EFFECTS OF 24-HOUR FASTING ON THE IN VITRO PHAGOCYTIC ACTIVITY OF NEUTROPHILS

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Abstract

The ability of the immune system to effectively protect the body from infection depends on various factors, one of which is proper diet. Fasting, the practice of withholding food for a period of time has been practiced all over the world mainly as part of a religious ritual. The main emphasis of this study is to elucidate the effects of fasting on the human innate immunity. This research aims to investigate whether fasting would cause a significant change in the neutrophilic phagocytic activity during a 24-hour religious fast. The study was performed on 20 healthy students who fasted for 24-hours. Blood samples were collected before and after the fasting period and analyzed for white blood cell count and phagocytic activity. The paired T-Test comparing before and after fasting values, showed that there is a significant increase in all of the measured parameters for phagocytosis constituting an average of 54.24% increase on the overall activity of neutrophils after the fasting period. Statistical analyses provided no evidence that the change in white blood cell count is related to the phagocytic activity which leadsto the conclusion that an increase in phagocytic index is associated with enhanced function rather than the decrease of number of leukocytes.

1. INTRODUCTION

Ancient cultures, according to the World Research Foundation, believed fasting - a partial or total abstention from all foods, or a select abstention from prohibited foods - could purify the soul. A growing number of practitioners of a type of alternative therapy called metabolic therapy as studied by Chiu, Bianchi, Franchi-Gazzola & Bussolati in 2012 believe that the body has environmental toxins and other harmful substances that can be removed by fasting or detoxifying the body.
They claim that fasting allows the body to focus energy on cleansing and healing itself. According to these practitioners, fasting helps the immune system work more efficiently, allowing more oxygen and white blood cells to flow through the body, help the body burn more fat, help increase energy, and allows other healing functions to improve. A study by the University of Berlin (2013) on new therapeutic approach to fight cancer revealed similar results.

However, fasting is not only practiced for health reasons. Almost all major religions of the world have a form of fasting incorporated in their beliefs (Brown & Mussett, 1984). While religious fast is partaken primarily for spiritual purposes, it also has the potential to greatly affect one's physical health (Desai, 2000). Accordingly, the health effects of religious fasting have recently been the subject of scientific inquiry, with most of the research being performed measuring health parameters during Ramadan, a time period in which Muslim pilgrims subject themselves to a partial fast wherein meals are only taken at the start and the end of a day thereby inducing a fast lasting for an average of 12 hours/day for 40 days (Latifinia, Vojgani, Ghargozlon & Sharifian, 2009)

A cross sectional study by Khazaei, Bo-kaeian & Jalili in 2013 involving 90 athletes during the month of Ramadan showed a positive increase of C4 and IgA levels among the participants. The increase in C4 and IgA demonstrated protective effects on an individual's immune system against infection. In another study by Hiramoto et. al. in 2008, it has also been observed that a nutritional stress of a 36-hour fast increased the number of neutrophils in the peripheral blood in both the elderly and young adult subjects.

A study conducted by Chia Wei et al. (2014) on the effects of prolonged fasting (48 – 120 hours of fasting) on the immune system of mice that were administered with chemotherapeutic drugs. The study showed that cycles of prolonged fasting protected hematopoietic cells from chemotoxicity and induced immune system regeneration, shifting stem cells from a dormant state to a state of self-renewal to reverse immunosuppression. Prolonged fasting also lowered levels of IGF-1, a growth-factor hormone that Longo and others have linked to aging, tumor progression and cancer risk.

Measurement of neutrophil parameters is an area of interest in immunology because neutrophils play a critical role in host defense. Neutrophils constitute an organism's first line of defense against external aggression and represent one of the key nonspecific host defense cell populations responsible for the phagocytosis of many microbial, bacterial, fungal and viral pathogens. (Stevens, 2010) Phagocytosis is defined as the ingestion of particles by cells, and this process involves the binding of (opsonized) particles to the surface of phagocytic cells, followed by the internalization and destruction of these particles. The killing and microbicidal functions of neutrophils are facilitated by the metabolic pathways involving the activation of NADPH oxidase system and the myeloperoxidase (MPO). (Todar, 2008)

