NEUTROPHIL FUNCTION (INNATE IMMUNITY) DURING RAMADAN

Afshineh Latifynia, Mohammad Vojgani, Mohammad Javad Gharagozlou, Sharifian R
Department of Immunology, School of Medicine, Medical Sciences/University of Tehran, Tehran, Iran

Background: Fasting during the month of Ramadan is one of the essential religious practices of Muslims. The aim of this study was to evaluate opsonisation, phagocytosis, and nitroblue tetrazolium (NBT) reduction by white blood cells in normal, healthy, male subjects under non-fasting (before Ramadan) and fasting (after Ramadan) conditions. **Methods**: In this study, 13 Muslim men, aged 28-54 years, whose health was confirmed by health application forms, gave blood samples one week before the beginning of the holy month of Ramadan and during the last week of Ramadan. Blood samples were tested for neutrophil phagocytosis, serum opsonisation power, and NBT reduction. **Results**: Despite a decline in the neutrophil phagocytic index and serum opsonisation index, the percentage of neutrophils participating in phagocytosis increased with fasting. In addition, there was an increase in the percentage of neutrophils demonstrating NBT reduction. Although there was a decrease in opsonisation of the serum, the increased percentage of opsonisation compensated for this defect. **Conclusion**: This study demonstrates the beneficial effect of fasting during Ramadan on neutrophil phagocytic function.

Keywords: Neutrophil, Ramadan, Nitroblue tetrazolium, Phagocytosis, Opsonisation

INTRODUCTION

Every year, millions of Muslims fast from dawn until sunset during the lunar month of Ramadan. Ramadan can occur in any of the four seasons and the hours spent fasting vary accordingly, from 11 hours to 18 hours (average 13 hours) a day. Fasting during Ramadan can be considered a controlled type of partial fasting. The common practice is to eat two meals instead of three, one before dawn and one after sunset. Generally, one would expect that limitation of total food intake could potentially lead to weight loss during the holy month. This is not always the case, however, because larger amounts and a greater variety of food are often consumed at the evening meal. In fact, we commonly find that people are several kilograms heavier at the end of Ramadan.[1]

We examined the effect of fasting during the month of Ramadan on innate immune function (phagocytosis, opsonization, and neutrophil tetrazolium reduction)[2] in a group of 13 males. The main emphasis of this study was to elucidate the effect of fasting during Ramadan on immunity, as little is known about this topic.[3] Intracellular organisms are destroyed by non specific immune system or innate immunity. The neutrophil is the main cell of this system that acts by phagocytosis and kills the organisms by inducing free radicals.

Phagocytosis is the main part of innate immune system. Since patients with disturbance in phagocytotic systems are generally prone to infections, recognition of defects in the microbicidal function of phagocytes is important. The innate immune system, including phagocytotic cells, is the first line of defense against microbial disease, especially intracellular pathogens.

Neutrophils are one of the most important phagocytotic cell types. Neutrophils follow chemotactic cues to locate sites of inflammation, migrating to infection sites in response to signals such as chemoattractants. Phagocytosis is defined as the ingestion of particles by cells, and this process involves the binding of particles to the surface of phagocytic cells, followed by the internalization and destruction of these particles. The coating of a microorganism with molecules that trigger its destruction by phagocytes is known as opsonization.[4] The reduction of nitroblue tetrazolium (NBT) by monocytes and neutrophils can be used as an indirect marker of the phagocytic activity of these cells.[4–8] NBT is a dye with low reduction potential that produces an intensely stained product formazan when reduced. NBT is easily phagocytosed by cells and is reduced to formazan inside the mitochondria.[9] Therefore, the aim of this study was to evaluate opsonization, phagocytosis, and NBT reduction in healthy males under non-fasting (before Ramadan) and fasting (during Ramadan) conditions.

MATERIAL AND METHODS

In this study, immune function was assessed during Ramadan. Blood samples were examined using quantification of opsonization, phagocytosis, and NBT reduction. The methods were standardised at the Department of Immunology, Tehran University of Medical Sciences.

