in which hibernation/fasting was induced for two weeks had a lower number of Clostridia (anaerobic intestinal bacteria) (14).

Research related to gastrointestinal tract morphology in fasting less than 24 hours is still rare. Rats that fasted for 24 hours showed a decrease in villous height, crypt depth, lymphocyte number, epithelia and lamina propria but not in PPs (12). However, there are studies related to the microbiota in intermittent fasting, and a systematic review shows that Ramadan fasting and other intermittent fasting can affect the composition of the gut microbiome, namely, by increasing the amount of *Lactobacilli* and *Bifidobacteria* and increasing the production of short-chain fatty acids. In addition, intermittent fasting decreases inflammation and improves health parameters (15, 16).

Meanwhile, the fasting observed by Muslims lasts only 12 to 22 hours, and there is a refeeding process before the next fasting day. Therefore, this study is the first to describe the effects of 14-hour, every-other-day intermittent fasting on the morphology of intestinal villi, duodenal GCs, PPs and the number of microbiota in the intestines of mice.

MATERIALS AND METHODS

Mice

Healthy and active male BALB/c mice, weighing 15 to 30 grams each and aged 9–10 weeks, were obtained from the Experimental Animal Laboratory of the Faculty of Medicine, Universitas Islam Indonesia. Ten mice were randomised and divided into two groups. After a one-week adaptation, the control group was fed an ad libitum (AL) diet of AIN-93 (a standard rodent diet that is 14% casein; 62% corn starch; 10% sucrose; 4% corn oil; 5% fibre; 3.5% mineral mix; 1% vitamin mix; and 0.25% choline bitartrate) (17). (This was the AL group.) In contrast, the fasting group (the F group) was restricted to eating and drinking for 14 hours (from 5 p.m. to 7 a.m.) on the first day and given standard AIN-93 AL on the second day, alternating this pattern for 56 days (to mimic Dawood fasting). During the study, the mice were kept in individual standard cages with a 12-hour light–dark cycle and a temperature of 23°C. The maintenance followed the animal welfare standards in the Experimental Animal Laboratory of Universitas Islam Indonesia and has been reviewed by the Ethics Committee of the Faculty of Medicine UII with the Approval Letter No. 48/Ka/Kom.Et/70/KE/IV/2018.

Organ Sampling and Embedding

On day 56, the mice were anaesthetised using ketamine, and abdominal surgery was performed. Two duodena were cut from below the pylorus of the stomach. Four slices (each measuring 1.5 cm) of the middle part of each jejunum and ileum were taken. Fixation using 10% formalin PBS, dehydration and clearing were conducted with serial alcohol and xylol, and paraffin was used for tissue embedding. The paraffin block was then sliced as thick as four μm.

Histological Analysis

The slices of jejunum and ileum were embedded in paraffin blocks and stained with haematoxylin and eosin. From all fields of view, the length of the intestinal villi (VL) and the area of PPs were measured based on a previous study (18) using Image J software. The mean area of PP was the total area of PP in each mouse divided by the number of PPs found. Two slices of the duodenum were stained with periodic acid-Schiff staining (19). Twenty duodenal villi in each field of view were counted from the cryptic base to the apical (20).

Microbiota Count

The ileum was aseptically massaged until the stool came out to be weighed. The stool was diluted 100- and 1,000-fold, and each dilution result was cultured on agar. The microbiota was quantified using the colony counter software after completing the incubation period at 37°C for 48 hours. Aerobic microbiota count is the number of colonies per plate quantified, between 30 and 300 colony-forming units (CFU). Large, small and spreading colonies were considered one unit of CFU/ml, and the quantification scale was numeric. The results were inserted into the following analytical formula: total plate count in a unit of CFU/ml = colonies of growing bacteria x amount (ml) x percent dilution (21).

Statistical Analysis

The VL, GC count, area of PPs and microbiota count data were displayed as mean plus standard deviation. To increase the validity of the data obtained, the researchers blinded all samples during measurement, thus leaving the researchers uninformed about the treatment and control groups. The differences between the two groups were analytically tested using the independent t-test (for parametric data) and the Mann–Whitney test (for non-parametric data), with a confidence level of 95% ($p < 0.05$).

RESULTS

Before treatment, mice were adapted for one week and given food and drink AL. After one week, the mice's body weight increased, indicating that experimental animals could adapt well. After the adaptation, all mice were randomised into two groups, the F group and the AL group. During the treatment, all mice in both groups appeared healthy and their body weight had increased. The observation of the mean VL in the jejunal mucosa of the F group (369.5338 ± 48.412 μm) indicated a slightly longer size but not much different ($p = 0.26$) from the AL group (307.1612 ± 61.160 μm) (Figure 1).

