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IMMUNE DIMORPHISM IN RELATION TO GENDER AND AGE

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ABSTRACT

Immune system is one of the crucial body systems that do not work in isolation and is affected by many factors like genetic, physiological and environmental. These factors tend to change the immune response due to the presence of sex hormone receptors on immune cells. The present study was done to determine the correlation of age, gender and immune status of normal healthy subjects leading to the basis of immune dimorphism. Serum samples of healthy male and female subjects were analyzed using following parameters- Total Leukocyte Count and Differential Leukocyte Count, Erythrocyte Sedimentation Rate and presence of free radicals- Nitrite levels and reduction of NBT dye. Results showed females had higher TLC, DLC, ESR as well as higher levels of free radicals as compared to males. But within females of two age groups i.e. cyclic and non- cyclic (menopausal females) variations in these values were observed. Levels of inflammation and immune response followed the trend: non cyclic females> cyclic females> males irrespective of age. It can be inferred from the study that gender and age affects the immune response with females showing a very strong immune response as compared to males.

KEYWORDS: Leukocyte profile, inflammation, nitrite levels, free radicals.

INTRODUCTION

Immune system is one of the crucial body systems that protect body against infections and it does not work in isolation. It is altered by many factors viz. genetic, physiological and environmental. From the literature it is apparent that immune system interacts with most of our body systems (Berczi and Nagy, 1998) and reproductive system is one of the most concerned factors Modulation of immune functioning and regulation of immune cells by sex steroids is related to the sex hormones i.e. estradiol, progesterone and testosterone (Yamaguchi *et al*., 2001). The interaction of reproductive system with that of immune system is attributed to the binding of sex hormones to the receptors on immune cells (Beato *et al*., 2011). The immune systems of males and females have important sex-based differences that depend on differential responses of immune cells to steroid hormones, such as estrogens, progesterone, and androgens. Humans show immunological variations throughout their life which are more prominent in females as than males (Kelly *et al*., 2008) as hormonal profile lead changes in immune response in a female during different reproductive phases (Bouman *et al*., 2004). This difference in hormone levels made females to show more vigorous immune response as compared to males leading to immune dimorphism (Ansar *et al*., 1985). Clinical studies show variations in leukocyte count with hormones (Apseloff *et al*., 2000) and in females by variations in cyclic phase (Madhura *et al*., 2014, Tikare *et al*., 2008). The present study was planned as the literature lacks the systematic study showing immune dimorphism in relation to gender and age.

METHODOLOGY

The study was conducted on 90 healthy subjects in the age group of 16-65 years and divided into following groups (Table 1).

Table1. Subjects divided on the basis of age and gender

F1	Females – cyclic (proliferative phase) 18-45 years of age
F2	Females non- cyclic 45-60 years of age
M1	Males – below 45 years of age
M2	Males – above 45 years of age

Subjects suffering from any health problem, taking drugs or hormone supplements, pregnant females and those that undergone ovariectomy was excluded from the study.

Blood samples were collected from different subjects with the consent of the subject according to the guidelines of Institutional Committee for Ethical

Clearance (ICEC) for which ethical clearance by **ICEC** has been given.

ICEC No. 149/DLS/HG and 150/DLS/HG
3 ml venous blood sample was withdrawn and put into vials with EDTA with their consent. The blood sample was subjected to following studies.

1. CELL VIABILITY TESTING
Tryphan blue staining of the blood samples was done distinguishing unstained viable cells from that of stained non-viable cells.

Calculation of cell viability
%Cell Viability = [Total Viable cells (Unstained) / Total cells (Viable +Dead)] X 100.

2. LEUKOCYTE PROFILE (Madhura, 2014)
To determine leukocyte profile of normal healthy subjects two measurements of white blood cells were done

1. Total number of white blood cells in (microliter) in blood
2. Percentage of each \type of white blood cells in blood sample known as differential cell count.

a. DLC (Differential leukocyte count)
Smear of blood stained with Leishman dye that stained the nucleus of WBCs was observed microscopically distinguishing cells from the morphology of nucleus.

b. TLC (Total leukocyte count)
Weak acid solution was added to blood sample that lyses red blood cells. Following adequate mixing, the stained specimen was introduced into a counting chamber where the white blood cells (leukocytes) in a diluted volume were counted.