Opsonization and phagocytosis

Reagents: Six percent Dextran in saline. Ice cold 0.87% ammonium chloride in this buffer. Phosphate-buffered saline (PBS) was prepared from 0.8 g/L NaCl, 200 mg/L KH_2PO_4, 1.15 g/L Na_2HPO_4, 200 mg/L KCl, 133 mg/L $CaCl_2.2H_2O$, and 100 mg/L $MgCl_2.6H_2O$. PBS was filter sterilized through 0.22 Mm vacuum filtration units.[10] Pooled normal human serum, type AB, was used

as control, and patient plasma was used for each patient. Staphylococcus bacteria were suspended in saline, washed twice, and re-suspended to achieve a concentration of 2×10^6 per ml. Venous blood (5–10 ml) was drawn and stored in heparinised tubes. Sedimented erythrocytes in dextran saline (0.5ml) were added to each volume of blood at room temperature. After 45–60 minutes, supernatant plasma was removed and transferred to conical centrifuge tubes, and one half volume of ice-cold ammonium chloride was added. The tubes were then rapidly inverted and the suspensions were centrifuged at 500 g for 10 min. The cells were re-suspended in ice-cold PBS and washed twice. Finally, the cells were re-suspended in normal saline to a concentration of 4×10^6 cells per 0.8 ml and 0.8 ml of this cell suspension was transferred to capped plastic test tubes and incubated for 5 min in a 37 ºC shaking water batch. At this time, 0.5 ml of normal human serum, type AB, was added to control tubes, and 0.5 ml of patient plasma was added to each patient tube. Then, 0.2 ml staphylococcus bacteria (Mackfarlin No. 2 tube) (2×10^6) were added, and the tubes were incubated for 30 minutes. Tests were conducted in duplicate. Blood smears were prepared on cover slips. Air dried cover slips were stained with Wright's stain. Cells were counted under oil-immersion, and roughly 100 cells were counted to obtain a reliable result. Index and percentage of phagocytosis are similar parameters and are explained by the following formulas:

$$\text{Opsonic/phagocytic percent} = \frac{\text{No. of phagocytic Neutrophils}}{\text{Total No. of Neutrophils}}$$

$$\text{Opsonic Index} = \frac{\text{No. of Staphylococci in Phagocytic Neutrophils}}{\text{Phagocytic Neutrophils}}$$

Note: A cell was considered a phagocyte and able to opsonize if it contained more than two staphylococci.[11]

Reagents for NBT test

PBS Buffer 8 g, NaCl 0.2 g, KCl 1.15 g, H_2SO_4 0.2 g, H_3PO_4, in 1,000 ml distilled water, 1 mg/ml PMA, 1 ml PMA+1.25 mg, NBT+17.5 mg, BSA 4-Wright solution.

Procedure

Between 0.5 and 1.0 ml of peripheral venous blood was drawn into heparinised tubes and mixed gently. For each sample, about 0.1 ml blood was transferred to a second tube, and 0.1 ml of buffered NBT solution was added to the second tube and mixed and incubated at 37 ºC for 30 min. About 0.05 ml of blood was transferred onto a concave microslide, and a cell smear was prepared on a coverslip. Coverslips were air-dried and stained with Wright's stain. Cells were counted under oil-immersion. A count of 100 cells was considered sufficient to provide a reliable estimate of percent NBT-positive cells within the total neutrophil population.[12]

Remarks

A cell was considered NBT-positive if it contained large bluish-black deposits or stippled granules in the cytoplasm. Formazan, the reduced form of NBT, has a refractile border when viewed under the microscope, which becomes visible when focusing up and down with the micrometer. This helps to differentiate it from other cytoplasmic structures.

Statistical Analysis

For statistical analysis, paired *t*-tests were performed using SPSS version 11.5 for Windows.

RESULTS

The study population consisted of males in medical school at Tehran University of Medical Sciences. Due to various limitations, including the needs for laboratory examinations, fresh blood to be examined immediately, and adequate blood volumes, most blood samples were unsuitable, leaving only 13 cases eligible for study. The subjects were aged 28–58 years (mean±SD 39.4±10.9 years).

