THE EFFECT OF OVERNIGHT FASTING ON SOME INFLAMMATORY RATIOS AND SERUM BILIRUBIN CONCENTRATION AMONGST A POPULATION OF STUDENTS IN UNIBEN

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CERTIFICATION

This is to certify that this project work on "**THE EFFECT OF OVERNIGHT FASTING ON SOME INFLAMMATORY RATIOS AND SERUM BILIRUBIN CONCENTRATION AMONGST A POPULATION OF STUDENTS IN UNIBEN"** was carried out by **LAWAL BUSHRA TEMITOPE,** with matriculation number: **BMS1802613**; in partial fulfillment for the Award of Bachelor of Science (B.Sc.) Degree in the Department of Physiology, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin City.

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ABSTRACT

Overnight fasting which is a form of Intermittent fasting (IF) is a dietary approach where individuals consume regular meals during specific intervals and then undergo extended periods with minimal or no energy intake for time periods that can range from 12hours to several days, on recurring basis which has same health benefits as overnight fasting. Inflammatory ratios, which are used in medical diagnostics are calculated by analyzing the level or ratios of certain blood markers, including neutrophils, fibrinogen, platelets and others, to assess the presence and severity of inflammation in the body. The aim of this study is to evaluate the effect of intermittent fasting on some inflammatory ratios and serum bilirubin concentration. Sixty (60) individuals including male and female within the age of 18-30 years were recruited for this study. This study was conducted in the University of Benin environment. About 10mls of blood samples were collected with minimal trauma via the cubital fossa using a sterile disposable syringe and needle. 5ml of blood were emptied into EDTA bottles for hematological analysis and 5ml of whole blood dispensed into lithium heparin bottle, spun and the supernatant dispensed into plain containers for bilirubin estimation. The results showed no significant difference in bilirubin, platelets, platelets-lymphocyte ratio during the fasting phase when compared to non-fasting phase but there was a significant increase in NLR in fasting phase compared to non-fasting phase coupled with a significant decrease in MLR in fasting individual. In Conclusion, this study has shown that intermittent fasting has a significant effect on some inflammatory ratios and no significant effect on bilirubin concentration.

CHAPTER ONE

1.0 INTRODUCTION

In recent years, there has been evidence showing that overnight fasting has same potential health benefits as intermittent fasting as long as fasting is observed properly. Intermittent fasting (IF), a dietary pattern that involves alternating periods of fasting and non-fasting. One of the proposed mechanisms behind the health benefits of IF is its ability to reduce inflammation in the body, which is thought to be a major contributor to the development of chronic diseases such as obesity, diabetes, and cardiovascular disease. Several studies have explored the impact of intermittent fasting on inflammatory markers and ratios. One commonly measured marker is C-reactive protein (CRP), which is produced by the liver in response to inflammation. High levels of CRP are associated with an increased risk of cardiovascular disease.

Research suggests that intermittent fasting may reduce CRP levels. A study published in the Journal of Nutritional Biochemistry in 2013 found that alternate-day fasting significantly decreased CRP levels in overweight adults. Another study published in the Journal of Aging and Disease in 2017 reported a reduction in CRP levels in obese individuals following an intermittent fasting regimen (Antoni *et al.,* 2017).

In addition to CRP, intermittent fasting has been found to affect other inflammatory ratios. One such ratio is the neutrophil-to-lymphocyte ratio (NLR), which is calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. A higher NLR is associated with increased inflammation and poor health outcomes.

A study published in the journal Nutrients in 2020 investigated the effects of intermittent fasting on NLR in overweight and obese women. The researchers found that intermittent fasting significantly reduced NLR, indicating a decrease in inflammation. Furthermore, intermittent fasting has been shown to modulate the production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha). These cytokines play a crucial role in promoting inflammation (Barnosky *et al.*, 2014).

Inflammation is a complex physiological response to injury or infection that involves the release of cytokines and other immune system mediators, cytokines are small proteins secreted by immune cells that act as messengers between cells, regulating various immune responses and inflammation. They play a crucial role in maintaining overall health and are involved in various physiological process.Several studies have explored the impact of intermittent fasting on cytokines and have suggested that it may have positive effects on immune function and inflammation

Intermittent fasting has been practiced for centuries in various cultures and religions as a form of spiritual or religious observance. In recent years, it has gained attention from the scientific community due to its potential health benefits beyond weight loss. Researchers have been studying its effects on various aspects of health, including metabolic health, inflammation, cardiovascular health, and now, bilirubin concentration (Cloffi *et al*., 2018).

While acute inflammation is a critical part of the body's defense against infection and injury, chronic inflammation can be damaging to tissues and organs over time. Chronic low-grade inflammation has been linked to the development of numerous chronic diseases, including metabolic syndrome, atherosclerosis, and cancer (De Cabo and Maffon, 2019).

IT'S IMPACT ON BILIRUBIN

Bilirubin is a compound produced during the breakdown of red blood cells and has potent antioxidant properties; it plays a crucial role in the body's waste elimination process and is excreted through bile. Elevated levels of bilirubin in the blood can indicate liver dysfunction or other health conditions (Anton *et al*., 2019).

The study of the impact of intermittent fasting on bilirubin concentration aims to investigate whether this dietary approach has any effect on bilirubin levels in the body. By analyzing blood samples before and after periods of intermittent fasting,

researchers can assess any changes in bilirubin concentration and determine if there is a correlation with the fasting regimen.Several studies have examined the effects of intermittent fasting on various aspects of liver function, including bilirubin levels. While the research is still limited, some studies have suggested that intermittent fasting may have a positive impact on liver health and may help regulate bilirubin concentration.Elevated levels of bilirubin in the blood, known as hyperbilirubinemia, can be indicative of liver dysfunction, such as hepatitis, liver cirrhosis, or certain genetic disorders like Gilbert's syndrome. It can also be a symptom of other health conditions, such as hemolytic anemia or gallstones. Previous research has suggested that bilirubin levels may be inversely associated with inflammation, and that increased bilirubin levels may confer protective effects against cardiovascular disease and other chronic diseases (Harvie *et al*., 2013).

Intermittent fasting has been shown to have various effects on the body that may potentially impact bilirubin concentration. For example, fasting triggers a metabolic switch from glucose utilization to fatty acid oxidation, leading to increased fat burning and ketone production. This metabolic shift can have positive effects on liver health and function. Additionally, intermittent fasting may improve insulin sensitivity and reduce inflammation, both of which can have indirect effects on liver function and bilirubin metabolism. Insulin resistance and chronic inflammation are known to contribute to liver diseases (Meyer *et al*., 1995).