Calculations
Cell number was counted according to the formula-
WBC per cu mm= **Average number of cells counted in four squares X Dilution factor**
Volume of chamber

3. DETERMINATION OF ERYTHROCYTE SEDIMENTATION RATE (ESR) BY WESTERGREN METHOD
ESR was performed by Westergren method as described by (Plebani, 2002).

WESTERGREN METHOD FOR ESR DETERMINATION
Anticoagulated whole blood was allowed to stand in a narrow vertical tube for a period of time. RBCs – under the influence of gravity - settled out from the plasma. The rate at which they settled was measured as the number of millimeters of clear plasma present at the top of the column after one hour (mm/hr).

4. SERUM NITRITE LEVEL
a. NITRITE STANDARD CURVE FORMATION
Griess reaction according to the modified method of Mathew, 1996 was employed to determine the nitrite levels. Griess reaction involves formation of a chromophere during the reaction of nitric oxide (NO_2) with sulfanilamide and heterocyclic amine of N-(1-napthyl) ethylenediamine (griess reagent) under conditions of low pH. During this reaction acidified nitrite undergoes diazotization with sulfanilamide to form diazonium salt. Diazonium salt then couples to N-(1-napthyl) ethylenediamine form a magenta colored compound with a characteristic absorption spectrum visible at 543nm.

Griess reagent
A. 0.2% (w/v) N-naphyleneethylenediamine
B. 2% (w/v) sulfanilamide prepared in 5% (v/v) ortho phosphoric acid.

Griess reagent- A:B - 1:1
Different concentrations of sodium nitrite ranging between 0.2 to 2 ppm and 10 to 100 ppm were prepared in PBS. 1:1 reaction mixture was prepared by adding each concentration and griess reagent. It was incubated at 37˚C for 10 minutes and absorbance was noted at 543nm followed by standard curve formation.

b. DETERMINATION OF SERUM NITRITE LEVEL
Determination of (NO) in serum was performed by the measurement of stable decomposition product nitrite (NO_2^-). Anticoagulated venous blood incubated for 5 mins at 37˚C was subjected to viability test by tryphan blue. Blood sample was centrifuged at 4000 rpm for 3 mins at 4˚C and serum was collected. Serum samples were subjected to griess reaction and absorbance was recorded at 543nm.

Calculations: Nitrite content was determined from the standard slope constructed from the known standard concentration of Sodium Nitrite with their corresponding absorbance value.

5. DETERMINATION OF THE PRESENCE OF FREE REDICALS IN BLOOD BY NITROBLUE TETRAZOLIUM DYE REDUCTION
The NBT reduction test was performed to measure the neutrophils function by modifying the method described by Hudson and Hay, 1989. Whenever a particle is ingested by a phagocyte, a respiratory burst is induced. The assay is based on the reaction that addition of yellow colour NBT dye to blood results in the formation of complex which can be phagocytosed by neutrophils and absorbance was measured spectrophotometrically at 520nm.

Calculation
% NBT reduction = **O.D of test (T) – O. D of control (C) X100**

O. D of control (C)

RESULTS

Prior to further analysis the percentage viability of blood cells was observed and was found to be 91.76%

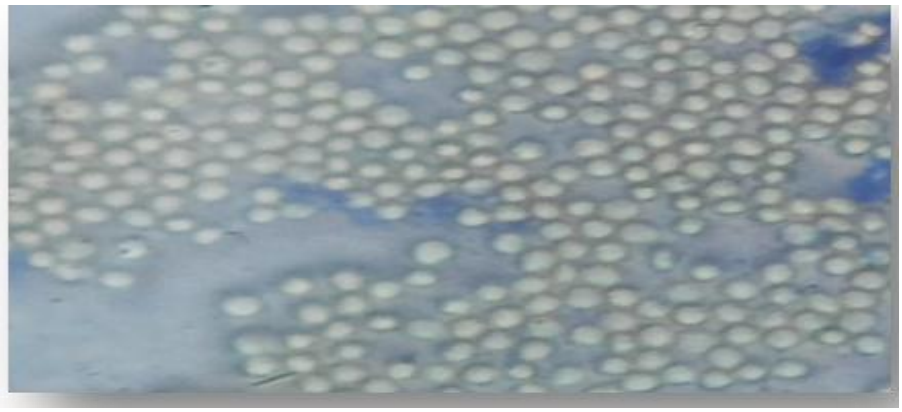

Fig. 1 Microscopic view of stained blood smear showing unstained cells as viable ones.

