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Original Article

Effects of pneumonia and malnutrition on the frequency of micronuclei in peripheral blood of pediatric patients

Khalid I Elsayh[1], Douaa M Sayed[2], Asmaa M Zahran[2], Khaled Saad[1], Gamal Badr[3,4]

[1]Department of Pediatrics, Assiut University, Assiut 71516, Egypt; [2]Department of Clinical Pathology, South Egypt Cancer Institute, Assiut University, Assiut 71516, Egypt; [3]Princess Al-Johara Al-Ibrahim Center for Cancer Research, College of Medicine, King Saud University, Riyadh, Saudi Arabia; [4]Laboratory of Immunology & Molecular Biology, Department of Zoology, Faculty of Science, Assiut University, 71516, Assiut, Egypt

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Abstract: The aim of this study was to evaluate the effects of bacterial pneumonia and malnutrition on the frequency of micronuclei (MN) in peripheral blood of pediatric patients through flow cytometric analysis. The study was an analytical case-control study carried out on 35 malnourished children with bacterial pneumonia and 20 well-nourished children with bacterial pneumonia, in addition to 20 healthy children as controls. Complete physical examination including; anthropometric measurement, Chest roentgenograms were done for all cases. Assessment of MN was done by FACSCalibur flow cytometry. The frequency of micronucleated reticulocytes (MN-RETs) was higher both in the malnourished children with pneumonia and well-nourished children with pneumonia than the controls. Within the malnourished children with pneumonia, patients with kwashiorkor had more micronucleated mature erythrocytes (MN-RBCs) and MN-RETs than patients with marasmus. In conclusion: Pneumonia is associated with an increased frequency of MN and this increment is more pronounced in children with severe malnutrition especially kwashiorkor group.

Keywords: Pneumonia, malnutrition, micronuclei, flow cytometry, children

Introduction

Malnutrition is the most important underlying risk factor for childhood death and considered one of the most serious health problems in developing countries. Malnutrition includes a variety of clinical conditions such as kwashiorkor, marasmus, wasting, stunting, and micronutrient deficiencies. Malnutrition commonly affects infants and young children under 5 years [1, 2]. More than 77 million children are born every year in the 36 countries with the highest burden of malnutrition (21 of these countries are in Africa, 13 in Asia and two in Latin America). Of these children, about 7.4 million die before the age of three years and a further 0.6 million die between the ages of three and five years [2, 3]. The major problems of protein energy malnutrition (PEM) include mortality and morbidity, for example, pneumonia, diarrhea and impaired thermoregulation. Also PEM in children may affect the adult size, intellectual ability and increase the risk of metabolic disorders and cardiovascular diseases [3]. PEM is almost exclusively an important problem of children in Egypt. Stunting was high but constant at 23% between the years 2000 and 2005 and it increased to 29% by 2008. Among the children that are stunted 50% were severely stunted. Children 18-23 months old had the highest levels of stunting (41%) with levels being slightly higher for boys. Wasting and underweight in the under 5 year olds were much less prevalent at 7% and 6% respectively [4]. Malnutrition can be classified as moderate or severe. Moderate malnutrition (MAM) is defined as a weight for height Z score (WHZ) between two and three standard deviations (SDs) below the mean. Severe malnutrition (SAM) is defined as a WHZ of more than three SDs below the mean, or a mid-upper arm circumference (MUAC) of less than 115 mm, or the presence of nutritional edema. MAM or SAM without bilateral pitting edema is termed *marasmus*. In the presence of bilateral pitting edema, the term *kwashiorkor* is used [5-7].

The increased incidence and severity of infections in malnourished children is largely due to the deterioration of immune function; limited production and/or diminished functional capacity of all cellular components of the immune system [8]. Pneumonia is one of the most important causes of death in children under the age of five years in developing countries. Up to two-thirds of malnourished children that are hospitalized are diagnosed with pneumonia; generally, the etiologic agent is S. pneumoniae. Despite the availability of antibiotics, mortality and morbidity rates remain high. Pneumonia is common in malnourished children and is frequently associated with fatal outcome, especially in children younger than 24 months of age. Studies consistently reported a two- to three fold greater risk of mortality in cases with pneumonia associated with malnutrition. Therefore, pneumonia and malnutrition are two of the biggest killers in childhood diseases [8-12].

