

# Structural Changes to Immune Organs in Rats after Intermittent Fasting Following a High Carb and Fat Diet

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**Abstract:** *Background:* A diet high in carbs and fat puts people at risk of obesity. Obesity causes changes in the immune system and increases the risk of premature ageing, including in the lymphoid organs — such as the thymus and spleen. Fasting is expected to improve the immune system. The purpose of this research is to determine the effects of intermittent fasting on images of the structure of the thymus, the number of fat cells, Hassall's corpuscles of the thymus, the area and density of pulp white spleen, and the number of leukocytes in Wistar rats (*Rattus norvegicus*) fed a diet high in carbs and fat.

*Methods:* An experimental study with post-test only control group design, with 15 male rat subjects aged 2.5 months were divided into three groups: first group had a diet that was ad libitum (AL); second group were given a diet high in fats and carbohydrates for 1 month then were fed ad libitum (HCL); and third group were given a diet high in fat and carbohydrates for 1 month continued with alternating 12 hour fasting periods for 72 days (F).

*Results:* Total Hassall's corpuscles of AL, HCL, and F groups were  $1.33 \pm 0.77$ ;  $2.58 \pm 1.35$ ; and  $0.69 \pm 0.27$ , respectively ( $p = 0.008$ ). Fat cells were not found in the thymus. The largest white pulp in the spleen was found in group F, followed by AL, while the smallest was found in group of HCL ( $p = 0.01$ ). The most depleted white pulp density was the HCL group. There is no significant difference in the number of leukocytes and different leukocyte count between the groups ( $p > 0.05$ ).

*Conclusion:* Intermittent fasting for 72 days affects the number of Hassall's corpuscles in the thymus and the width of white pulp in the spleen of Wistar rats.

**Keywords:** Intermittent fasting, thymus, spleen, leukocytes, high carbohydrate diet, high fat diet.

## 1. INTRODUCTION

Lifestyle changes are an important issue in developing countries, as many people eat a diet high in carbs and fat. A diet high in carbs and fat increases the risk of obesity, a health problem that is increasing. Obesity has been recognised as a risk factor for many chronic and serious diseases such as cardiovascular disease, diabetes mellitus, stroke, heart failure, gout and cancer. Research in Austria of 42,099 men and women who are stably obese showed an increase of 1.57 in death rate among women and 1.46 among men [1]. Obese patients are also prone to infections [2].

Obesity causes a variety of degenerative diseases as the mechanisms of the body change, which leads to ageing. The process is initiated by an increase in oxidative stress and increased inflammatory reactions. This results in insulin resistance, a decreased intake of nutrients and oxygen to various organs of the body, and a decline in organ function. Instead, diet restriction

is thought to have the opposite effect. It prevents ageing by boosting immunity as it inhibits inflammatory reactions [3].

Fasting is one way to restrict diets. Ramadan fasting decreases the incidence of inflammation characterised by decreased interleukin-2 (IL-2), tumour necrosis factor- $\alpha$  (TNF  $\alpha$ ) and IL-8 [4], decreased oxidative stress [5] and increased levels of endorphins in the body [6]. This situation improves the immune system and indirectly inhibits ageing. Intermittent fasting affects the transcription of genes that prolong life [7].

The thymus and spleen are organs involved in the immune system. Thymus plays a role in activating the maturation of T lymphocytes. T lymphocytes play an important role in the immune system, increasing the activity of B lymphocytes macrophage and forming a memory cell [8]. Obesity damages the regeneration of the immune system, especially the ability of the thymus to produce immune cells. Obesity also causes involution of the thymus and significantly defects the responsiveness of T cells, reduces the ability of dendritic cells to present antigens to T cells; the

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decrease in cells of the natural killer cytotoxic is also significant [3]. Obesity causes the thymus structure was dominated by fat cells, affecting the immune system [3]. Along with age, the thymus can decrease in size and function, sometimes even undergo the process of involution, and replaced with fatty connective tissue [9]. Decreased size of the thymus causes the thymic epithelial cells to undergo fibrosis that forms Hassall's corpuscles [10].