1. LEUKOCYTE PROFILE

a. TLC (TOTAL LEUKOCYTE COUNT)

White blood cells (leukocytes) in a diluted volume were counted and the results are shown in Table 2 and Figure 2.

Table 2. Total leukocyte count of different groups

Groups	Number of Subjects	TLC cells/micro lit	Standard Error
F1	25	**9743**	479.01
F2	25	8414	194.48
M1	20	5917	177.85
M2	20	5814	208.65

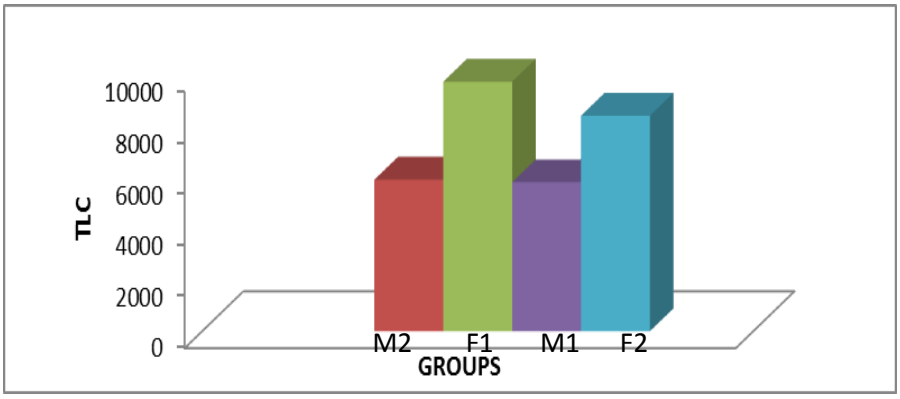

Figure 2 Total leukocyte counts of healthy males and females

Females of both age groups showed higher TLC as compared to males and within females of different age groups menstruating females were shown to have higher TLC.

b. DLC (DIFFERENTIAL LEUKOCYTE COUNT)

The results of differential leukocyte count in different groups are shown in Table 3, Figures 3 and 4.

Table 3 Differential leukocyte count of different groups

GROUPS	NUMBER OF SUBJECTS	NEUTROPHILS %	LYMPHOCYTES %	MONOCYTES %	EIOSINOPHILS %
F1	25	**63.43**	**31**	2.5	2.4
F2	25	**68.85**	**25.42**	3.15	2.57
M1	20	**60**	**29**	3	3
M2	20	**61.14**	**28.71**	3	2.4

- **GENDER BASED IMMUNE DIMORPHISM IN SUBJECTS OF SAME AGE**

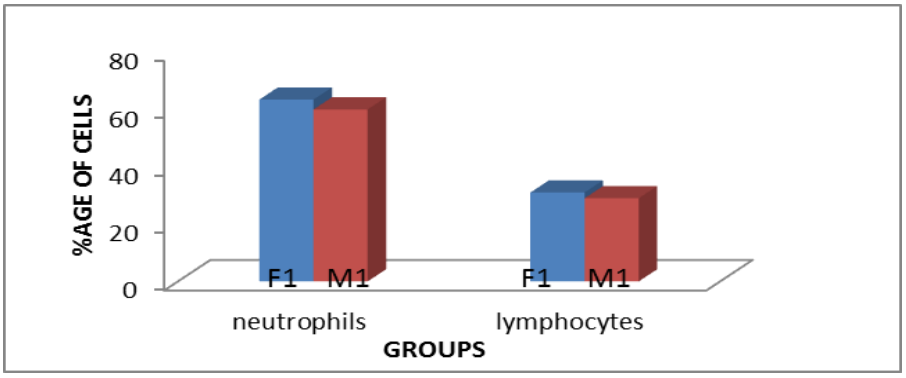

Figure 3 DLC in Subjects of age group 18-45 Years

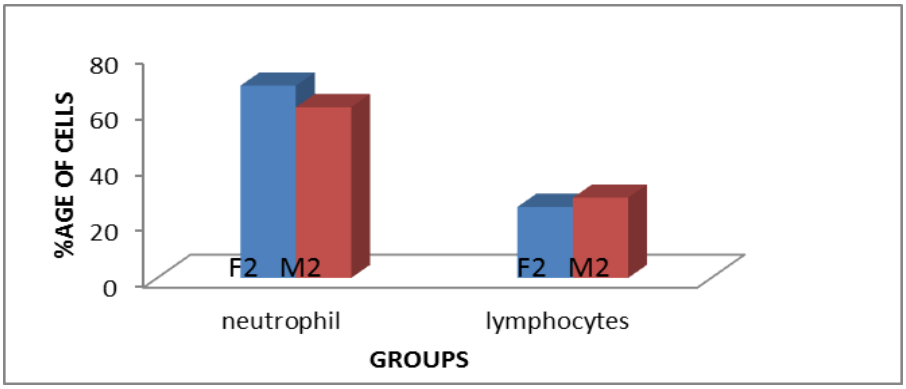

Figure 4 DLC in Subjects of age group 45-60 Years

In both the groups females have higher number of neutrophils and lymphocyte count as compared to males.