For example, a study published in the Journal of Clinical and Translational Hepatology in 2018 found that intermittent fasting could improve liver function and reduce bilirubin levels in patients with non-alcoholic fatty liver disease (NAFLD). Another study published in the journal Nutrients in 2019 reported similar findings, suggesting that intermittent fasting may help reduce bilirubin levels and improve liver health in individuals with metabolic syndrome. Another study published in the journal Clinical and Experimental Hepatology in 2020 reported similar findings, suggesting that intermittent fasting could be a potential therapeutic approach for reducing bilirubin levels in patients with NAFLD (Ma *et al*., 2021).

However, it is important to note that the research on the impact of intermittent fasting on bilirubin concentration is still relatively limited. Most studies have been conducted on animal models or small human populations, making it difficult to draw definitive conclusions. Additionally, the specific mechanisms by which intermittent fasting affects bilirubin metabolism are not yet fully understood and require further investigation. However, it is important to note that more research is needed to fully understand the relationship between intermittent fasting and bilirubin concentration. The existing studies have been small-scale and conducted on specific patient populations, making it difficult to generalize the findings to the broader population (Ziberna *et al*., 2021).

1.1 **AIM OF STUDY**

The aim of this study is to evaluate the impact of intermittent fasting on some inflammatory ratios and bilirubin concentration in both male and females in the University of Benin.

1.2 **STATEMENT OF THE PROBLEM**

Despite the growing interest in IF and its potential health benefits, there is limited research on the impact of IF on inflammatory ratios and bilirubin levels. While some studies have suggested that IF may reduce inflammation and increase bilirubin levels, others have found conflicting results.

Given the potential health benefits of IF and the need for more research in this area, this study aims to investigate the impact of IF on some inflammatory ratios and bilirubin concentration.

1.3 **JUSTIFICATION**

Recent research has revealed the possibility of serum bilirubin to also function as an endogenous regulator of inflammatory responses. With an increase in stress comes a possible increase in inflammation but the research knowledge of the impact of intermittent fasting on the inflammatory indices and serum bilirubin concentration remains minimal in our society.

1.4 **RESEARCH QUESTIONS**

1. Was there a difference in the blood sugar level during the fasting phase from the random phase?

2. Was there a difference in serum bilirubin concentration during the fasting phase compared to the non-fasting phase?

3. Was there a difference in platelet concentration during the fasting phase compared to the non-fasting phase?

4. Was there a difference in NLR during the fasting phase compared to the non-fasting phase?

5. Was there a difference in PLR during the fasting phase compared to the non-fasting phase?

6. Was there a difference in MLR during the fasting phase compared to the non-fasting phase?

7. Was there a difference in fibrinogen to platelet ratio during the fasting phase compared to non-fasting phase?

1.5 **SPECIFIC OBJECTIVES**

1. To determine the fasting blood sugar using a standard glucometer machine.

2. To determine serum bilirubin concentration using standard ELISA technique.

3. To determine platelet concentration using a standard hematological autoanalyser machine.

4. To determine the neutrophil to lymphocytes ratio concentration.

5. To determine the platelet to lymphocytes ratio concentration.

6. To determine the monocyte to lymphocytes ratio concentration.

7. To determine the fibrinogen to platelets ratio concentration.

CHAPTER TWO

2.1 Intermittent Fasting

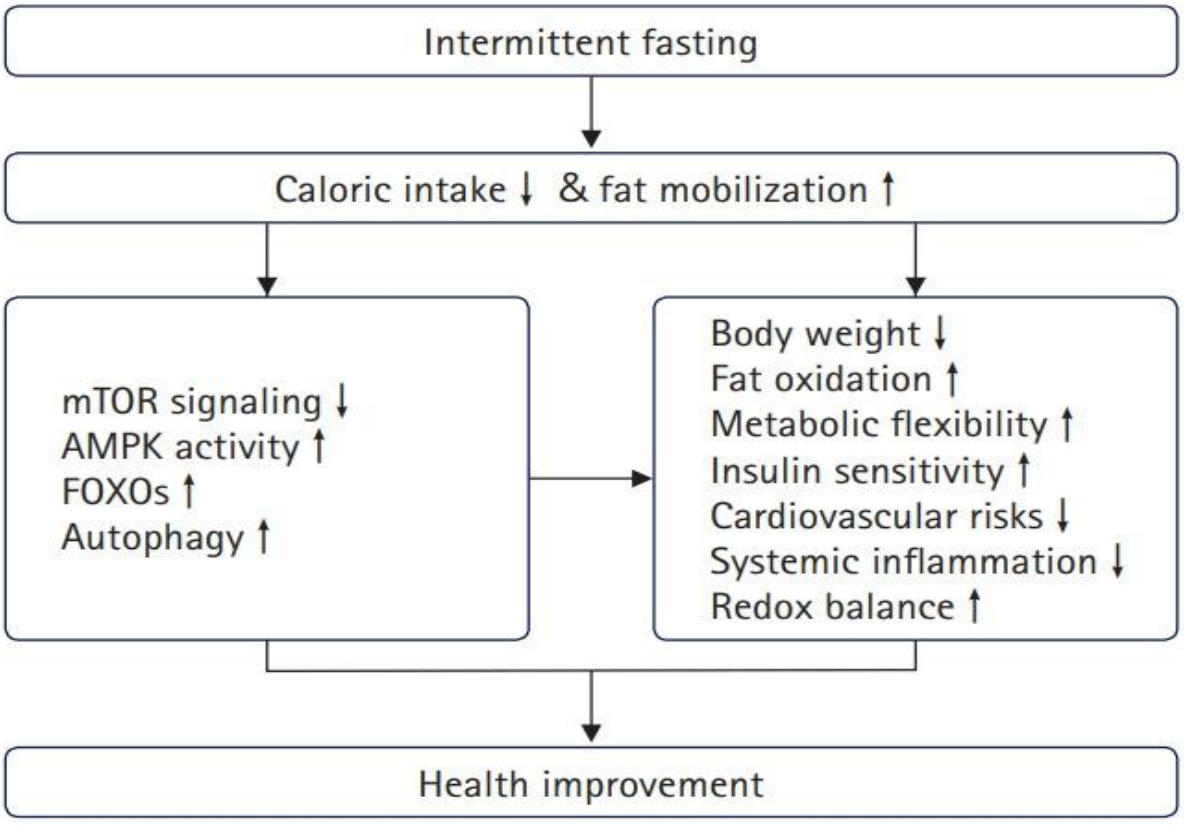
WHAT IS INTERMITTENT FASTING(IF)?

Intermittent fasting (IF) is a dietary approach where individuals consume regular meals during specific intervals and then undergo extended periods with minimal or no energy intake for time periods that can range from 12hours to several days, on recurring basis (Anton *et al*., 2018).