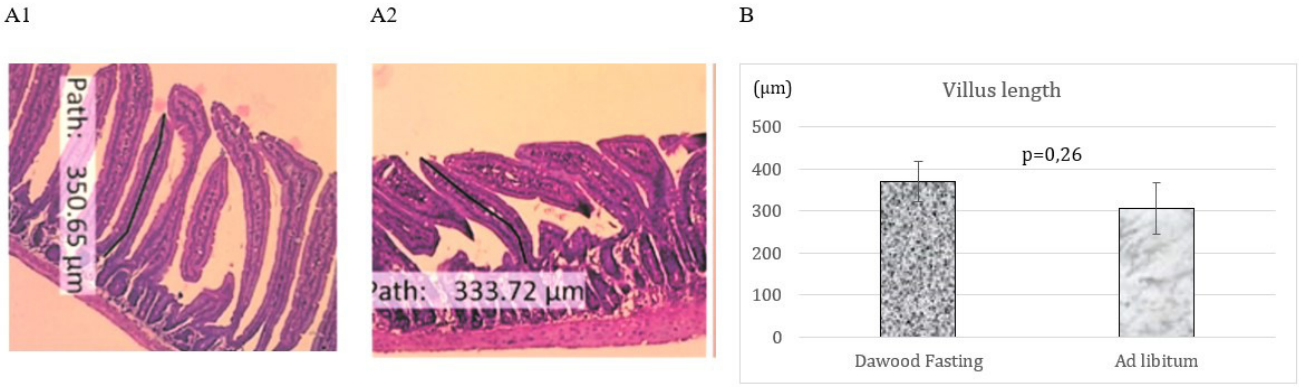

Figure 1: (A) Observation of the villus length of jejunal mucosa in 100x magnification (haematoxylin and eosin staining) between the Dawood fasting group (A1) and the ad libitum diet group (A2). (B) Mean villus length.

The microscope observations showed that GCs spread from the base to the apex of the duodenal surface and appeared red. The mean number of GCs in the F group (10.42 ± 1.27) was slightly higher compared to the AL group (9.15 ± 2.44), but there was no significant difference (p = 0.33) (Figure 2). The mean area of PPs of the F group (0.164 ± 0.069 mm2) was wider than that of the AL group (0.159 ± 0.089 mm2) but not significantly different (p = 0.8) (Figure 3). Meanwhile, the number of microbiota in the F group (1.85 ± 0.97 CFU/ml) was less than that of the AL group (2.22 ± 1.43 CFU/ml), but the independent t-test showed no significant differences (p = 0.64) (Figure 4).

DISCUSSION

This study showed that the Dawood fasting did not change the VL, GC count or the number of gastrointestinal PPs. Dawood fasting differs from long-term fasting, which can cause intestinal mucosal atrophy, as it does not last for more than 24 hours and is followed by refeeding before the next fast. Previous research on healthy mice intermittently fasted for 24 hours also showed no difference in VL. However, in

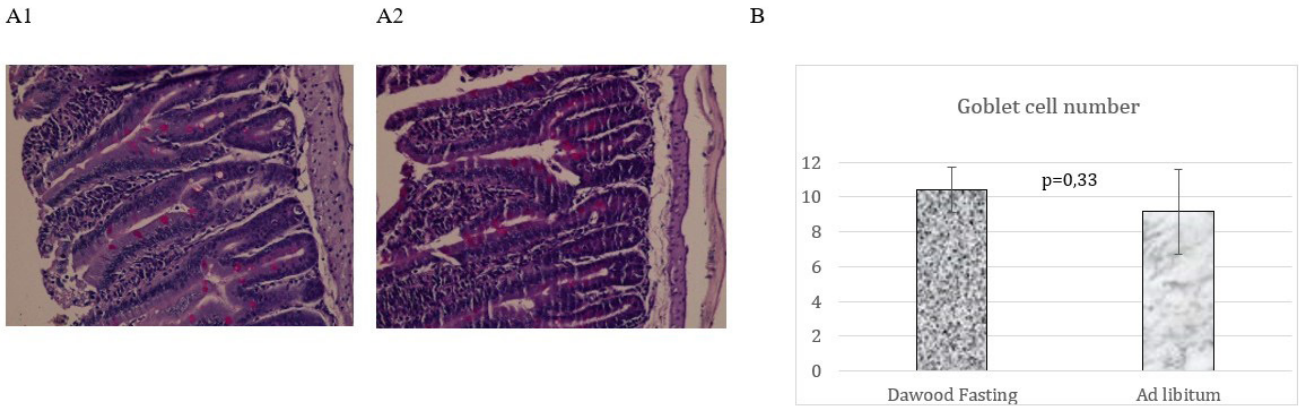

Figure 2: (A) Histological condition of duodenum with periodic acid-Schiff staining in which the goblet cells appear red between the Dawood fasting group (A1) and the ad libitum diet group (A2). (B) The results of the mean goblet cell count between the mice with Dawood fasting (56 days) and those with the ad libitum diet.

