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Apoptosis in Peripheral Blood Neutrophils in Patients with Active Tuberculosis: A Case Control Study

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ABSTRACT

Apoptosis is defined as programmed or controlled cell death which is often regulated. It is as important as cell production. This process is a form of cell suicide which occurs within individual cells. Apoptosis is induced in patients with active tuberculosis by activation of circulating polymorphonuclear neutrophils(PMN). To evaluate the Mycobacterium tuberculosis induced apoptosis in the peripheral blood neutrophils of patients with active tuberculosis. To compare the percentage of apoptotic neutrophils in tuberculosis versus normal controls. The present study included 40 cases of tuberculosis and 10 controls. The study included 4 groups: Group 1: Sputum positive TB. Group 2: Sputum negative, X-ray positive TB. Group 3: Extra pulmonary tuberculosis. Group 4: Healthy volunteers. Samples collected in EDTA tubes, centrifuged in PCV tubes, buffy coat smears stained with Leishman stain and accretion orange dyes, examined under oil immersion light microscopy and fluorescence microscope at a final magnification of X 1000. The percentage of apoptotic neutrophils were determined by counting the number of neutrophils showing features of apoptosis. The percentage of apoptotic neutrophils were increased in peripheral smears of patients with pulmonary and extra pulmonary tuberculosis. There was no significant increase in apoptotic neutrophils in peripheral smears of healthy volunteers. The Fishers Exact test was used for comparison between the groups and a 'p' value of <0.001 was considered statistically significant. Mycobacterium tuberculosis induces apoptosis in peripheral blood neutrophils. Increased circulating apoptotic neutrophils in patients with pulmonary and extra pulmonary tuberculosis correlated positively with disease activity when compared to healthy volunteers.

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Key Words

Apoptotic neutrophils, pulmonary, extra pulmonary tuberculosis, leishman stain, acridine orange

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INTRODUCTION

Apoptosis is a programmed cell death, which is well regulated. It can occur as a physiological or as a pathological process. It involves individual cells. The inflammatory response is regulated in the neutrophils by the process of apoptosis. When human neutrophils are infected with Mycobacterium tuberculosis (M-TB), rapid cell death occurs by apoptosis[1]. Thus, human neutrophils play a significant protective role in the acute phase of mycobacterial infection as they act as first line of defense mechanism encountered by the organisms. Intracellular growth of mycobacteria is thus limited by neutrophil apoptosis. Many studies have observed that the degree of neutrophil apoptosis is directly related to the bacillary load, which in turn helps in limiting the spread of tuberculosis[1]. Powerful mediators of inflammation are released by the neutrophils which display bactericidal responses causing injury to the endothelial cells and producing structural damages too[2]. Thus these cells should be removed from the inflamed site or else they may damage the tissues or progress to chronic inflammation[3]. In our previous study we observed apoptotic leucocytes where in infections accounted for 39.6%. Among these infections, pulmonary TB accounted for 25%[4]. Hence, we undertook this case control study to identify apoptotic neutrophils in patients with active tuberculosis and to ascertain the clinical significance of their presence. This study was undertaken to know whether chronic infections like tuberculosis accelerate the rate of apoptosis in neutrophils.

Aims of the Study:

- To evaluate the apoptosis in the peripheral blood neutrophils in patients with active tuberculosis.
- To compare the percentage of apoptotic neutrophils in tuberculosis versus normal controls.

Study Design:

Source of Data: The samples for the present study comprised of 50 cases of both control (10) and study group (40) were obtained from the RNTCP center at institution.

MATERIALS AND METHODS

Age and sex matched normal healthy volunteers were taken as controls. The patient population (study group) as well as controls included males and females, ranging from 1yr-70yrs. The samples were divided into 4 groups.

- **Group 1:** Sputum positive TB.
- **Group 2:** Sputum negative, X-ray positive TB.
- **Group 3:** Extra pulmonary tuberculosis.
- **Group 4:** Healthy volunteers.

The blood samples were collected from cases as well as controls using EDTA as an anticoagulant. Peripheral blood smears were made and stained with Leishman stain and Acridine Orange stains. Additionally, samples were taken into PCV tubes, centrifuged for 30 mins at 3000 rps in a centrifuge. Smears were made from buffy coat and stained with Leishman stain and Acridine Orange stain. Examined under oil immersion light microscopy followed by fluorescent microscope at a final magnification of X 1000. The percentage of apoptotic neutrophils were determined by counting the number of neutrophils showing features of apoptosis. Subsequently the buffy coat smears were also examined under fluorescence microscope for confirmation of apoptotic neutrophils.

The criteria used for detection of apoptosis was:

- Cell shrinkage.
- Nuclear cytoplasmic changes (peripheral condensation of chromatin along the nuclear membrane, nuclear fragmentation, formation of cytoplasmic blebs, membrane bound apoptotic bodies, cytoplasmic vacuoles)[4-6].