Recently, many studies have reported that intermittent CR (intermittent fasting, IF) may improve dietary adherence; thus, IF has emerged as an alternative intervention for prolonged CR, with similar benefits in body weight reduction and chronic illness control. IF originated from religious traditions, such as Ramadan fasting (Hoddy *et al.,* 2020). Muslims fast during the daytime (approximately 15 hours between sunrise and sunset) for a month during the Ramadan period every year. Ramadan fasting has been reported to improve human health. IF involves reduced or no caloric intake in an intermittent pattern, such as short periods of very restricted caloric intake or fasting interspersed with normal caloric intake. Thus, dieter intake is 0 to 500 kcal/day on fasting days. The fasting time varies from several hours per day to a complete day. The most studied IF interventions include 2 days of CR or fasting per week (5:2 diet) and alternate-day fasting

(ADF)(Grajower and Horne, 2019). One of the most popular variants of IF is time-restricted feeding, in which energy intake is limited to 12 to 16 hours each day and normal caloric intake during the other hours. In this review, we evaluate the results mainly from ADF and 5:2 diet trials. Weight reduction is the primary mechanism underlying the beneficial effects of IF. As shown in the results from CR, weight reduction per se reduces fasting plasma insulin levels, cardiovascular risk factors, and body inflammatory status by regulating metabolic signaling pathways, including those involving forkhead box O (FOXO), mechanistic target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), and autophagy (Hwangbo *et al.,* 2020). During the fed state, signaling pathways for nutrient sensing and cellular growth (e.g., mTOR) are activated. Stress-responsive signaling pathways (e.g., FOXO and AMPK) are activated by fasting, resulting in the protection from cell damage and inhibition of cell proliferation. An additional mechanism of IF is the metabolic switch between fed and fasting states. Fasting, especially repetitive fasting, induces organisms to shift their metabolic phase, which improves metabolic conditions and extends health expectancy (Stekovic *et al.,* 2019). de Cabo and Mattson reported that fasting optimizes cellular use of fuel sources, favoring ketone bodies and fatty acids over glucose, which ameliorates the blunting of metabolic flexibility observed in obesity and type 2 diabetes mellitus (T2DM) and improves mitochondrial function. Furthermore, fasting activates

autophagy and defense mechanisms against oxidative and metabolic stress and suppresses inflammation (Zarrinpar *et al.,* 2014). These effects of IF are similar to those of aerobic exercise. Fasting induces glucose and amino acid deprivation, stimulating AMPK activity and suppressing mTOR signaling, which are important nutrient-sensing signaling pathways. These changes inhibit FOXO-dependent gene transcription, resulting in the induction of autophagy and oxidative defense mechanisms (Green *et al.,* 2022) (Fig. 1). During IF, the body activates pathways for rejuvenation and repair.



Source: Pubmed central (PMC)

2.1.1 Effects of intermittent fasting on inflammation and redox balance

Macrophages infiltrate hypertrophied adipose tissue and produce proinflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α), which induce insulin resistance and atherosclerosis and are linked to low-grade systemic inflammation (Shoelson *et al.,* 2006). The plasma concentrations of these inflammatory cytokines parallel the degree of obesity and are positively correlated with insulin resistance. Systemic inflammation is linked to the pathogenesis of T2DM, cardiovascular diseases, and some types of cancers. Thus, systemic inflammatory markers can predict the development of these metabolic disorders. Body weight reduction decreases adipose tissue macrophages, reduces proinflammatory cytokines, and improves insulin resistance and systemic inflammatory status (Xu *et al.,* 2003). Several clinical trials have shown that IF intervention improves inflammatory status in obese subjects and is associated with reductions in plasma levels of IL-6, TNF-α, C-reactive protein (CRP), and interferon-γ. Wang revealed that IF intervention decreased CRP levels without changes in IL-6 and TNF-α compared with the corresponding levels in controls in a systematic review of 18 randomized controlled trials. However, there have been some inconsistent studies. Liu reported that IF increased macrophage infiltration in adipose tissue by fasting in overweight or obese women, which may be associated with elevated adipose tissue lipolysis (Liu *et al.,* 2019).

2.2 History of Inflammation

WHAT ARE INFLAMMATORY RATIOS?

Inflammatory ratios, which are used in medical diagnostics are calculated by analyzing the level or ratios of certain blood markers, including neutrophils, fibrinogen, platelets and others, to assess the presence and severity of inflammation in the body (Barnosky *et al.*, 2014).

The record of inflammation dates back to nearly first century AD by Celsus. Initially it was reported as a mechanism of tissue reaction or response to injury that gave rise to rubor (redness, due to hyperemia), tumor (swelling, due to greater permeability of the microvasculature and leakage of several proteins into the interstitial space), calor (heat, associated with the greater blood flow and the metabolic activity of the cellular inflammatory mediators), and dolor (pain, in part due to alterations in the perivasculature and related nerve endings). The fifth characteristic of inflammation, known as functio laesa (dysfunction of the organs involved), was revealed from the writings of Rudolf Virchow in the 1850s. Then, in the late nineteenth century, Elie Metchnikoff introduced the new concept of phagocytosis, a fundamental attribute of the innate immunity, after studying the engulfment of particulate matter by protozoa and also researching the ingestion of foreign bodies by blood leukocytes. Subsequently, Metchnikoff was awarded the

Nobel Prize for Physiology or Medicine in 1908, along with Paul Ehrlich, for his work and discovery of humoral immunity, a component of adaptive immunity (Li *et al.,* 2007).

2.2.1 Acute and Chronic Inflammation

Inflammation is mainly divided into two types—acute and chronic inflammation. If the inflammation winds up in less than 48 h, then it is acute inflammation (e.g., abscess), and if it rests for greater than 48 h (i.e., weeks, months, or years), then it is chronic inflammation. ing pattern recognition receptors (PRRs) on their surface, which recognize/identify pathogen-associated molecular patterns (PAMPs) expressed exclusively on the outer surface of the pathogens (Agrawal *et al.,* 2014). These immune cells after activation release inflammatory mediators, and subsequently the cardinal signs of rubor, calor, tumor, and dolor are visible. Vasodilation and increased permeability result in an exudation/seepage (edema) of fluid and plasma proteins into the tissue space and migration and extravasations of mainly neutrophils along a chemotactic incline created by the locally inhabited cells to reach the location of injury in the tissue. Inflammatory mediators increase the sensitivity of the tissues to pain (hyperalgesia), and a resultant neurological reflex in response to pain leads to loss of function (functio laesa). A number of biochemical cascade systems, namely, the complement system (mostly activated by bacteria) and coagulation and fibrinolysis systems (mostly activated by