Spleen, one of the secondary lymphoid organs, functions to activate cluster of differentiation 4 (CD4) lymphocyte cells. With ageing, it decreases the number of lymphocytes in the white pulp and increases reticular cells and macrophages [11]. Otherwise, diet restrictions in obese rats can cause a decrease in the weight of the spleen, but increase the CD4+ T cells. In addition, diet restrictions can decrease the production of pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and Prostaglandin E2 (PGE2) [12].

White blood cells or leukocytes are cells have a role in the immune system. Nilsson *et al.* [13] showed that the number of white blood cells can be used as a predictor of survival. An increase in white blood cell count in the blood is also associated with increased mortality. The aim of this research is to discover the effects of intermittent fasting on differences in the number of fat cells in the thymus, Hassall's corpuscles of the thymus, the white pulp area of the spleen, the density of the white pulp of the spleen, and the number of leukocytes in Wistar rats on a high carb and high fat diet.

## 2. MATERIAL AND METHODS

### Research Design

This study is an experimental study with a post-test control group design. It assesses the difference in the structure of the thymus, spleen and leukocyte count in the group of rats on free diet (ad libitum), rats on high carbs and fat diet that have fasted, as well as rats on high carbs and fat diet that have not fasted. The study protocol was approved by the Research Ethics Committee of the Medical Faculty of Medicine and Health Universitas Islam Indonesia number 02/Ketua/KEFKUII / III / 2015.

### Research Subject

The subjects were 90 days old female Wistar rats weighing between 120 to 220 grams. The sample size of the study was based on the formula [14] :E = total

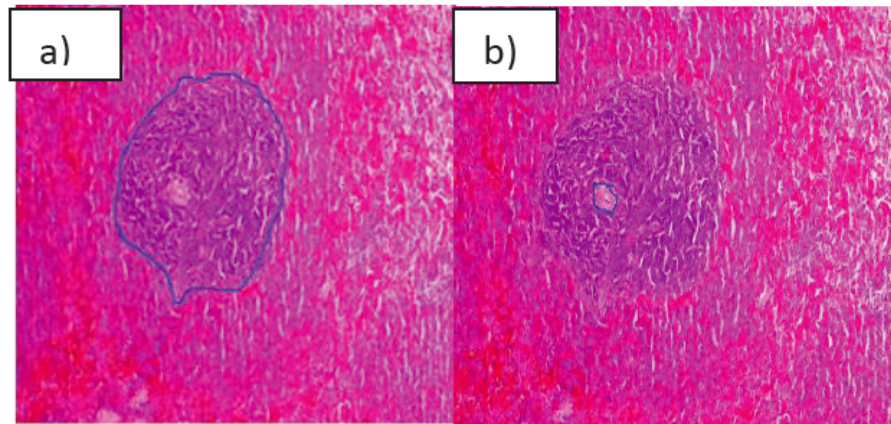
number of animals - the total number of groups. As many as 15 rats were divided into three groups: the control rats had an ad libitum diet (AL) in the form of standard rat feed weighing 20 grams. The first group of rats were treated with a diet high in carbs and fat (HCL) for 1 month followed by an AL diet for 72 days. The HCL diet consisted of 40 grams of standard feed and 8 grams of egg yolk. Treatment group II were rats fed with a high carb and fat diet for one month, followed by continual fasting (F) for 72 days. Fasting means intermittent fasting; rats that have been adapted to day and night were fasted for 12 hours from 6 pm until 6 am on the first day, not fasted on the second day, fasted again on the third day, and not fasted on the fourth day onwards. Drinking was provided AL. During the study, we maintained appropriate animal welfare in one rat cage. It was set at room temperature, with a light dark cycle for 12 hours, in laboratory test animals, Faculty of Mathematics and Natural Sciences, Islamic University of Indonesia.

### Tissue Observation

Prior to the removal of tissue, blood samples were drawn from an orbital vein of the rats to count the number of leukocytes and differentials of the leukocytes. Sample of blood, spleen and thymus tissue were preceded by giving anaesthesia to all rats. The spleen and thymus were fixed, with a small portion in the same area taken for all groups. The stages of the preparation of the histological specimens are as follows: dehydration with serial alcohol, embedding with paraffin, cutting as many as one slice, and staining haematoxylin eosin for all tissues both spleen and thymus. For Leukocyte count, observation was completed manually using Turk colouring.