Neutrophils are also known to be involved in the synthesis and release of immunomodulatory cytokines that influence both T cell and B cell activities. (Pyne, 1994)
Like every other cellular function, phagocytosis is an energy requiring mechanism that is inhibited by an inadequate supply of glucose (Segal, 2005). However, it has also been noted that excessive glucose in the blood may also decrease phagocytic activity (Van Oss, 1971). This phenomenon can be observed in patients with poorly managed cases of diabetes mellitus. DM patients are characterized by elevated blood glucose levels and lowered resistance to infection. Diabetes mellitus patients present physiological impairments, including diminished immunological function and inflammatory responses (chemotaxis, phagocytosis and killing), leading to higher susceptibility to bacterial and fungal infections. (Hotamisligil, 2006) Studies done by Wilson & Reeves in 1986, Alba-Loureiro et al in 2006 & Kempf et. al in 2007 have suggested that a possible cause of these weaker immune responses is neutrophil dysfunction caused by hyperglycemia. Similar findings have been observed in obese animal models. (Nabi, Islam, Rahman & Biswas, 2005; Slavov, Dzhelebov, Andonova & Girginov, 2009 & De Sourza Ferreira, 2012)

Since most of the study centers on the effects of prolonged fasting on the immune system, this study explored the effects of 24-hour fasting on one of the major functions of human innate immunity – neutrophil phagocytosis.

II. MATERIALS AND METHOD

Sampling

This study was approved by the Department of Medical Laboratory Science of the Adventist University of the Philippines (AUP), and written informed consent was obtained from all subjects before the beginning of the study. The group of subjects included in this study consisted of 20 healthy AUP college students (5 females and 15 males). The selected group was ideal in this study for two reasons. (1) All of the subjects are Christians with the majority being Seventh-day Adventists and were committed to observing a religious fast during the testing period. This enabled the research to obtain results from this event. (2) Since all the participants are students enrolled in AUP and residing in the dormitories, it can be considered that the similarities in their demographics like diet, levels of stress, physical exertion may have limited possible confounding factors.

Phagocytic Index Evaluation

Blood samples from the subjects were collected 2-hour postprandial for the non-fasting state, and a 24-hour fasting blood specimen for the fasting state. Each subject served as his own control and the 2-hour postprandial value was the control for each subject. Blood samples were collected in heparin tubes for the phagocytic assay, and EDTA tubes for complete blood count (CBC).

A sample of 0.9 ml freshly drawn heparinized blood was mixed in siliconized tubes and stoppers with 0.1 ml of the opsonized bacterial suspension of Staphylococcus aureus. This was rotated mechanically end to end at 37°C for 30 minutes. Blood smears were prepared on glass slides. (Latifinya et. al, 2000 and Heit, B, 2001) Prepared blood smears from the patients were stained with Wright stain. Cells were counted under oil-immersion, and 40 cells were counted to obtain a reliable result.
Blood smears were also prepared from the heparinized blood samples prior to bacterial inoculation to serve as ‘check slides’.

These slides were viewed to inspect for presence of toxic granulations. The Phagocytic Index was recorded as the mean number of bacteria in the first 40 neutrophils viewed under the microscope while the Phagocytic Percentage is the percentage of PMNs that has participated in ingestion of more than three staphylococci. Phagocytic Activity, which serves as the overall picture of Neutrophilic Phagocytosis, is then computed by finding the product of Phagocytic Index and Phagocytic Percentage. Parameters for phagocytosis are explained by the following formulas. (Hellum, 1977)

\[
\text{Phagocytic percent} = \frac{\text{No.of phagocytic Neutrophils}}{\text{Total No.of Neutrophils Observed}} \\
\text{Phagocytic Index} = \frac{\text{No.of Staphylococci in Phagocytic Neutrophils}}{\text{Total No.of Neutrophils Observed}} \\
\text{Phagocytic Activity} = \frac{\text{Phagocytic \%}}{\text{Phagocytic Index}}
\]

This procedure for Phagocytic Activity has been reviewed and evaluated by UERM’s chief pathologist, Dr. Araceli P. Jacoba, MD, FPSP.

**Preparation of Bacterial Suspension**

*Staphylococcus aureus* pure culture was purchased in UP Diliman Department of Microbiology, subcultured at 37oC in Blood Agar Plate and harvested during exponential growth. A 1:10 ratio of pooled Serum to Bacterial suspension was prepared and incubated for 30 min at 37oC under continuous agitation to opsonize the bacteria with antibodies from the serum sample.