necrosis), work in conjunction with inflammatory mediators to continue the inflammatory response (Ansar *et al.,* 2013). However, the short half-life of inflammatory mediators is coterminous with the inflammation-activating signal. Acute inflammatory outcomes like resolution, abscess, and ulcer (fistula, sinus) may lead to a chronic inflammation (Ansar *et al.,* 2013). An unresolved acute-phase inflammatory response leads to the development of chronic inflammation by persistent injury or infection (e.g., ulcer, tuberculosis (TB)) and prolonged exposure to toxic agents like silica and autoimmune diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Chronic inflammation leads to tissue destruction, fibrosis, and necrosis. Neutrophils are the major cell types in acute inflammation while mononuclear cells (mostly lymphocytes, macrophages, and plasma cells) participate in chronic inflammation (Ansar *et al.,* 2009). The outcomes of acute inflammation are resolution, abscess, and ulcer (fistula, sinus) and may expand to a chronic inflammation. In chronic inflammation, the outcomes are tissue destruction, fibrosis, and necrosis. Acute inflammation is perpetuated by any immune cells previously present in the tissue. Acute inflammation is initiated mostly by the cells like DCs, macrophages, Kupffer cells, mastocytes, and histiocytes. These cells bear on their outer surfaces PRRs, which recognize the PAMPs expressed exclusively on the surface of the pathogens. These immune cells after activation with the pathogen releases inflammatory mediators

and subsequently showed the cardinal signs (rubor, calor, tumor, and dolor) of inflammation (Bae *et al.,* 2012). Vasodilation and increased permeability result in an exudation/leakage of fluid and subsequent edema. Release of plasma proteins into the tissue and migration and extravasations of leukocytes (mainly neutrophils) are the next steps of reactions. These reactions take place along a chemotactic flow generated by the locally inhabited cells to reach the locale of injury/assault in the tissue. Inflammatory mediators enhance the sensitivity of the affected tissues to pain (hyperalgesia), and a resultant neurological reflex in response to pain leads to loss of function (functio laesa) of the tissue. Additionally, to continue the inflammatory response with the help of the inflammatory mediators, a number of biochemical cascade systems are activated. These systems are the complement system (chiefly activated by bacteria), kinin system, and coagulation and fibrinolysis systems (chiefly activated by necrosis)(Ogita *et a.,* 2015). Once the stimulus of acute inflammation is removed, the inflammatory mediators, having short half-lives, are disgraced in the inflamed tissue. Thus an acute inflammatory response requires the constant stimulation.

2.2.2 Mechanism of Inflammation

Inflammatory reactions involve a series of biochemical and cellular changes, the extent of which is associated with the spread of the initial trauma. The mechanism of inflammation takes place in four phases—vasodilation, exudation (edema),

emigration of cells, and chemotaxis. Inappropriate stimulation of inflammatory responses is the primary cause of many known diseases, and inflammatory reactions are, as a result, also an imperative target for drug development (Li *et al.* 2007). The most prolific systemic expression of inflammation is a hike of body temperature and a number of biochemical changes known as the acute-phase reaction which steers to the production of acute-phase proteins from the liver. The local inflammatory reaction is portrayed by an early increase in blood flow to the locale of injury, increased vascular permeability, and the sequential and directional influx and careful accumulation of various effector cells from the peripheral blood flow at the place of injury. Influx of nonantigen-specific but prominent destructive cells (neutrophils) is one of the initial stages of the inflammatory response/reactions. These cells mount a phagocytic response which is rapid but nonspecific. An exudation/leakage of plasma into the lesion in the initial stage is also seen (Iwalewa *et al.* 2007). At a subsequent stage, macrophages, monocytes, and different cells of varied lineages of lymphocytes (T and B cells of specific subsets) appear at the locale of injury. These cell types are related with more tightly regulated, antigen-specific immune responses/reactions, and once they are stimulated, they also produce several protective inflammatory molecules. Inflammatory cells articulate greater numbers of cell adhesion molecules like glycoproteins and cell surface proteins. Endothelial cells are also stimulated during

the early phase of the inflammatory response/reactions and thereafter express, among many other things, several adhesion molecule counter-receptors. The controlled expression of these molecules allows for the prominent trafficking of blood-circulating leukocytes to a locale of inflammation. Cellular attachment of some immune cells to the wall of the endothelial cells lining the blood vessels around the inflammatory site stops them from being swept away from the site of tissue damage or infection. This is a crucial stage required for the consequent emigration of these immune cells into the surrounding medium of inflammatory tissues (extravasation) (Libby 2007). Once leukocytes have appeared at a locale of inflammation or infection, they release inflammatory mediators, which regulate the later accumulation and stimulation of other cells. However, in inflammatory responses initiated by the innate immune system, the final control is expressed by the antigen itself, in the identical way as it regulates the immune response itself. For this valid reason, the cellular accumulation at the locale of autoimmune reactions (antigen itself ultimately cannot be eradicated) or in chronic infection is quite distinct from that where the antigenic stimulus is rapidly cleared from the inflammatory sites (Delves *et al.* 2006). In homeostasis and control of inflammation, four major plasma enzyme systems are there, which have an important role. This enzyme system includes the complement cascade, the clotting system, the plasmin (fibrinolytic) system, and the kinin system.

2.3 Formation of Bilirubin

Bilirubin is derived from two main sources. Roughly, 80% of bilirubin is made from the breakdown of hemoglobin in senescent red blood cells, and prematurely destroyed erythroid cells in the bone marrow. The remainder originates from the turnover of various heme-containing proteins found in other tissues, primarily the liver and muscles. These proteins include myoglobin, cytochromes, catalase, peroxidase, and tryptophan pyrrolase (Ngashangva *et al.,* 2019). About 4 mg/kg body weight of bilirubin is produced daily.

2.3.1 Metabolism of Bilirubin

Albumin binding: Once bilirubin is released into the plasma, it is taken up by albumin which serves as its transporter throughout the body. The binding affinity for albumin to bilirubin is extremely high, and under ideal conditions, no free (non-albumin bound) unconjugated bilirubin is seen in the plasma. To a lesser degree, especially in states of hypoalbuminemia, binding also occurs with high-density lipoprotein. The binding of albumin limits the escape of bilirubin from the vascular space minimizes glomerular filtration and prevents its precipitation and deposition in tissues (Dosch *et al.,* 2019). When the albumin-bilirubin complex reaches the liver, the highly permeable hepatic circulation allows the complex to reach the sinusoidal surface of the hepatocyte. This allows the pigment to disassociate from

the albumin and enter the liver. This process is relatively inefficient with the first pass clearance of bilirubin being approximately 20%. This inefficient process allows for always having the ability to measure a concentration of unconjugated bilirubin bound to albumin in the venous circulation. The binding of albumin to bilirubin is reversible. Hepatic transport mechanisms: Bilirubin is taken up into the hepatocytes from the liver sinusoids by two different mechanisms: passive diffusion and receptor-mediated endocytosis. The process of passive diffusion is not energy-consuming and as a result, follows a concentration gradient making the flow bi-directional (Hind and Stec, 2018). Active transporter uptake of unconjugated bilirubin from the hepatic sinusoids is mediated by carrier proteins that are not well understood. A majority of the unconjugated bilirubin entering the hepatocytes is extracted in the periportal region. A fraction of conjugated and unconjugated bilirubin within the hepatocyte is transported back into the sinusoidal space, and this fraction is once again taken up downstream to the sinusoidal flow. The uptake is mediated by the 1A and 1B members of the organic anion transporting polypeptide family (OATP). These polypeptides are encoded by the genes: SLCO1B1 and SLCO1B3. Conjugated bilirubin that escapes reuptake into the hepatocyte is excreted in the urine. Bilirubin binding to glutathione S-transferases, which by itself increases net uptake, minimizes the efflux of internalized bilirubin.