Apoptotic Neutrophils (ANs) Were Arbitrarilysemi Quantitated:

- Number/100 neutrophils-peripheral smear and acridine orange smears.
- Number/500 neutrophils-buffy coat smears.
- A finding of 1-3 apoptotic neutrophil in entire smear was considered as “few”, 4-5/smear as “moderate” and 6 or more/smear as “many”[4,6].

Statistical Analysis: We have used proportions and Fishers Exact test.A ‘p’ value <0.001 was statistically significant.

Inclusion Criteria:

- Pulmonary TB-only freshly diagnosed cases.
- Extrapulmonary TB-TB meningitis, cutaneous TB, Genitourinary and Gastrointestinal TB, TB lymph nodes, TB bone etc.

Exclusion Criteria:

- Patients on treatment for tuberculosis, old cases and treatment defaulters
- Patients with other co-existent infections like HIV, acute bacterial infections.
- Patients with underlying disease process - any malignancy.

Ethical Clearance: Informed consent was obtained from all healthy volunteers as well as from patients who participated in this study as per the Ethical Committee norms. Reports were dispatched to the patients.

RESULTS AND DISCUSSIONS

Our sample size was 50 cases (40 cases and 10 controls). The total leucocyte count ranged between

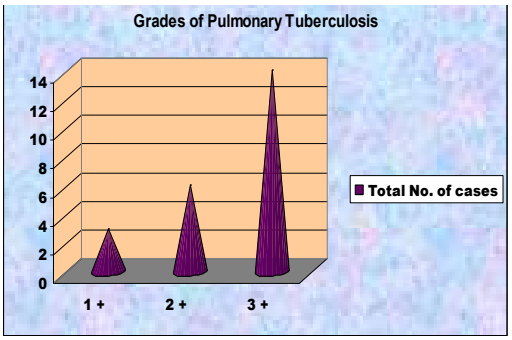
4,000-24,000/cmm. The patient population included 35 males and 15 females, ranging from 5yrs-75yrs of age.

Table 1: Age and Sex Distribution of Cases

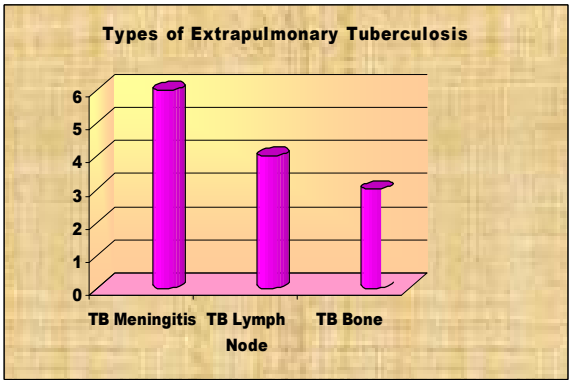
	Cases		Controls	
Age-yrs	**Males**	**Females**	**Males**	**Females**
1-10	3 (75%)	1 (25%)	-	-
11-20	3 (75%)	1 (25%)	1 (100%)	
21-30	5 (42%)	7 (58%)	2 (67%)	1 (33%)
31-40	6 (86%)	1 (14%)	-	2 (100%)
40 &>	11 (85%)	2 (15%)	4 (100%)	-
Total	28 (70%)	12 (30%)	7 (70%)	3 (30%)

Table 2: Group Distribution of Cases

Groups	**Total No. of Cases**
Pulmonary	
Group 1 (sputum+ve)	23 (46%)
Group 2 (X-ray+ve)	4 (8%)
EXTRAPULMONARY	
Group 3	13 (26%)
Healthy Volunteers	
Group 4	10 (20%)
Total	**50 (100%)**



Graph 1: Grades of Pulmonary Tuberculosis



Graph 2: Types of Extra Pulmonary Tuberculosis

Table -3: Semiquantitative Distribution of Apoptotic Neutrophils

Group 1(23 cases) (Sputum+)	**Few (1-3)**	**Moderate (4-5)**	**Many (>6)**	**Total**
1+	1(33%)	**2 (67%)**	-	3 (13%)
2+	-	**6 (100%)**	-	6 (26%)
3+	-	-	**14 (100%)**	14 (61%)
Group 2 (4 cases) (X-ray+)	2 (50%)	2 (50%)	-	4 (100%)
Group 3 (13 cases) (Extrapulmonary) TB Meningitis	-	**4 (67%)**	2 (33%)	6 (46%)
TB Lymph Node	-	**3 (75%)**	1 (25%)	4 (31%)
TB Bone	-	**3 (100%)**	-	3 (23%)
Group 4 (10 cases) (Healthy Volunteers)	4 (40%)	-	-	