### Results Analysis

The structure of the thymus, spleen and leukocyte count were observed by two observers who did not know the groups treatments of the rats. Image J software was used to calculate differences in the amounts of Hassall's corpuscles, the size of the white pulp of the spleen, and the density of the white pulp (the number of rat with depleted of white pulp). The mean area of white pulp was measured by the size of the white pulp in all field of vision, minus the width of the central artery (Figure 1). It was then divided by the number of white pulps. Observations were made using a light microscope at 400X magnification. The differences were analysed by ANOVA. If it did not qualify, it was analysed by the Kruskal Wallis test.



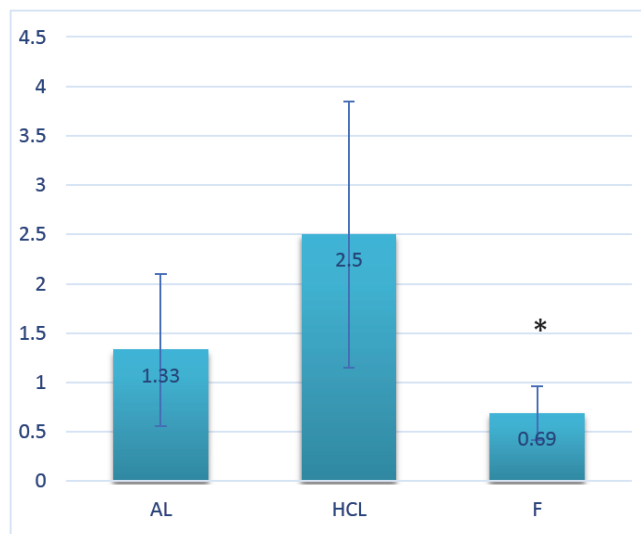
**Figure 1:** White pulp of the spleen: **a)** area of white pulp (blue line); **b)** area of the central artery (blue line).

**3. RESULTS**

Table 1 describes the characteristics of the study subjects according to their weight before research and after the groups were randomised. Statistical analysis showed no significant difference in body weight between the groups of rats.

**Table 1: Body Weight (BB) of Rat Initial after Randomised**

Group	Mean initial BW	P Value
ALdiet	160±24,5	0,39
High carbs and fat diet then AL	164±8,94	
High carbs and fat diet then fasting	176±16,73	



**Figure 2:** Mean Hassall's corpuscles (p=0.008).

\*There are significant differences between F and HCL Group (p=0,002).

The histological image of the thymus structure in all the groups did not reveal any fat cells. There is a

difference in the average number of corpuscles (p = 0.008) between the groups. The lowest average number of Hassall's corpuscles was in F Group. Otherwise, the highest average number of Hassall's corpuscles were in the HCL group (Figures 2 & 3).

In the calculation, the highest average area of white pulp is in the group that fasted (F), and the lowest average is in the HCL group (Figure 4). The statistical test result with ANOVA showed that there was a difference between the groups (p = 0.01).

All the white pulp in the HCL was depleted. There were two rats in the fasted group and one rat in the control group in which white pulp was depleted (Figures 5 & 6).