- **AGE BASED IMMUNE DIMORPHISM IN SUBJECTS OF SAME GENDER**

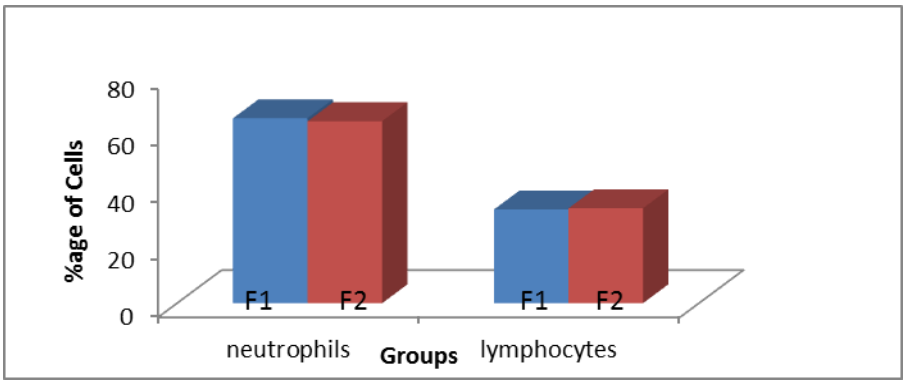

Figure 5 Age based immune dimorphism in females of different age group

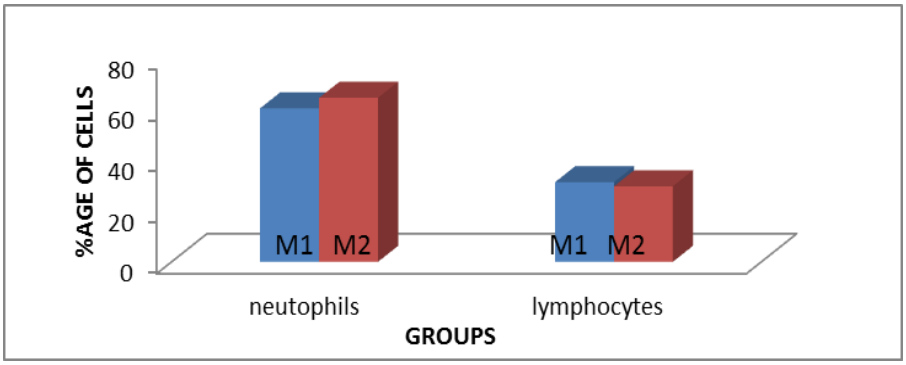

Figure 6 Age based immune dimorphism males of different age group

Non cyclic females showed higher neutrophils count and lower lymphocytes count as compared to cyclic females and there is slight difference in neutrophils and lymphocytes count in males with higher count in case of aged males.

2. ERYTHROCYTE SEDIMENTATION RATE DETERMINATION

The results of ESR are shown in table 4 figure 7.

Table 4 ESR of different age groups

Groups	Number of Subjects	ESR(mm/hour)
F1	25	11.68
F2	25	**17.8**
M1	20	7.85
M2	20	9.5

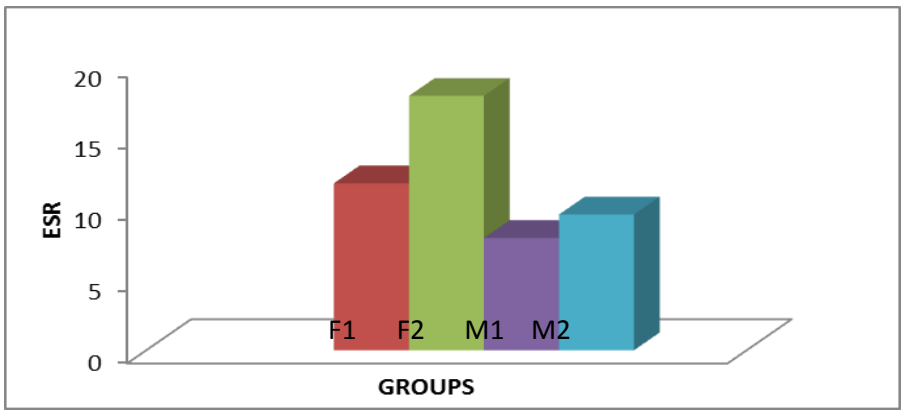

Figure 7 ESR of male and females of different age groups

Males of both age groups have lower ESR as compared to females and within females of menstruating females have lower ESR as compared to menopausal females