The “coated” bacteria was centrifuged and resuspended in NSS. It was then washed three times with NSS and suspended in Hanks balanced salt solution (HBSS) and adjusted to a final bacterial concentration of 18% transmittance at a wavelength of 420 nm. (Ordeonez et al., 2008)

**Medium**

Hanks balanced salt solution containing 7g of NaCl, 350 mg of NaHCO2, 350 mg of KCL, 200 mg of MgSO4 * 7H2O, 55 mg of Na2HP04 * 2H2O, and 55 mg of KH2PO3 per liter without addition of the usual amount (0.09 %) of glucose at pH 7.4.

**Complete Blood Counts**

Complete Blood Count was determined using Horiba Pentra XLR hematology analyzer that utilizes Double Hydrodynamic Sequential System Principle.

**III. RESULTS**

The study population, aged 19–27 years old, consisted of 15 male and 5 female college students from AUP. Twenty-six people volunteered for the study but due to various limitations and circumstances (including the lack of transportation, volunteers with CBC parameters outside the normal range and volunteers that prematurely broke their fast) some blood samples were considered unsuitable, leaving only 20 volunteers eligible for study.

For statistical analyses, a paired t-test was used to compare the phagocytic index, phagocytic percent and phagocytic activity...
Effects of 24-Hour Fasting on the Vitro Phagocytic Activity of Neutrophils

between the samples collected and processed before and after fasting. Statistically significant changes were seen both in phagocytic index: \( r = 4.371, p < 0.05 \) and phagocytic percent: \( r = 4.539, (p < 0.05) \) as displayed in Table 1. The mean phagocytic index before fasting was 6.12±0.90 and 8.16±1.70, \( (p < 0.05) \) after the fasting period. Additionally, the mean phagocytic percent was 78\%±6\% and 87\%±5\% \( (p < 0.000) \) before and after fasting, respectively.

Table 1

<table>
<thead>
<tr>
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<th>Before Fasting</th>
<th>After Fasting</th>
<th>95% CI for Mean Difference</th>
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<tbody>
<tr>
<td>Phagocytic Index</td>
<td>M = 6.12</td>
<td>M = 8.16</td>
<td>df = 19</td>
</tr>
<tr>
<td></td>
<td>SD = 0.90</td>
<td>SD = 1.70</td>
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<tr>
<td>Phagocytic percent</td>
<td>0.78</td>
<td>0.87</td>
<td>df = 19</td>
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<td></td>
<td>0.06</td>
<td>0.05</td>
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Since both Phagocytic Index and Phagocytic Percentage have shown significant increase after the fasting period, it naturally followed that Phagocytic activity, the computed value derived from the product of both of the fore-mentioned parameters, was also shown to have significantly increased at the end of the 24-hour fasting period.

Results in Table 2 show a statistically significant difference in mean phagocytic activity before and after fasting. Mean Phagocytic activity was reported to be 4.80±0.89 and 7.08±1.66 before and after fasting, respectively \( (p < 0.05) \). This constitutes an average of 54.24\% increase on the overall activity of Neutrophils after the fasting period.

Table 2

<table>
<thead>
<tr>
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<th>Before Fasting</th>
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<th>95% CI for Mean Difference</th>
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<tbody>
<tr>
<td>Phagocytic Activity</td>
<td>M = 4.80</td>
<td>M = 7.08</td>
<td>df = 19</td>
</tr>
<tr>
<td></td>
<td>SD = 0.89</td>
<td>SD = 1.66</td>
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A Pearson product-moment correlation coefficient (PPMCC) was computed to assess the relationship between the change in WBC Count and the change in Phagocytic Activity. Results in Table 3 show that change in WBC is not correlated with change in
Phagocytic Activity, $r (20) = -0.352, p = 0.128$.

Table 3

<table>
<thead>
<tr>
<th>Change in WBC Count</th>
<th>Change in Phagocytic Activity</th>
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<tr>
<td></td>
<td>$r$</td>
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<tr>
<td>Change in WBC Count</td>
<td>$-0.352$</td>
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Statistical analyses in Table 3 provided no evidence that the number of WBCs is related to the phagocytic activity. This would lead to the conclusion that an increase in phagocytic index is associated with enhanced function rather than the decrease of number of leukocytes.