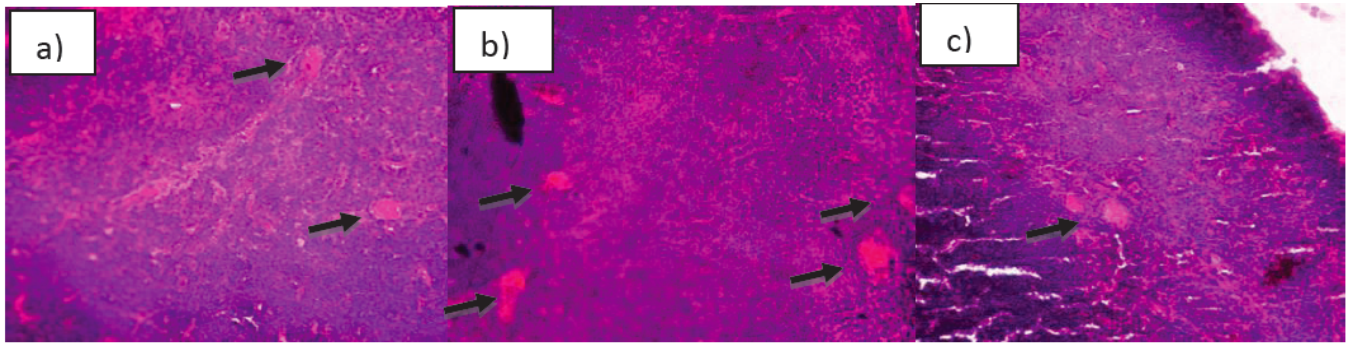
There was no significant difference between the groups in the number of leukocytes (neutrophil, eosinophils, basophils, lymphocytes and monocytes) and differential leukocyte count after treatment (Table 2). Basophils were not found in any rats (basophils: 0).

**4. DISCUSSION**

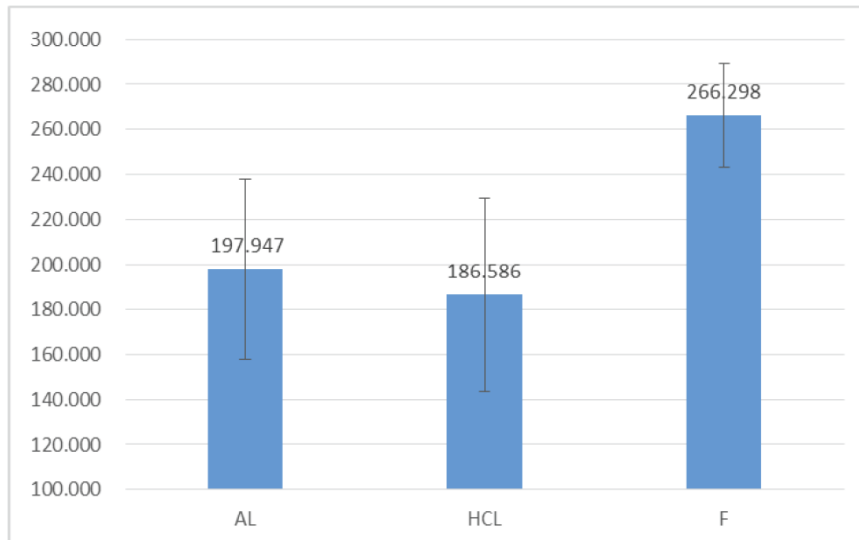
**Total Hassall's Corpuscles of Thymus**

The total number of Hassall's corpuscles in the rats on a HCL diet was higher than in the rats on an AL diet, although they were not statistically significant. Yang *et al.* [15] mentions that, when compared with a control rat, rats on a high-fat diet tend to undergo thymus involution, with decreased cortical and medullary cellularity, a decreased number of thymocytes, thymic epithelial cells and an increased apoptosis thymocyte. The process of involution of the thymus and apoptosis thymocyte along with increasing the number of Hassall's corpuscles [10].

Obesity induces an inflammatory response in tissue. Inflammation induces epithelial mesenchymal