The immune system, in response to infection, first consumes the innate and then subsequently acquired host defense functions. Both processes involve activation and propagation of immune cells and synthesis of an array of molecules requiring DNA replication, RNA expression, protein synthesis and secretion and therefore consume additional anabolic energy. Mediators of inflammation further increase the catabolic response. Consequently, the nutritive status of the host critically determines the outcome of infection [13]. During cell division the genetic material (nuclear DNA) is replicated and divided equally resulting in two identical daughter cells. As replication errors, double-strand DNA breaks and mitotic spindle apparatus dysfunction may result in unequal distribution of the genetic material or chromosomal loss, which produces daughter cells with abnormal DNA and extra-nuclear chromatin. When a chromosome is lost, the genetic material is excluded and not correctly incorporated into the daughter cell nucleus; the daughter nuclei that are missing genetic information are smaller than the primary nucleus and are termed *micronuclei* (MN), which easily visible under the optical microscope. The detached genetic material may be derived from whole chromosomes or, more frequently, of acentric chromosome fragments, they are excluded from the nuclei of new mitotic cells during anaphase. MN that result from the loss of an entire chromosome are termed aneugenic, while MN that are missing chromosomal fragments and do not have centromeres are termed clastogenic [14-16]. The relationship between malnutrition and genetic damage has been got an attention in both human and animal models studies. The MN assay may be useful in detecting chromosome damage induced by several factors [16].

The aim of this study was to evaluate the effects of bacterial pneumonia and malnutrition on the frequency of MN in peripheral blood of pediatric patients through flow cytometric analysis.

Materials and methods

The study was approved by the Faculty of Medicine Ethic Committee for the Scientific Research Conduct, Assiut University, Assiut, Egypt. An informed written consent in accordance with Assiut University Ethical Committee guidelines was taken from guardians of all cases and controls.

Patients

The target population was children aged 2-60 months who were attending Pediatric Hospital, Assiut University, Egypt. This study was an analytical case-control study carried out on 35 malnourished children with bacterial pneumonia and 20 well-nourished children with bacterial pneumonia admitted to our hospital. The study also included 20 healthy children as controls. The mean age of first group (malnourished children) was 14.39±6.89 months; they were 16 boys (46%) and 19 girls (54%). Twenty six patients (74%) were marasmic; 14 patients had MAM with WHZ between two and three standard deviations (SDs) below the mean and 12 patients had SAM with WHZ of more than three SDs below the mean, and/or a MUAC of less than 115 mm. The rest of the malnourished group (9 patients; 26%) had kwashiorkor. Among the malnourished children, 14 were breast fed (40%), 15 patients (43%) were artificially fed and 6 (17%) were fed both. The clinical signs and symptoms of malnutrition, as well as weight, height deficits, MUAC and Z score were used to determine the type and severity of malnutrition according to the established values [5-7].

The mean age of the second group (well-nourished children with bacterial pneumonia) was

14.22±5.64 months; they were 8 boys (40%) and 12 girls (60%). Eight patients (40%) were artificially fed and 12 (60%) were breastfed. Diagnosis of bacterial pneumonia was based on established clinical criteria; {fever, cough, chest wall in-drawing and/or difficult breathing and tachypnea (respiratory rate >50 cycles / minute in infants 3 to 12 months old; >40/min in children 12 to 60 months old), lobar or bronchopneumonic consolidation demonstrated by X-ray and positive microbiological tests as blood culture [17-19].

The control group included 13 females and 7 males, with a mean of 12.80±5.94 months. They come to outpatient's clinic in our hospital for routine care. All had normal anthropometric measurements for age and sex.

We exclude patients with age less than 2 months or more than 5 years, patients with bronchial asthma and children with wheezing whose indrawing resolves after bronchodilator therapy, patients with viral pneumonia, and patients with history of prematurity or low birth weight and patients with recent hospitalization. Also we exclude blood samples from patients with negative microbiological tests for bacteria. All cases were subjected to the following data collection or examination.

History: Thorough history including age, sex, duration of symptoms, immunization status and developmental milestones.

Clinical examination: Complete physical examination including; anthropometric measurement (weight and height) of the children were measured to determine their nutritional status. Weight was measured in kg (to the nearest 100 grams) using an electronic digital scale and its accuracy was periodically verified using reference weights. Length was measured in cm (measured to the nearest mm) using a pediatric measuring board. Children were measured in a recumbent position. We used a software program for assessing growth and development of the children to make comparisons to the reference standards [20]. The software program combines the raw data on the variables (age, sex, length and weight) to compute a nutritional status index such as weight-for-height, weight-for-age and height-for-age.