Figure 1 illustrates the percent increase in all of the parameters for phagocytosis. It can be noted that the increase in Phagocytic Activity was principally due to the increase in the Phagocytic Index rather than the Increase in the Phagocytic Percentage.

![Figure 1](image1.png)

*Figure 1*. Bar graph showing the percent increase in parameters on phagocytosis before and after fasting period.

Figure 2.1 shows scatter plots that graphically illustrate a strong positive correlation between phagocytic index and phagocytic activity while Figure 2.2 illustrates a weak positive correlation between the phagocytic percentage and phagocytic activity. This further supports that the change in phagocytic activity is mainly due to the phagocytic index rather than the phagocytic percentage.

![Figure 2](image2.png)

*Figure 2.1* Scatter plots representing before and after fasting values of phagocytic index (y
Table 4 shows the summary of Complete Blood Count values of all participants. These values were within the laboratory normal range for both male and females during each of the time periods in which blood was drawn. Furthermore, it also exhibits significant trends in CBC parameters following the 24-hour fasting period.

Analysis of subjects' CBC showed that Total WBC count, RBC, Hemoglobin and Hematocrit were significantly increased at 0.05 significance level. Percentage and absolute counts of Neutrophils were also increased at the end of the fasting period while Lymphocyte, Monocyte and Eosinophil percentages and absolute counts have all significantly decreased. Other statistical results did not have significant differences.

Table 4

<table>
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<th>Descriptive Statistics and T-test Results for Blood Count</th>
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<tbody>
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<td>Before Fasting</td>
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Figure 2.2 Scatter plots representing before and after fasting values of phagocytic percentage (y axis) versus phagocytic activity (x axis).
The above results for index of and percent phagocytosis, for example, demonstrate that roughly 480 staphylococcus would be phagocytised by 100 PMNs before fasting, while after fasting, this number would reach 708. Therefore, it seems that after fasting, the immune system may respond more actively to infection (by gram positive bacteria, for example) than before fasting.

Since a significant increase in WBC count was observed before and after fasting, PPCMC was performed to establish that the increase in WBC was not the cause of the increase in phagocytic activity. The result showed no correlation between the increase in WBC count and phagocytic activity, therefore the increase in phagocytic activity was due to...
After 24-30 hrs, liver glycogen stores are mostly depleted and glucose levels drop and maintained at 75-80 mglL (Warade, 2014). This marginal decrease in glucose levels after 24-hour fasting can be implicated to cause a positive increase in phagocytic activity where lowering of blood glucose levels of diabetic patients or experimental animals has been reported to have significant correlation with improvement of neutrophil functional activity (Van Oss, 1971).

A very recent study conducted in the University of California (published June of 2014) has shown intermittent, prolonged fasting induces changes that trigger stem cell-based regeneration of new immune system cells. In particular, "prolonged fasting reduced the enzyme PKA, shifting stem cells from a dormant state to a state of self-renewal". In mice, it was described that fasting cycles "flipped a regenerative switch," changing the signaling pathways for hematopoietic stem cells, which are responsible for the generation of blood and immune systems. (Chia-Wei et al, 2014) These findings support this study’s data that revealed a significant increase in CBC pa-
rameters like RBC count, Hemoglobin, Hematocrit, WBC count including the relative count for all 5 WBC types – neutrophils, lymphocytes, monocytes, basophils and eosinophils.

V. CONCLUSION

This study was intended to determine if there is a relationship between a 24-hour religious fasting on neutrophilic phagocytosis. The study found that 24-hour fasting significantly enhanced the neutrophils capacity to engulf bacteria, which may be an important beneficial effect of religious fasting. These observations become meaningful when it is recognized that phagocytosis is the rate limiting step in the reduction of viable organisms. Thus, diet may play a key role in the control of resistance to infection.

VI. RECOMMENDATIONS

The design of future studies that would employ the use of more analytic test methods which makes use of flow-cytometric test systems that simultaneously measures phagocytosis and production of reactive oxygen species (ROS) of neutrophils is recommended to confirm and to have a more accurate reading of the parameters that have been measured in this study. This would also give the future researchers a more complete set of data that would describe neutrophil activity under fasting conditions. The researchers also recommend a follow-up study regarding the duration of the beneficial effects on the neutrophil phagocytic activity after the 24-hour fasting period.

REFERENCES


Fasting differentially modulates the immunological system: its mechanism and sex differ