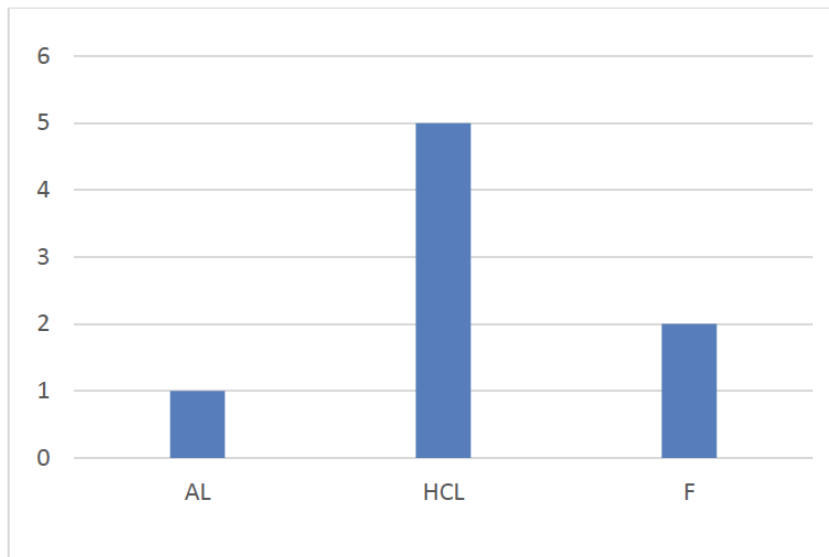


**Figure 3:** Hassall's corpuscles of the thymus (black arrow): a) AL group; b) HCL group; c) F group.



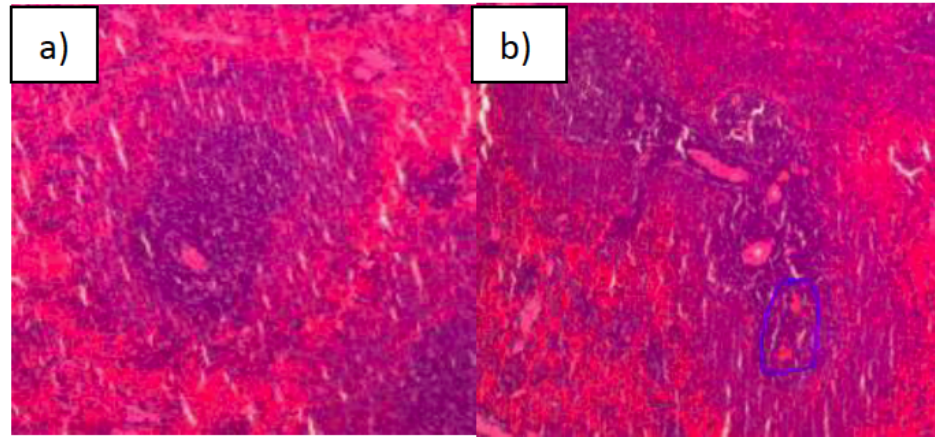
**Figure 4:** Mean width of white pulp (p=0.01).

There are significant differences between F and HCL (p=0,005) and between F and AL(p=0,021).



**Figure 5:** The number of rat with depleted of the white pulp.

There are significant differences (p=0,024) between AL and HCL group.



**Figure 6:** The density of white pulp: **a)** Fasting group (high density); **b)** HCL group (low density), there was area with depleted.

**Table 2: The Number of Leukocyte and Differential Leukocyte Count**

K	JL	N	E	L	M
AL	10100±8636	24±6.48	0.5±0.58	69.5±8.39	6±2.16
HCL	9883±3392	21.33±11.95	1.33±1.52	74.75±11.95	5.33±0.58
F	8710±1561	21.8±4.66	1.8±1.3	68±4.66	7.6±4.45
<i>p</i>	0.975	0.549	0.546	0.558	0.546

Note: K: Group, AL: ad libitum group, HCL: high carb&fat group, F: fasting group, JL: leukocyte count/mm<sup>3</sup>, N: neutrophil(%), E: eosinophil(%), L: lymphocyte(%), M: monocyte(%).

transitions in the thymus cells through mechanisms of epithelial mesenchymal transition (EMT). The transition process results in a decrease in the number of flattened epithelial reticular cells (cell type IV) in the thymus. It is characterised by fibrosis formation, keratinocytes and calcification of the epithelial cells in the thymus that are seen as formations of Hassall's corpuscles [16].

There were fewer Hassall's corpuscles in group F. Yang *et al.* [16] noted that a rat with caloric restriction can inhibit fatty thymus, experience an increase of cellularity cells in the cortex and medulla of the thymus, and inhibit thymic epithelial cells that differentiate into fibroblasts and adipocytes. It causes Hassall's corpuscles to not be formed.