Chest roentgenograms: Posteroanterior view in older children and anteroposterior view in infants and a lateral view were taken, to document the presence of infiltrates or consolidation.

Collection of blood samples and processing: Blood samples were collected by the researchers themselves at the first day of admission before any medical treatment. The complete blood count was done on Celltac E automated hematology analyzer (Nihon Khoden Corporation, Tokyo, Japan).

Flow cytometric assessment of micronuclei: The blood samples were collected in heparinized tube. Sample staining was performed as proposed by Dertinger et al and Cervantes-Ríos et al [21-23]. The peripheral blood was diluted (1:2) in BBS (bicarbonate-buffered saline solution: 0.9 g NaCl + 0.0444 g $NaHCO_3$ in 100 mL distilled H_2O) at pH 7.5. 100 µL of the diluted sample were removed and fixed by vigorous shaking in cryogenic tubes containing 2 mL of ultra cold (-70°C) methanol. The samples were stored at -70°C for at least 24 h prior to staining for further analysis. The samples were removed from the freezer and resuspended. One mL was removed, washed with BBS at 4°C and centrifuged at 600 × g for 5 min at 4°C. The pellet was resuspended and 25 µL were aliquoted into three polypropylene tubes that contained 80 µL of RNase (1 mg/mL, *from Glory science Company, TX, USA*). The first tube was used to measure the auto fluorescence of the cells. In the second tube, 5 µL of anti-IgG1-FITC was added. In the third tube, we added 5 µL of anti-CD71-FITC to label the reticulocytes (RETs) and 5 µL of anti-CD61-PE to identify platelet aggregates. All monoclonal antibodies were purchased from Becton Dickinson Biosciences (*San Jose, California, USA*). The three tubes were incubated in the dark at 4°C for 40 minutes and then at room temperature for 90 minutes. After incubation, 5 µL of propidium iodide (fluorescent DNA staining dye) which used to detect micronuclei was added. The sample was analyzed by FACSCalibur flow cytometry with Cell Quest software (Becton Dickinson Biosciences). A total of 500,000 events were acquired per sample. The gating strategy was performed as shown in **Figure 1**. The following erythrocyte populations were detected; mature erythrocytes, micronucleated mature erythrocytes (MN-RBCs), reticulocytes (RETs) and micronucleated reticulocytes (MN-RETs).