2.4 Neutrophils

Neutrophils, also known as polymorphonuclear (PMN) leukocytes, are the most abundant cell type in human blood. They are produced in the bone marrow in large numbers, ~1011 cell per day. Under homeostatic conditions, neutrophils enter the circulation, migrate to tissues, where they complete their functions, and finally are eliminated by macrophages, all in the lapse of a day. Neutrophils are important effector cells in the innate arm of the immune system (Mayadas *et al.*, 2014). They constantly patrol the organism for signs of microbial infections, and when found, these cells quickly respond to trap and kill the invading pathogens. Three main antimicrobial functions are recognized for neutrophils: phagocytosis, degranulation, and the release of nuclear material in the form of neutrophil extracellular traps (NETs) (Figure 2). These functions were considered, until recently, the only purpose of neutrophils. However, current research by investigators in several fields of neutrophil cell biology has revealed that neutrophils possess a much diverse repertoire of functional responses that go beyond the simple killing of microorganisms. Neutrophils respond to multiple signals and respond by producing several cytokines and other inflammatory factors that influence and regulate inflammation and also the immune system (Nauseef and Borregaard, 2014). Nowadays it is recognized that neutrophils are transcriptionally active complex cells that produce cytokine, modulate the activities of neighboring cells and

contribute to the resolution of inflammation, regulate macrophages for long-term immune responses, actively participate in several diseases including cancer, and even have a role in innate immune memory (Netea *et al*., 2016). The multitude of neutrophil functional responses is induced by transcriptional activation and by changes in expression of surface molecules or activity. These phenotypic changes are usually detected in only a subset of neutrophils, suggesting that great neutrophil heterogeneity exists (Beyrau *et al*., 2012). Neutrophils display different phenotypes from the time they leave the bone marrow and enter the circulation (fresh neutrophils) to the time they disappear from the circulation (aged neutrophils). This shift in phenotype is known as aging, since it takes place within a single day, and results in various neutrophils with distinct properties (Adrover *et al.*, 2016). In addition, the microenvironment in different tissues can induce neutrophils to acquire specialized functions. Thus, the fact that neutrophils can display many functional phenotypes further supports the existence of several neutrophil subsets.

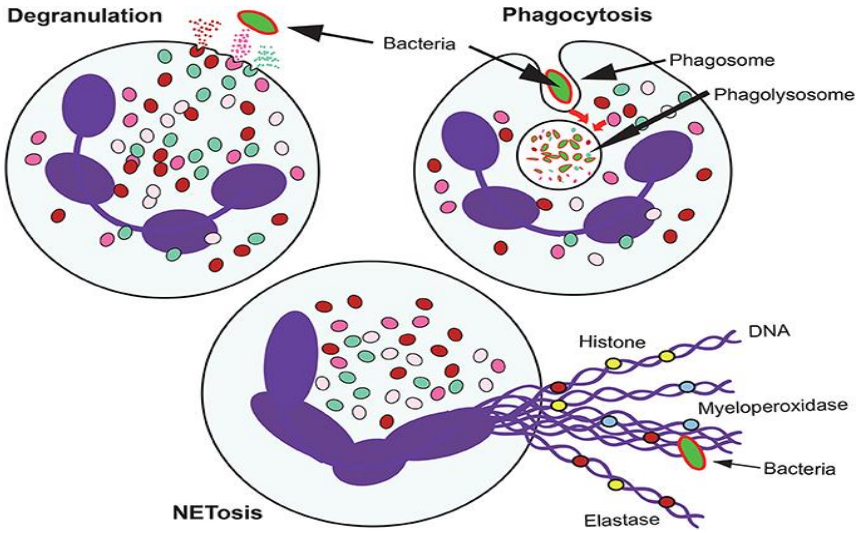


FIGURE 2. Antimicrobial mechanisms of neutrophils.

Source: frontiersin.org

2.4. 1 Neutrophil Life Cycle

Neutrophils represent about 70% of all leukocytes and more than 1011 cells are produced every day in the bone marrow. From there, neutrophils enter the blood where they circulate until they leave into tissues. Once neutrophils reach the end of their lifespan within tissues, they are cleared mostly by macrophages through the process of phagocytosis (Bratton and Henson, 2011). Despite this impressive turnover, the number of neutrophils in circulation remains relatively constant thanks to a fine balance between production and elimination. In addition, neutrophils actively change to be able to perform special functions at different times or places.

2.5 Lymphocytes

A lymphocyte is a type of white blood cell (leukocyte) in the immune system of most vertebrates (Janeway *et al.,* 2001). Lymphocytes include T cells (for cell-mediated, cytotoxic adaptive immunity), B cells (for humoral, antibody-driven adaptive immunity) and Innate lymphoid cells (ILCs) ("innate T cell-like" cells involved in mucosal immunity and homeostasis), of which natural killer cells are an important subtype (which functions in cell-mediated, cytotoxic innate immunity). They are the main type of cell found in lymph, which prompted the name "lymphocyte" (with cyte meaning cell). Lymphocytes make up between 18% and 42% of circulating white blood cells (Cohn *et al.,* 2014).

2.5.1 Types of lymphocytes

T cells and B cells: T cells (thymus cells) and B cells (bone marrow- or bursa-derived cells) are the major cellular components of the adaptive immune response. T cells are involved in cell-mediated immunity, whereas B cells are primarily responsible for humoral immunity (relating to antibodies). The function of T cells and B cells is to recognize specific "non-self" antigens, during a process known as antigen presentation. Once they have identified an invader, the cells generate specific responses that are tailored maximally to eliminate specific pathogens or pathogen-infected cells. B cells respond to pathogens by producing large quantities

of antibodies which then neutralize foreign objects like bacteria and viruses. In response to pathogens some T cells, called T helper cells, produce cytokines that direct the immune response, while other T cells, called cytotoxic T cells, produce toxic granules that contain powerful enzymes which induce the death of pathogen-infected cells (Omman and Kini, 2020). Following activation, B cells and T cells leave a lasting legacy of the antigens they have encountered, in the form of memory cells. Throughout the lifetime of an animal, these memory cells will "remember" each specific pathogen encountered, and are able to mount a strong and rapid response if the same pathogen is detected again; this is known as acquired immunity.