Fasting causes a negative energy balance and can extend life by improving orexigenic factors such as neuropeptide Y (NPY), agouti gene-related protein (AGRP), and ghrelin. It also reduces anorexigenic hormones, such as leptin in rats. Increased ghrelin due to dietary restrictions is associated with reduced inflammation, as well as inhibition of the release of pro-inflammatory cytokines from T cells, monocytes, and endothelial cells and increased thymopoiesis. This improves the epithelial cells of the thymus and prevents

involution of the thymus [3]. Besides that, fasting can also reduce the number of adipocytes in the thymus, increase the number of naive T cells, and prevent the loss of thymic epithelial cells in the cortex and medulla. Fasting can also reduce fibroblasts through the EMT process that reduces the formation of Hassall's corpuscles [16].

Diet restrictions can also increase adenosine monophosphate (AMP) count, which activates protein kinases (AMPK). AMPK inhibits nuclear factor kappa B (NF-κB) which increases the transcription of pro-inflammatory cytokines, so that AMPK controls reduction of the inflammatory response [3]. Based on research by Hyunwon *et al.* [16], calorie restriction can also inhibit the increase in activated XOR (xanthine oxidoreductase) that produce reactive oxygen species. XOR is one of the key regulators in causing an ageing thymus through peroxisome proliferator-activated receptor (PPAR), by accelerating the transition of thymic stromal cells and fibroblasts into adipocytes.

### Splenic White Pulp

The HCL group has the lowest average area of white pulp of the spleen when compared to the other groups. The group also experienced the most depletion. Previous research found that obese rats

have greater spleen volume than the control group rats due to sinusoidal dilation in red pulp [17]. However, there was a decline in the size of the white pulp in obese rat. This is because obesity causes an increase in oxidative stress and the activation of mitochondrial apoptosis cascade pathways that lead to cell death [18].

Otherwise, group F rats had the widest white pulp and most were not depleted. This is consistent with previous research that dietary restrictions in obese rat can cause weight loss, but will increase spleen CD4+ T cells [3]. Likewise, Wang *et al.* [12] illustrate that diet restriction causes a decrease of spleen weight, but increases a number of CD4+T cells, so they are higher than controls. Other research by Silva *et al.* [19] demonstrates that the number of white pulp that contain a lot of B and T lymphocytes increased in the group that were on a restricted diet.

Diet restrictions are shown to reduce pro-inflammatory cytokines, such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and PGE2 which can delay the onset of ageing [12]. In this study, fasting tends to inhibit a decrease in the size of the white pulp. The reduction in white pulp is about 80% occurred in 30 month olds rats compared with 4 month old rats [11]. A decrease in the number and area of white pulp and red pulp occurs in rats aged 35-42 days when compared with rats aged 7-14 days; this is caused by cell apoptosis [20].

### The Leukocyte Count

The results of this study have shown there are no significant differences in the number of leukocytes and differential leukocyte counts between the groups. It is similar to a previous study that found the number of lymphocytes T, B and NK cells did not differ before and after the fasting of Ramadan in winter [21]. However, the numbers of leukocytes and lymphocytes percentages do tend to be lower in the fasting of the AL and HCL Group. Previous research on those who fasted during Ramadan found that the number of leukocytes was significantly lower after fasting than before fasting [4]. In addition, a previous study showed that diet restrictions for 6 months showed significant decreases in the numbers of leukocytes [22], but research by Latifynia [23] demonstrates that although the number of neutrophils tends to decrease, the phagocytic ability of these cells increased during Ramadan. Decreases in leucocytes and lymphocytes in this study are within normal limits. People that have a normal or low range value of leukocytes have a longer longevity prognosis than people who have a higher number of leukocytes [13].

### LIMITATIONS

The process of giving a diet high in carbs and fat was over a short period of time. Therefore, there was not a significant weight difference between rats in the AL diet group and those in high in carbs and fat diet group.

### CONCLUSION

The thymus and spleen organs in Wistar rats that fasted intermittently for 72 days (a day of fasting and a day not fasting) had a structure that was better at preventing ageing. Fasting is likely to decrease and increase the number of Hassall's corpuscles in the broad white pulp of the spleen.

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### CONFLICT OF INTEREST DISCLOSURES

The author does not have any conflicts of interest.

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