The results of opsonization assays showed that the index correlation coefficient before and after Ramadan was close to zero. The mean±SD opsonization indixes before and after Ramadan were 4.82±1.16 and 4.03±0.8, respectively (p=0.027*) Additionally, the mean opsonization percentages were 69.3% and 73.5% before and after Ramadan, respectively. After Ramadan, the mean index of opsonization decreased slightly, and the mean percent opsonization increased, but both remained within the normal range. For statistical analyses, paired t-tests were used, and statistically significant changes were not seen Opsonisation percent: t=-1.009, (p=0.337). The correlation coefficient for the phagocytic index before and after Ramadan was 0.21. The mean phagocytic index before Ramadan was 4.73±1.39 and afterward, it was 4.82±1.03, (p=0.866). Additionally, the mean phagocytic percent before Ramadan was 60.16, while that after Ramadan was 68.61, which are both within the normal range (p=0.037*). These data demonstrate that the mean index of phagocytosis decreased slightly, while mean percent of phagocytosis increased after Ramadan however, both stayed within normal range. The correlation coefficient for percent phagocytosis before and after Ramadan was 0.08. The results of the NBT assays showed that in all subjects, the percent of neutrophils that reduced nitroblue tetrazolium and produced free radicals was normal. The correlation coefficient for percent NBT reduction before and after Ramadan was 0.9. This change was not statistically significant (p=0.562).

Table-1: Phaocytosis variation during the period of Holy Ramadan

Variable	Mean	Standard Deviation	*p*-value
Phagocytic Index Before Ramadan	4.73	1.38	0.866
Phagcytic Index After Ramadan	4.82	1.03	
Phagcytic Percent Before Ramadan	60.161	13.27	0.037*
PhagcyticPercent After Ramadan	68.61	10.0	
Opsonization Index Before Ramadan	4.82	1.16	0.027*
Opsonization Index After Ramadan	4.03	0.86	
Opsonization Percent Before Ramadan	69.30	14.97	0.333
Opsonization Percent After Ramadan	73.53	10.42	
NBT Percent Before Ramadan	96.46	3.90	0.562
NBT Percent After Ramadan	96.92	3.90	

** Statistically significant*

DISCUSSION

Polymorphonuclear (PMN) cells play an essential role in host defense. A variety of microbicidal systems are involved in phagocytosis; some are dependent on oxygen and others are effective in its absence.[2,13] Neutrophils can be stimulated not only by foreign antigenic substances, but also by C5a from the complement system, certain bioactive lipids,[14] bacterial products, endogenous cationic proteins[15] and by neutrophil activating factor (NAF) produced by cells of the Mononuclear Phagocytose System (MPS).[15] Certain lymphokines secreted from activated T cells, such as leukocytic migration inhibition factor (LIF) and GM-CSF,[16–18] have been shown to be potent stimulators of neutrophil activity.[19,20] In some circumstances, enhanced granulocytic function may be detrimental to the host.[21] White blood cells can be stimulated with complement-opsonized zymozan, C5a, formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe), and PMA.[1] Proper function of the immune system depends on the right cells being at the right place at the right time.[21] Attachment of phagocytes to particles may happen nonspecifically, as a result of the particle itself, or may occur in the presence of an opsonin, in the process known as immune adherence. IgG or activated complement components (i.e., iC3b) may act as a bridge between a target molecule and a receptor-bearing phagocyte, thereby greatly increasing the efficiency of the phagocytic process.[22] Binding of a particle to complement receptors (CR1, CR2) or to FCgR alone promotes optimal contact with the phagocytic cell, and may induce ingestion.[23] Ingestion of a particle results from extension of an advancing pseudopod over and around the particle surface, such that fusion of the pseudopod with the bulk of the cell eventually occurs. The vacuole resulting from the closure of the membrane is referred to as a phagosome. Respiratory burst[13,24–26] refers to the enhanced oxidative metabolism initiated by the release of contents of secondary granules. Two enzymes derived from the hexose monophosphate (HMP) shunt, designated G6PD and 6PGD, participate in the formation of NADPH oxidase, a plasma membrane bound enzyme unique to phagocytes. NADPH is used in the production of hydrogen peroxide (H_2O_2) and certain oxidizing radicals (e.g., OH^-) by the so-called myeloperoxidase (MPO)-independent system. Alternatively, a superoxide radical may react with MPO. MPO systems include activation and inactivation of secreted neutrophil proteases and inactivation of toxins and other mediators of inflammation. Our results showed that not only before Ramadan, opsonization, phagocytosis and phagocytes' function from normal subjects were in normal range but also those to be left over normal range after Ramadan. Phagocytic opsonization in the presence of C3b, IgG, or IgA was significantly decreased after fasting, from 4.85 to 4.0 ($p<0.001$). However, the percent of phagocytes was increased after fasting ($p<0.037$). These results demonstrate that, before Ramadan, in a normal, healthy subject, 71% of neutrophils each phagocytosed 4.85 staphylococci, resulting in the removal of limited 344 staphylococci, and after fasting in normal, healthy subjects, 72.1% of neutrophils each phagocytosed 4.0 staphylococci, removing 288 staphylococcus. Opsonization Index before Ramadan was 4.82 and declined to 4.03 after Ramadan fasting ($p<0.027$), but the opsonization percent before Ramadan was 69.3% and increased to 73.53% after Ramadan fasting. This would mean the number of phagocytosed staphylococci was 272 before Ramadan and 275 after fasting in normal, healthy subjects. Statistical analysis of the neutrophil respiratory burst in normal, healthy subjects before and after fasting showed that there was a significant correlation ($p<0.0001$), with the percent of neutrophils showing a positive respiratory burst being 96.4% before Ramadan, and 97.7% after Ramadan (Table-1).