3. DETERMINATION OF SERUM NITRITE LEVELS

a. STANDARD CURVE FORMATION

Standard slope was constructed from the known concentrations of sodium nitrite with their corresponding absorbing values. The results are graphically represented in figure 8.

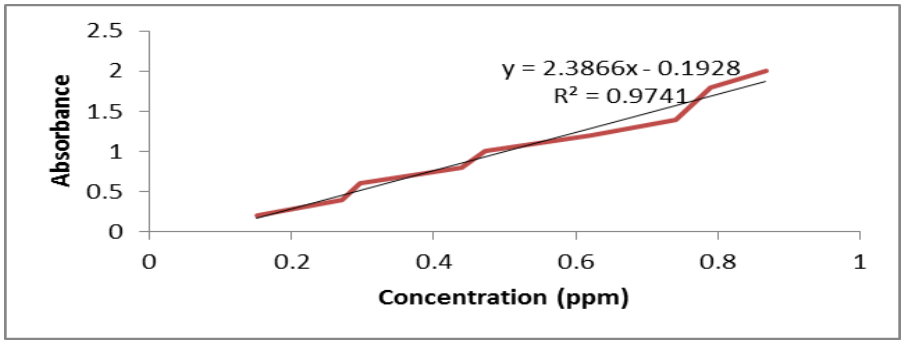

Figure 8 Standard curve for sodium nitrite

5. DETERMINATION OF NITRIC OXIDE SYNTHASE ACTIVITY FROM SERUM NITRITE LEVELS: Determination of (NO) in serum will be performed by the measurement of stable decomposition product nitrite (NO_2) employing the Griess reaction. The nitrite levels in healthy are shown in Table 5 and Figure 9.

Table 5 Serum nitrite level in subjects divided into different groups

Groups	Number of subjects	Serum nitrate level (ppm)	SD
F1	5	276.68	67.9
F2	5	**317.58**	75.21
M1	5	112.97	84.05
M2	5	132.38	19.16

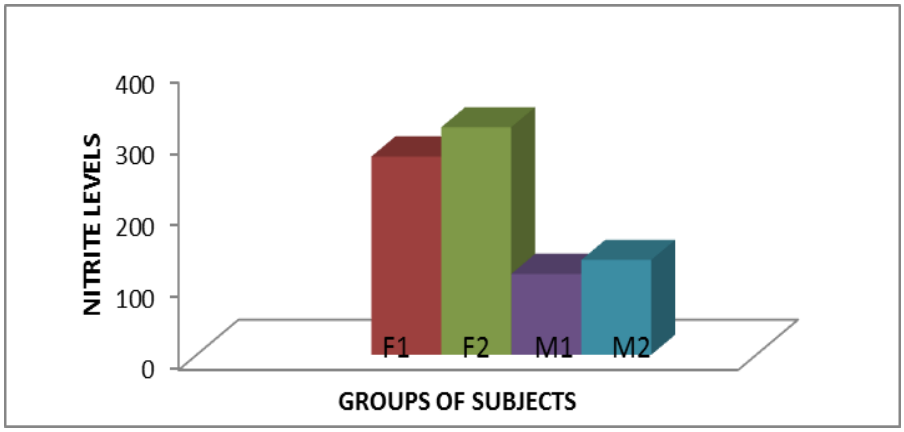

Figure 9 Serum nitrite level in subjects divided into different groups

Within healthy subjects non cyclic females have shown highest nitrite levels as compared to menstruating females and males of both the groups. But maximum levels of nitrates were observed in diabetic subjects.

6. DETERMINATION OF THE PRESENCE OF FREE REDICALS IN BLOOD BY NITROBLUE TETRAZOLIUM DYE REDUCTION

The NBT reduction was measured spectrophotometrically at 520 nm using dioxan as blank. Results are shown in Table 6 and Figure 10.