Acridine orange stainedsmears were examined under fluorescence microscope. Apoptotic neutrophils with apoptotic bodies were easily identified as bright yellow fluorescence. Findings were consistent with the counts obtained on Leishman stain. Fishers Exact test showed significant difference between apoptotic neutrophils among cases when compared to controls with 'p' value <0.001. Mycobacterium tuberculosis (Mtb) is the causative agent of Tuberculosis. As per the India TB report 2019, the incidence of Tuberculosis is 27 lakhs[7]. Incidence in Karnataka is 6.2% with a death rate of 4%[8]. The burden of Tuberculosis is around 89% in the age group ranging between 15-69[7]. Our study also showed increased cases in the ages ranging between 20 and beyond. (**Table 1)** There was increased incidence of TB in males (70%) as compared to females (30%) in our study**. (Table 1)**. The most common site of tuberculosis are the lungs referred to as "Pulmonary" tuberculosis. If it affects any other organ it is known as "Extra pulmonary" tuberculosis. Our study included 27 cases (54%) of pulmonary tuberculosis. We divided them into Group 1 and Group 2. Group 1 was sputum positive which were 23 cases (46%) and Group 2 were sputum negative but X-ray positive-4 cases (8%). **(Table 2)**. The sputum positive smears (Group 1 cases) were stained with Ziehl Neelsen (ZN) stain and were graded into 1+, 2+ and 3+based on RNTCP guidelines for grading[9]. **(Graph 1)** The peripheral blood smears of these patients stained with Leishman as well as Acridine orange stains were examined for apoptotic neutrophils. We observed >6 (many) apoptotic neutrophils in grade 3+category which proved that greater the bacillary load greater the presence of apoptotic neutrophils. However grades 1+ and 2+ showed moderate apoptotic neutrophils (ANs). **(Table -3) (Fig.1 showing apoptotic neutrophils)** Pulmonary tuberculosis showed increased occurrence of apoptotic neutrophils in a study by Narasimha[7]. Extra pulmonary TB was around 13 cases (26%), in which majority was TB meningitis followed by TB lymph node and TB of the bone. **(Table 2)** The apoptotic neutrophils ranged from moderate to many in these cases. **(Table 3) (Graph 2)** However the number of apoptotic neutrophils were very few in healthy volunteer controls. In our study apart from Leishman stain which is routinely used for peripheral blood smears we also employed acridine orange stain to identify apoptotic neutrophils. To study more number of leucocytes we also used buffy coat smears followed by acridine orange staining and examination under fluorescent microscope. Apoptotic neutrophils with apoptotic bodies showed bright yellow fluorescence. **(Fig 2)**. A significant protective role is played by human neutrophils in acute phase of mycobacteria infection[10]. Infection can remain dormant. Active immune response occurs to prevent progression to active disease. Upon Mtb infection, the macrophage become anergic and polymorphonuclear neutrophils (PMNs) enter apoptosis[1]. Infected apoptotic PMNs act as potent activators of inflammatory response. They generate reactive oxygen

species (ROS)[11] which kills few bacteria and helps in containing infection till the macrophage accumulate[12]. They also release other mediators of inflammation such as cytokines and chemokines which aid in attracting other immune cells to the site of infection[10]. PMNs have a short life span which is cleared by neighbouring phagocytes. Mechanism resolves inflammation and modulates tissue regeneration[1]. Mtb induced apoptosis inPMNs is not dependant on phagocytosis of the bacteria. Certain factors such as inflammatory mediators[13], products released by the bacteria[14], hypoxic conditions[15] and expression of Fas/Fas ligands[16] are postulated to either promote or suppress apoptosis. A study by Perskvist *et al* showed that infection with Mtb promoted apoptosis in human neutrophils by activation of Caspase-3 as well as altering the expression of Bax/Bcl-xLvia an oxygen dependant pathway[1]. Another study by Aleman et al demonstrated involvement of Toll-like receptor 2 and p38 Mitogen Protein Kinase in triggering apoptosis in peripheral neutrophils in patients with active tuberculosis. They suggested two mechanisms for induction of TB-PMN. The first mechanism occurs with low ratio of bacteria to PMN when serum opsonins and M.tuberculosis are limited. Second mechanism takes places at places with high bacillary load such as lungs, wherein M. tuberculosis phagocytosis is due to enhanced apoptosis[17]. This was convincingly seen in our study too where in patients with 3+pulmonary tuberculosis had many apoptotic neutrophils in the peripheral blood smears.

CONCLUSION

Mycobacterium tuberculosis induces apoptosis in peripheral blood neutrophils. This represents an important host defense mechanism which is aimed at selective removal of infected neutrophils. Increased circulating apoptotic neutrophils were seen in pulmonary and extra pulmonary TB, correlated positively with disease activity. Immunofluorescence aided in confirmation of apoptotic neutrophils, can be used as an additional special technique for detecting apoptotic leucocytes.

Take Home Message: Peripheral blood smears should be reported with caution. A finding of 3 or >of apoptotic neutrophils should raise suspicion of ongoing infectious process. This can serve as a guide to the clinician in detecting any active disease process. However the findings should be correlated with adequate clinical details and other relevant investigations.

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