Natural killer cells: NK cells are a part of the innate immune system and play a major role in defending the host from tumors and virally infected cells (Cohn *et al.,* 2014). NK cells modulate the functions of other cells, including macrophages and T cells, and distinguish infected cells and tumors from normal and uninfected cells by recognizing changes of a surface molecule called MHC (major histocompatibility complex) class I. NK cells are activated in response to a family of cytokines called interferons. Activated NK cells release cytotoxic (cell-killing) granules which then destroy the altered cells (Janeway *et al.,* 2001). They are named "natural killer cells" because they do not require prior activation in order to kill cells which are missing MHC class I.

Dual expresser lymphocyte – X cell: The X lymphocyte is a reported cell type expressing both a B-cell receptor and T-cell receptor and is hypothesized to be implicated in type 1 diabetes (Ahmed *et al.,* 2016). Its existence as a cell type has been challenged by two studies. However, the authors of original article pointed to the fact that the two studies have detected X cells by imaging microscopy and FACS as described (Japp, 2021). Additional studies are required to determine the nature and properties of X cells (also called dual expressers).

2.6 Platelets

The blood platelets are the smallest cells of the blood, averaging about 2 to 4 μm in diameter. Although much more numerous (150,000 to 400,000 per cubic millimetre) than the white cells, they occupy a much smaller fraction of the volume of the blood because of their relatively minute size. Like the red cells, they lack a nucleus and are incapable of cell division (mitosis), but they have a more complex metabolism and internal structure than have the red cells. When seen in fresh blood they appear spheroid, but they have a tendency to extrude hairlike filaments from their membranes (Netea *et al.,* 2016). They adhere to each other but not to red cells and white cells. Tiny granules within platelets contain substances important for the clot-promoting activity of platelets. The function of the platelets is related to hemostasis, the prevention and control of bleeding. When the endothelial surface (lining) of a blood vessel is injured, platelets in large numbers immediately attach

to the injured surface and to each other, forming a tenaciously adherent mass of platelets. The effect of the platelet response is to stop the bleeding and to form the site of the developing blood clot, or thrombus. If platelets are absent, this important defense reaction cannot occur, and protracted bleeding from small wounds (prolonged bleeding time) results. The normal resistance of capillary membranes to leakage of red cells is dependent upon platelets (Burel, 2020). Severe deficiency of platelets reduces the resistance of the capillary walls, and abnormal bleeding from the capillaries occurs, either spontaneously or as the result of minor injury. Platelets also contribute substances essential for the normal coagulation of the blood, and they cause the shrinking, or retraction, of a clot after it has been formed. Platelets are formed in the bone marrow by segmentation of the cytoplasm (the cell substance other than the nucleus) of cells known as megakaryocytes, the largest cells of the marrow. Within the marrow the abundant granular cytoplasm of the megakaryocyte divides into many small segments that break off and are released as platelets into the circulating blood. After about 10 days in the circulation, platelets are removed and destroyed. There are no reserve stores of platelets except in the spleen, in which platelets occur in higher concentration than in the peripheral blood. Some platelets are consumed in exerting their hemostatic effects, and others, reaching the end of their life span, are removed by reticuloendothelial cells (any of the tissue phagocytes). The rate of platelet production is controlled but not so

precisely as the control of red cell production (Abbas, 2003). A hormonelike substance called thrombopoietin is believed to be the chemical mediator that regulates the number of platelets in the blood by stimulating an increase in the number and growth of megakaryocytes, thus controlling the rate of platelet production.

2.7 Monocytes

Monocytes are a type of leukocyte or white blood cell. They are the largest type of leukocyte in blood and can differentiate into macrophages and monocyte-derived dendritic cells. As a part of the vertebrate innate immune system monocytes also influence adaptive immune responses and exert tissue repair functions. There are at least three subclasses of monocytes in human blood based on their phenotypic receptors.

2.7.1 Functions of Monocytes

Monocytes are mechanically active cells and migrate from blood to an inflammatory site to perform their functions. As explained before, they can differentiate into macrophages and dendritic cells, but the different monocyte subpopulations can also exert specific functions on their own. In general, monocytes and their macrophage and dendritic cell progeny serve three main functions in the immune system. These are phagocytosis, antigen presentation, and

cytokine production (Evers *et al.,* 2022). Phagocytosis is the process of uptake of microbes and particles followed by digestion and destruction of this material. Monocytes can perform phagocytosis using intermediary (opsonising) proteins such as antibodies or complement that coat the pathogen, as well as by binding to the microbe directly via pattern recognition receptors that recognize pathogens. Monocytes are also capable of killing infected host cells via antibody-dependent cell-mediated cytotoxicity. Vacuolization may be present in a cell that has recently phagocytized foreign matter.

2.7.2 Differentiation into other effector cells

Monocytes can migrate into tissues and replenish resident macrophage populations. Macrophages have a high antimicrobial and phagocytic activity and thereby protect tissues from foreign substances. They are cells that possess a large smooth nucleus, a large area of cytoplasm, and many internal vesicles for processing foreign material. Although they can be derived from monocytes, a large proportion is already formed prenatally in the yolk sac and foetal liver (Murphy and Weaver, 2018). In vitro, monocytes can differentiate into dendritic cells by adding the cytokines granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 4. Such monocyte-derived cells do, however, retain the signature of monocytes in their transcriptome and they cluster with monocytes and not with bona fide dendritic cells (Robbins *et al.,* 2008).

CHAPTER THREE

MATERIALS AND METHOD

Research Materials

Materials used for this study includes

ELISA kits

Centrifuge

Test tubes/racks

Timer

Cotton wool

Lithium Heparin bottles

Plain sample bottles

EDTA bottles

Questionnaires

70% alcohol

Syringes and needles

Hand Gloves

Tourniquet

Glucometer

3.1. Study Area

This study was carried out in the university of Benin and environs in Ovia North East local government area, Benin City, Edo state.

3.2. Study Design

This study was divided into two phases; the fasting phase (test phase) and non-fasting phase (control phase). During the fasting phase, participants were made to fast or deprived of food and water for 12 hours while the control phase involved maintaining regular eating pattern. A properly structured questionnaire was administered to every participant to obtain basic health, demographic details and lifestyle eating patterns.

3.3. Study Participants

sixty (60) apparently healthy individuals both male and female within the age of 18-30 years were recruited for this study, only participants who gave informed consent participated in this study.

3.4. Sample Size Determination

Sample Size

The sample size for this study would be determined using the prevalence value from previous studies. Sample size will be estimated using the sample size expression (Araoye, 2004).

$$N = \frac{(z \times z) \times p \times q}{C \times c}$$

- Where;
- n = the desired sample size.
- c = permissible error (0.05).
- Z = the standard normal deviation, usually set at 1.96, which correspond to the 95% confidence interval.
- p = the proportion in the target population estimated to have a particular characteristic. In this case, a reasonable estimate will be 0.08 (8%).
- q = 1.0−p = 1.0−0.08 = 0.92
- n =120.

$$N = \frac{(z \times z) \times p \times q}{C \times c}$$

$$= \frac{(1.96\text{x } 1.96) \times 0.08 \times 0.92}{0.05\text{x}0.05} = 113.1$$

A total of 120 sample size comprising of 60 test samples and 60 control samples were employed for this research.