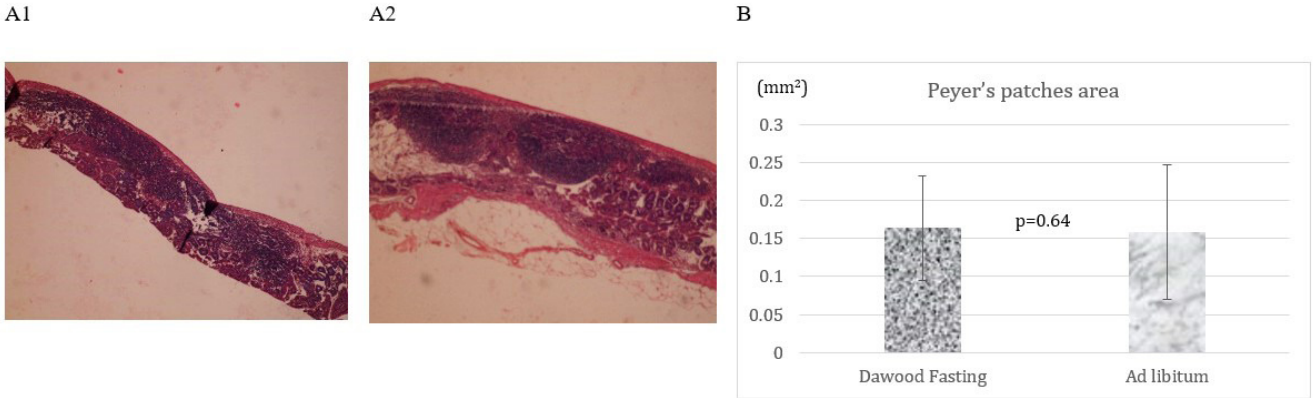

Figure 3: (A) Histological condition of Peyer's patches (haematoxylin and eosin staining) between mice with Dawood fasting (A1) and those with ad libitum diet (A2). (B) The results of the mean area of Peyer's patches and the statistical test show insignificant differences.

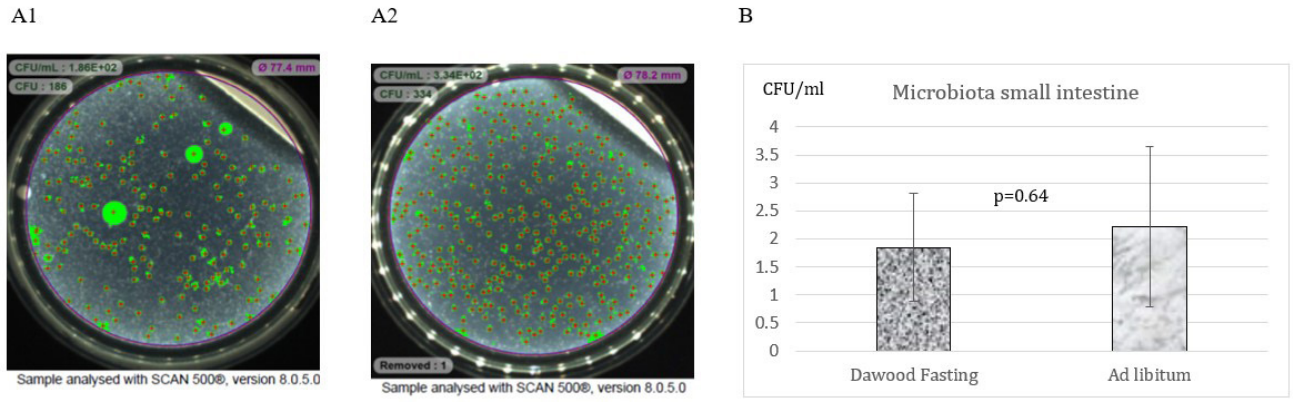

Figure 4: (A) Microbial colony of the ileum on agar plates after incubation at 37°C for 48 hours in mice with Dawood fasting (A1) and ad libitum diet (A2). (B) Calculation of the mean microbiota count (CFU/ml).

diabetic mice, the villi were significantly longer than in the AL diet mice (22). Dawood fasting can inhibit cell death due to the increased formation of antioxidants, including superoxide dismutase, glutathione peroxidase and catalase (23). The antioxidants formed during fasting for fewer than 24 hours can prepare the body to cope with oxidative stress during the refeeding phase. Fasting followed by refeeding prevents an increase in the CELF1/Myc-mRNA complex that inhibits the synthesis of the Myc protein, thereby preventing the atrophy (24).