Table 6 Percentage of free radicals in blood by NBT reduction

Groups	Number of subjects	Percentage reduction	SD
F1	5	99.99	30.6
F2	5	184.17	62.4
M1	5	28.95	9.76
M2	5	40.29	7.68

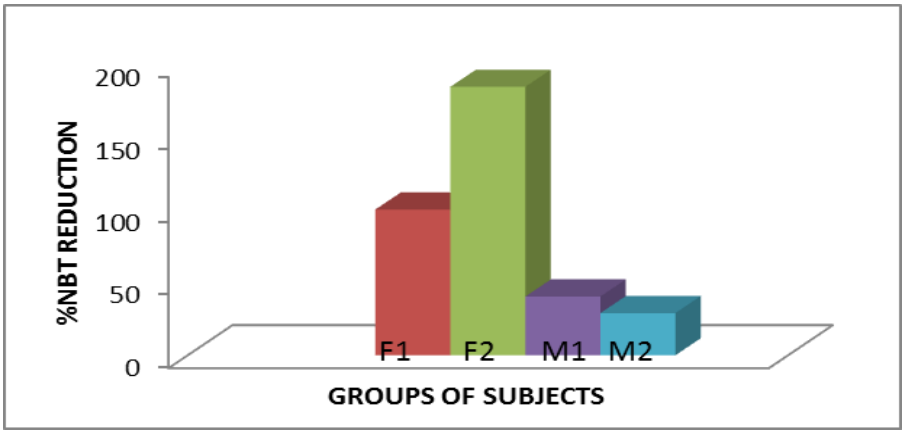

Figure 10 Percentage of free radicals in blood by NBT reduction

Presence of free radicals as inferred by the reduction of NBT dye is highest in diabetic subjects followed by healthy cyclic and non cyclic females. Healthy male subjects have shown least NBT dye reduction. Within healthy females the %age NBT dye reduction is higher in non cyclic females as compared to cyclic females.

As determined by 2 way ANOVA test all the results were statistically significant with P value <0.005.

DISCUSSION

The interaction of immune system with reproductive system results in physiological and biochemical changes in human body. This work was done to study the effect of age and gender on immune profile of normal healthy subjects leading to the basis of immune dimorphism. TLC, DLC, ESR, Serum Nitrite Levels and percent NBT dye reduction tests were performed to evaluate the immunological state of healthy subjects divided on the basis of sex and age.

Clinically relevant effect of hormones on the leukocyte count was observed in human subjects. The results were analyzed and comparison was made between different groups. Cyclic females in proliferative phase showed highest TLC count followed by menopausal females and males. Our study corroborates the findings of Bouman, 2004 and Kelly, 2006 where a significant high Total

WBC count in cyclic females was observed. The differential cell count results showed variations in females with higher lymphocytes count in cyclic females and higher fraction of neutrophils in postmenopausal females indicating inflammatory state in the later group of females. Apseloff, 2000 also reported that menstrual cycle affects the differential leukocyte count. ESR, a simple non-specific screening test for inflammation is higher in menopausal females indicating increase in inflammatory condition with aging in females compared to males. Hardly any study is available which shows the correlation of the effect of aging and sex on the levels of free radicals showing inflammatory conditions in body. In our study Nitric oxide (NO) levels and NBT reduction tests were carried out as a measure of free radicals. Nitric oxide is a short-lived signaling molecule that plays an important role in a variety of physiologic functions serves as a potent immunoregulatory factor following T cell activation. It is very unstable due to the presence of free electron and decay into nitrites and nitrates. The concentration of nitrite was measured in serum of all the subjects. Post menopausal females showed highest serum nitrite levels followed by cyclic females and then males. Human neutrophils contain mobile granules fusible with the cell membrane which plays important role in phagocytosis by producing reactive oxygen species. NBT dye particles acting as foreign particles are ingested and reduced into formazon complex that can be detected easily. NBT dye reduction has suggested maximum free radicals presence in menopausal females which might be responsible for the decrease in antioxidant capacity and in turn affects the immune response.

CONCLUSIONS

The results of the present study make us to conclude that there is a positive correlation of inflammation with age and gender leading to the immune dimorphism in the two sexes and different age groups. The effect of age on the immune response was more evident in the post menopausal females making the females more susceptible to inflammatory/autoimmune diseases.

CONFLICT OF INTERESTS

The author has no conflicts of interests.

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