3.5. Selection Criteria

3.5.1. Inclusion Criteria

- Individuals with no medical history of coagulation or other blood related disorders.
- Participants who were willing to participate in the study, able to comply with the intermittent fasting protocol for the duration of the study.

- Participants aged between 18-30 years.

3.5.2. Exclusion Criteria

- individuals who are unable to comply with the study protocol, including the intermittent fasting regime.
- Individuals with a history of liver disease or other chronic medical conditions that will interfere with the study results.
- Individuals below 18 years.

3.6. Ethical Approval

Ethical approval was sought from the Health Research Ethics committee, College of Medical Sciences, University of Benin, Benin City, Edo state with Approval number CMS/REC/2023/406.

3.7. Sample Collection

Under aseptic condition, sterile needles were used to obtain blood samples from the fingertip and applied to the glucose strip indicator region of the glucose strip and accurately inserted into the glucometer for both random and fasting blood glucose analysis.

About 10mls of blood samples were collected with minimal trauma via the cubital fossa using a sterile disposable syringe and needle. 5ml of blood were emptied into EDTA bottles for hematological analysis and 5ml of whole blood dispensed into

lithium heparin bottle, spun and the supernatant dispensed into plain containers for bilirubin estimation.

3.8. Laboratory Analysis.

3.8.3. Fasting and Random Blood Glucose Estimation

- An Accuchek test strip was inserted into the glucometer after which the device automatically turned on.
- A clean disposable lancet was used to puncture the side of the Finger tip of the participants.
- The tip of the test strip was gently used to touch the blood droplet until the strip absorbed an adequate amount of blood.
- The glucometer analysed the blood sample and the result was displayed on the screen.

3.9 Statistical Analysis

The statistical values obtained were presented graphically in form of bar charts Statistical analysis was done using Graph Pad prism version 8.1 statistical package and relevant statistical values were obtained. Paired Student t-test was used and data were presented as mean ± standard error of mean (SEM). Values of $P<0.05$ were considered statistically significant.

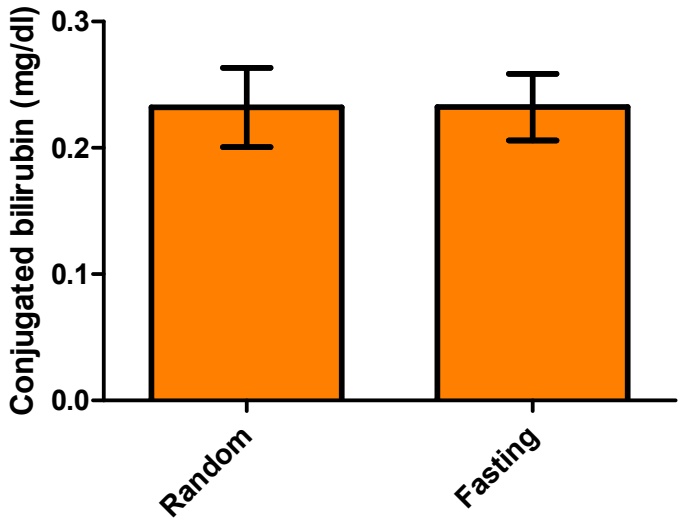
CHAPTER FOUR

Table 1: Comparing the mean values of inflammatory ratio and bilirubin during intermittent fasting and random

Parameters	**Random**	**Fasting**	**P-values**
Total bilirubin (mg/dl)	0.4400 ± 0.07	0.5516 ± 0.06	0.2308
Conjugated bilirubin (mg/dl)	0.2320 ± 0.03	0.2323 ± 0.03	0.9950
Platelet/Lymphocyte	66.71 ± 7.43	54.80 ± 4.75	0.1637
Neutrophil/Lymphocyte	0.461 ± 0.08	0.827 ± 0.07	0.0011
Monocyte/Lymphocyte	0.203 ± 0.015	0.169 ± 0.09	0.0395
Fibrinogen/Platelet	0.021 ± 0.003	0.025 ± 0.003	0.3370
Glucose level (mg/dl)	91.50 ± 3.58	75.81 ± 1.75	< 0.0001

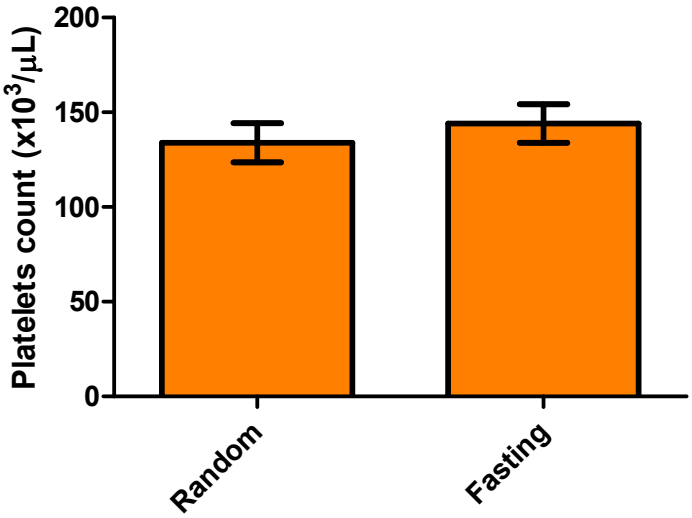
$P < 0.05$ indicates significant difference.

Fig I: graph of conjugated bilirubin changes during intermittent fasting and random intake



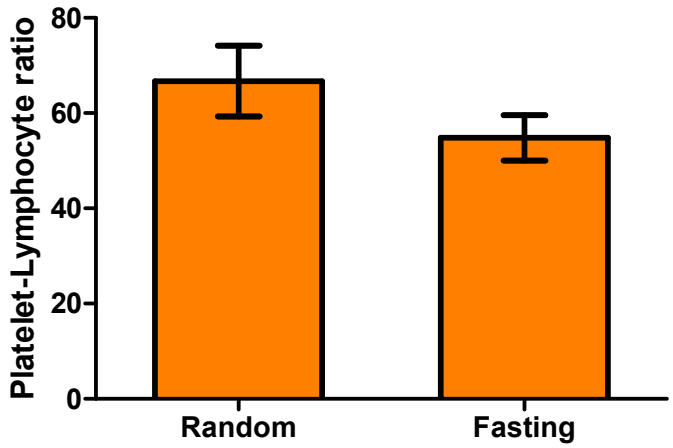
There was no significant difference in intermittent fasting compared with random intake of food.