Although this study showed no structural differences in the intestine, previous studies have found that fasting can activate the CREB and CRTC proteins, which will increase energy homeostasis by stimulating the expression of short neuropeptide F (sNPF) through intestinal enterocytes' sNPF receptors and preventing the tight junction's disintegration. Accordingly, fasting could maintain the intestinal epithelium's integrity (25). The refeeding maintains the homeostasis state of the intestinal epithelium (26) and ensures that the number of dormant intestinal stem cells returns to the pre-fasting baseline (27). Although in this study, the VL in the F group was more than that of the AL group (insignificant), previous studies show that intermittent fasting can reduce the incidence of mis-differentiation and hyperplasia in the intestine. In addition, it improves the function of the gut barrier to inhibit infection or absorption of toxic compounds (28). Alternate-day fasting, such as Dawood fasting (24 hours), can even inhibit the growth of colon cancer in mice (29).

The number of GCs in the F group was higher (but insignificant) than that of the AL group. Although the tight junction between epithelial cells becomes the barrier that protects the intestinal mucosa, the mucins produced by GCs can physically limit the epithelial cells to the luminal environment of the intestine, thereby producing a protective layer along the mucosa to limit bacteria and maintain intestinal permeability (30). Moreover, GCs can obtain antigens, presenting them to the antigen-presenting cell and inducing specific immune responses (31). The results of this study indicated that despite the fasting state, the number of GCs remained sufficient to protect the intestinal epithelial cells. Previous research has shown diabetic mice have fewer GCs than non-diabetic mice, but the number increases after being treated with 24-hour intermittent fasting. Peptidoglycan or bacterial product in plasma is decreased in mice with intermittent fasting, and it can prevent increased gut permeability and intestinal barrier dysfunction (22).

The area of PPs in the F group was not different from that of the AL group. It follows a previous study which found that the number of lymphocytes among short-term fasting mice (24 hours) does not differ from those eating AL (12). Although a 36-hour fast can reduce the size of PPs and their cell count, both increase after refeeding in 48 hours (32). In this study, the area of PPs tended to be broader (insignificant), indicating that the body defence could be expected to remain available despite the fast. Our previous study showed that the lymphoid tissues in the spleen of rats treated with Dawood fasting are broader than those in the groups with an AL, high-carbohydrate and high-fat diet (33).

Although the mean bacterial colony count in the F group was less than that of the AL group, the value was statistically insignificant because the fast was observed in less than 24 hours. Fasting can lead to an energy crisis in microorganisms due to reduced nutrient availability. Many animals reduce the size of their intestine during fasting, thus resulting in a crisis of habitat for microbiota (34). Long-term fasting (five days) can reduce bacterial load of Drosophila melanogaster in the gut (28). The number of bacterial colonies in the faeces of rats with intermittent fasting (24 hours) infected by Salmonella typhimurium is lower than in the control group. At the same time, the total intestinal IgA is higher than that of the AL diet group (35–37). Similar to previous research, microbiota richness was not changed in intermittent fasting, but bacterial diversity improved in fasting animals (22, 34). Unfortunately, this study did not observe bacterial diversity. In the 24-hour intermittent fasting, the number of good bacteria, including *Lactobacillus*, increases (22, 37). Such good bacteria also increase significantly in number after the

treatment of 8-week calorie restriction compared to the AL diet treatment (38). *Lactobacillus* is a good bacteria that greatly assists the digestion of food, especially vitamins, stimulates the immune system and inhibits the growth of harmful bacteria (39–40). The limitation of this study was that there was no examination of the types of intestinal microflora, mainly whether there were more *Bacteroidetes* than *Firmicutes* or whether there was an increase in the number of good bacteria, such as *Lactobacillus*. In addition, this study does not describe the overall gastrointestinal morphology, and the number of subjects was inadequate.

CONCLUSION

Dawood fasting did not change the histopathological condition of intestinal villi (VL, GC count, area of PPs and number of microbial colonies) in BALB/c mice. Therefore, people with no serious illnesses who participate in Dawood fasting need not be concerned about changes in the gastrointestinal tract.

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