In other studies, no statistically significant changes were observed in mean body weight, total cholesterol, or LDL levels. The mean HDL cholesterol level, however, increased significantly during Ramadan.[3] Other authors have also shown that eating behaviours during Ramadan may contribute to improved nutritional status in people at risk for nutritional deficiency.[1] Additionally, it has been demonstrated that, in patients with well controlled and moderately controlled type 2 diabetes mellitus, fasting

during Ramadan can cause a reduction in serum fructosamine. This fasting does not cause an increase in the production of beta hydroxybutyrate, however.[27] These results reflect separate host responses, and additional, independent measurements of these effects will lead to a more meaningful understanding of host defence and immune system.

Immune complexes and other factors in the serum may also interact with granulocytes to alter cell surface receptors and subsequent metabolic activity. In some circumstances, enhanced function of granulocytes may be detrimental to the host. In this study, white blood cells were stimulated with complement-opsonized zymozan, C5a, F-Met-Leu-Phe, and PMA.

There are some important notes to make regarding our study: (a) Opsonins play an important role in the phagocytic system, increasing phagocytic potential. (b) When the phagocytic index decreases in normal, healthy subjects, as may occur after fasting during Ramadan, the percent of phagocytes immediately increases. This can be equated to maintenance of normal body physiologic homeostasis. Additionally, in normal subjects, fasting during Ramadan does not negatively affect opsonization, phagocytes, or respiratory burst. Furthermore, fasting leads to an increase in the percent of phagocytes and the percent of phagocytes showing respiratory bursts. The respiratory burst is an important step for innate immunity. As such, NBT reduction test and its modification are useful for detection and differential of bacterial disease.[9] We conclude that Ramadan fasting does not have a negative effect on the components of innate immunity studied in this article (phagocytes, opsonization, respiratory burst) that would increase susceptibility to bacterial diseases. The above results for index of and percent phagocytosis, for example, demonstrate that, without opsonin factors, roughly 272 staphylococcus would be phagocytised before fasting, while after fasting, this number would reach 275.5. Therefore, it seems that after fasting, the immune system may respond more actively to infection (by gram positive bacteria, for example) than before fasting. While the number of staphylococci phagocytised in the presence of opsonins after fasting (288.4) was less than that before fasting (344.0), these numbers are nonetheless greater than the 272 and 275.5 bacteria phagocytised in the absence of opsonin factors before and after fasting, respectively.

CONCLUSION

The accumulation of phagocytised staphylococci increases after fasting, as does respiratory burst and killing of bacteria, although these remain within normal range. Therefore, the innate immune response to intracellular pathogens does not decrease, in fact increases after fasting, which may be an important, beneficial effect in Muslims fasting during Ramadan.

ACKNOWLEDGEMENT

The authors would like to thank Farzan Institute for Research and Technology for technical assistance.