Fig II: graph of platelets count changes during intermittent fasting and random intake



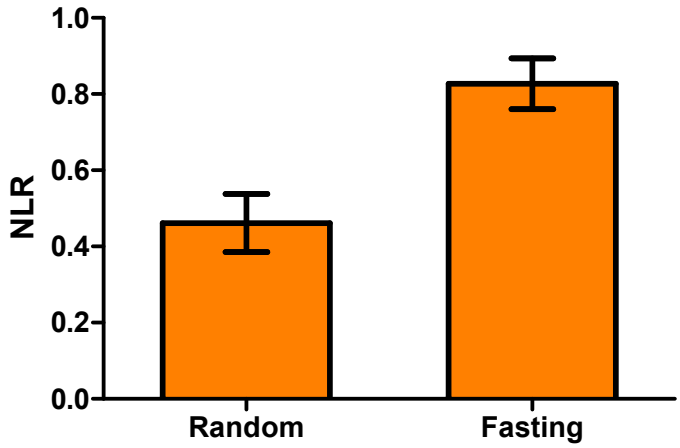
There was no significant difference in intermittent fasting compared with random intake of foo

Fig III: graph of platelets-Lymphocyte ratio changes during intermittent fasting and random intake



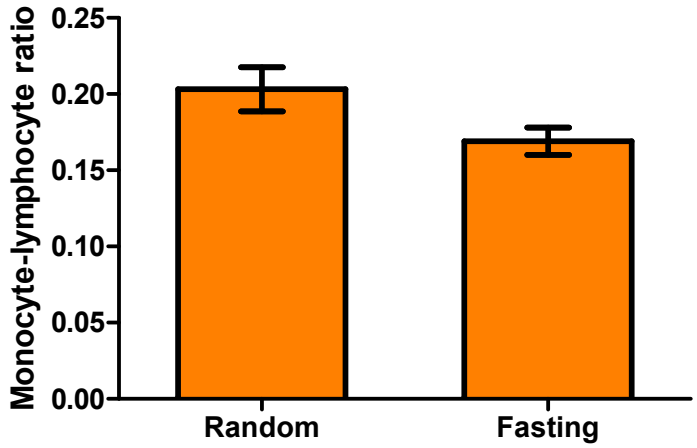
There was no significant difference in intermittent fasting compared with random intake of food

Fig IV: graph of Neutrophil-Lymphocyte ratio changes during intermittent fasting and random intake



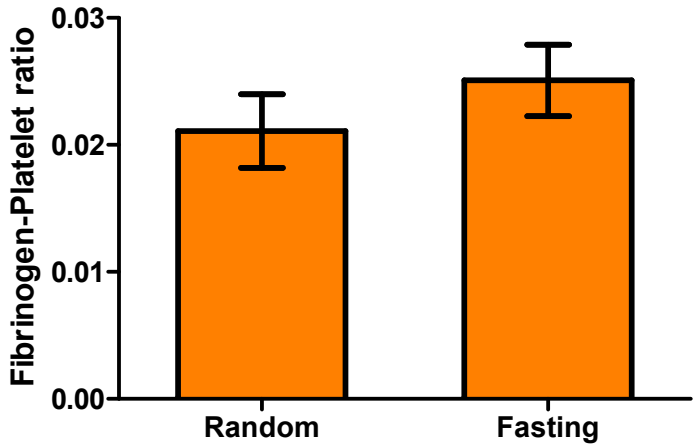
There was a significant increase in intermittent fasting compared with random intake of food

Fig V: graph of Monocyte-Lymphocyte ratio changes during intermittent fasting and random intake



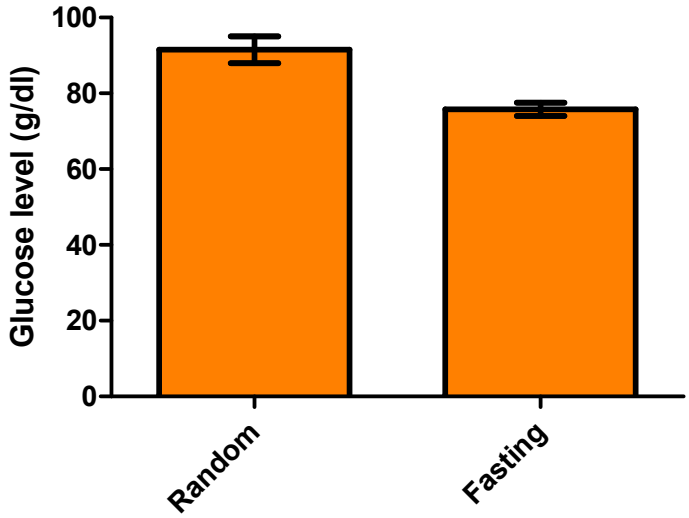
There was a significant decrease in intermittent fasting compared with random intake of food

Fig VI: graph of Fibrinogen-Platelet ratio changes during intermittent fasting and random intake



There was no significant difference in intermittent fasting compared with random intake of food

Fig VII: graph of glucose level changes during intermittent fasting and random intake



There was a significant decrease in intermittent fasting compared with random intake of food

CHAPTER FIVE

5.1 DISCUSSION

The goal of this study is to investigate the effect of intermittent fasting on some inflammatory ratios and serum bilirubin concentration in humans, the inflammatory ratios in this study included neutrophil-lymphocytes ratio, monocytes-lymphocyte ratio, platelets to lymphocytes ratio, and also fibrinogen to platelet ratio. The results of this study revealed no significance difference in bilirubin concentration as shown in Fig I, Conjugated bilirubin which is commonly used to asses liver's function and ability for the liver to form bilirubuin, the lack of significant difference in this marker indicates that intermittent fasting is unlikely to be detrimental to the liver in terms of bilirubin metabolism, the results also showed no significant difference in platelets counts, platelets-lymphocyte ratio during the fasting phase when compared to non fasting phase as seen in Fig II and Fig III, but there was a significant increase in NLR in fasting phase compared to non-fasting phase probably due to stress and could also indicate inflammation,(this marker could also stand as the negative effect of intermittent fasting on inflammatory ratio)as seen in Fig IV which is not consistent with a research carried out in 2014 by barnosky et al which said there was a decrease in NLR probably because the research was done on overweight and obese women (Barnosky *et al*., 2014). There was also a significant decrease seen in MLR in fasting individual which indicates a

reduction in inflammation (here we can also say intermittent fasting has a positive effect on inflammatory ratios). Another notable finding in this study is that there was a significant decrease in glucose levels among participants who practiced intermittent fasting compared to those with a random food intake pattern. This result is consistent with previous research that has demonstrated the positive effects of intermittent fasting on blood glucose regulation. Intermittent fasting may enhance insulin sensitivity and promote better glucose control, which is beneficial for individuals at risk of or managing type 2 diabetes. These findings support the notion that intermittent fasting can be a valuable dietary strategy for improving metabolic health.

5.2 Conclusion

In Conclusion, this study has shown that intermittent fasting has a significant effect on some inflammatory ratios, either to increase or reduce inflammation and no significant effect on bilirubin concentration.

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