REFERENCES

1. Aadil N, Houti IE, Moussamih S. Drug intake during Ramadan. BMJ 2004;329:778–82.
2. Alam M, Ranadive NS, Pruzanski W. Influence of neutrophil cationic proteins on generation of superoxide by human polymorphonuclear cells during phagocytosis. Inflammation 1987;11:131–42
3. Gharbi M, Akrout M, Zouari B. Food intake during and out side Ramadan. East Meditrr Health J 2003;1-2:131–40.
4. Hellum KB. Nitroblue tetrazolium test in bacterial and viral infections. Scand J Infect Dis 1977;9:269–76.
5. Ku HP. Increased production of hydrogen peroxide and expression of CD11/CD18 on alveolar macrophages in patients with active pulmonary tuberculosis. Tube Lung Dis 1996;77:468–75.
6. Nakagawara A, Kayashima K, Tamada R. Sensitive and rapid method and determination of superoxide generating activity of blood monocytes and its use as a probe for monocyte function in cancer patients. Gann 1989;70:829–33.
7. Schluger NW, Wiliam NR. The host immune response to tuberculosis. Am J Respir Crit Care Med 1998;157:679–91.
8. Schlesinger LS. Role of mononuclear phagocytesin M.tuberculosis pathogenesis. Invest Med 1996;44:312–23.
9. Dubaniewicz A, Dubaniewicz A, Hoppe A. The spontaneous and stimulated nitroblue tetrazolium (NBT) tests in mononuclear cells of patients with tuberculosis. Ann Acade Med Biaiost 2004;49:252–5.
10. Mathews SE, Warinner JE, Weeks BA. Assays of immune function in fish macrophages. In: Stolen JS, Fletcher TC, Anderson DP, editors. Techniques in fish immunology. Vol-1. New Jersey: Fair Haven;1990. p.155–63.
11. Garondahi G, Jahannisson A, Demmers S. Influence of age and plasma treatment on neutrophil phagocytosis and CD_{18} expression in foals. Vet Microbiol 1991;62:241–54.
12. Levinsky RJ, Harvey BAM, Rodeck CH F Soothill. Phorbol myrestate acetate stimulated NBT test: a sample method suitable for antenatal diagnosis of chronic granulomatous disease. Clin Exp Immunol 1983;54:595–8.
13. Gabig TG, Babior BM. The killing of pathogens by phagocytes. Ann Rev Med 1981;32:313–26.
14. Peveri P, Walz A, Dewald B, Baggiolini M. A novel neutrophil-activating factor produced by human mononuclear phagocytes. J Exp Med 1988;167:1547–59.
15. Uysal AR, Erdogan MF, Sahin G Kamel N, Erdoğan G. Clinical and metabolic effects of fasting in 41 type-2. Diabetic patients during Ramadan. Diabetes Care 1998;21:2033.
16. Lopez AF, Williamson DJ, Gamble JR, Begley CG, Harlan JM, Klebanoff SJ, *et al*. Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. J Clin Invest 1986;78:1220–8.
17. Naccache PH, Faucher N, Borgeat P, Gasson JC, DiPersio JF, *et al*. Granulocyte-macrophage colony-stimulating factor modulates the excitation-response coupling sequence in human neutrophils. J Immunol 1988;140:3541–6.
18. Weisbart RH, Kwan L, Golde DW, Gasson JC., Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants. Blood 1987;69:18–21.
19. Kowanko IC, Ferrante A. Stimulation of neutrophil respiratory burst and lysosomal enzyme release by human interferon-gamma. Immunology 1987;62:149–51.

20. Taetle R, Honeysett JM. Gamma-interferon modulates human monocyte/macrophage transferrin receptor expression. Blood 1988;71:1590–5.
21. Zagroski-kiprokowicz D. Nitroblue tetrazolium test in course of ulcerative olictis. Rocz Akad Med Bialymst 1997;42:69–74.
22. Fries LF, Siwik SA, Malbran A, Frank MM.. Phagocytosis of target particles bearing C3b-IgG covalent complexes by human monocytes and polymorphonuclear leucocytes. Immunology 1987;62:45–51.
23. Willis HE, Browder B, Feister AJ Mohanakumar T, Ruddy S. Monoclonal antibody to human IgG Fc receptors. Cross-linking of receptors induces lysosomal enzyme release and superoxide generation by neutrophils. J Immunol 1988;140:234–9.
24. Curnutte JT, Babior BM. Chronic granulomatous disease. Adv Hum Genet 1987;16:229–97.
25. Repo H. Defects in phagocytic functions. Ann Clin Res 1987;19:263–79.
26. Rossi F. The O2-forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. Biochim. Biophys Acta 1986;853:65–89.
27. Steele RW. Clinical applications of chemiluminescence of granulocytes. Rev Infect Dis 191;13:918–25.

Corresponding Author:

Afshineh Latifynia, PO Box 13185-1678, Tehran, Iran. **Tel:** +98-21-66439463, **Fax:** +98-21-66919206
Email: alatifynia@sina.tums.ac.ir, swt_f@yahoo.com