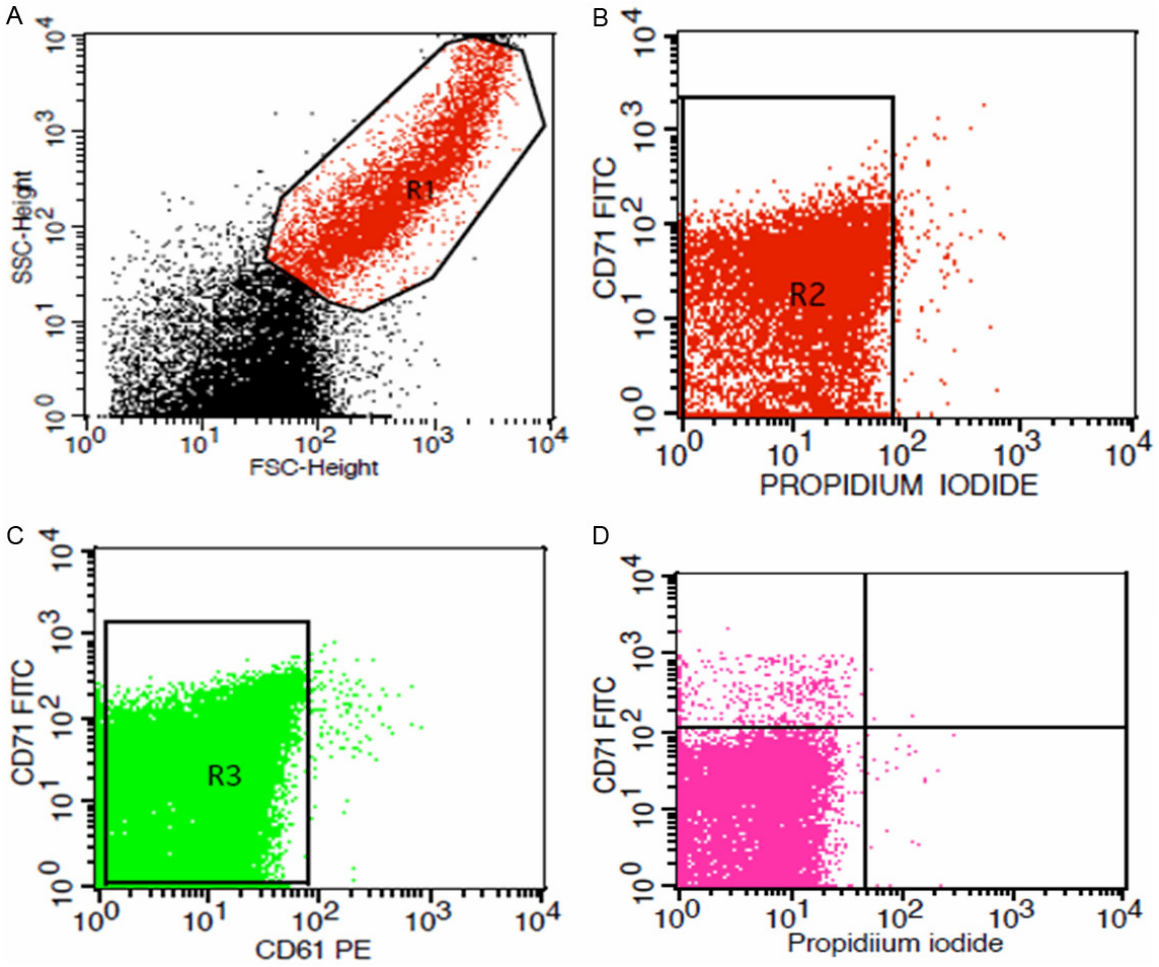

Figure 1. Flow Cytometric Assessment of Micronuclei. A: Forward and side scatter histogram was used to define the gate (R1) that excluded events corresponding to subcellular-sized particles. B: R2 gate was used to exclude nucleated cells based on their high PI expression. C: R3 gate was used to exclude cells that express platelet-specific antigen, CD61. D: Representative plot of erythrocyte populations: in the lower left quadrant, mature erythrocytes; lower right quadrant micronucleated mature erythrocytes (MN-RBCs), upper left quadrant, reticulocytes (RETs) and upper right quadrant micronucleated reticulocytes (MN-RETs).

Statistical analysis

Data analysis was done by statistical package for social sciences (SPSS), version 16. All data were expressed as the mean ± SD of mean. Differences between the patients and the controls were examined for statistical significance using One Way ANOVA. A P value of ≤0.05 denoted the presence of a statistically significant difference. Pearson correlation coefficient was used to examine the correlations among different studied parameters.

Results

Baseline characteristics of the patients and the controls were shown in **Table 1**. Malnourished children with pneumonia had significantly lower serum protein and albumin levels compared to the well-nourished children with pneumonia and the controls. No significant difference in serum protein and albumin between well-nourished children with pneumonia and controls.

The frequencies of RETs, MN-RETs and MN-RBCs in the patients and the controls were shown in **Table 2**. As regard the RETs frequencies; the well-nourished children with pneumonia had a significant increase in the frequency of RETs compared to the malnourished children with pneumonia and the controls. The frequency of RETs was not significantly different between the malnourished children with pneu-

Table 1. Baseline characteristics of patients and the controls

Item	Malnourished children with pneumonia (35)	Well-nourished Children with pneumonia (20)	Controls (20)	*P*-value[1]	*P*-value[2]	*P*-value[3]
Age (months)	14.39±6.89	14.22±5.64	12.80±5.94	NS	NS	NS
Six (male/female)	16/19	8/12	7/13	NS	NS	NS
Weight (kg)	5.87±1.56	10.07±1.79	10±1.57	0.000	0.000	0.990
WBCS (10^9/L)	12.03±1.78	15.89±5.87	6.22±1.28	0.000	0.000	0.000
Hemoglobin (g/dL)	7.1±2.08	8.71±1.31	12.16±0.91	0.003	0.000	0.000
Platelet count (10^9/L)	140.27±54.89	284.55±145.26	234.50±62.07	0.000	0.001	0.193
Protein (g/dL)	4.77±1.68	8.03±0.96	7.77±0.66	0.000	0.000	0.797
Albumin (g/dL)	2.23±0.4	4.04±0.59	4.00±0.61	0.000	0.000	0.964

[1]Malnourished children with pneumonia vs. controls. [2]Well nourished children with pneumonia vs. controls. [3]Malnourished children with pneumonia vs. well nourished children with pneumonia. Data are presented mean ± SD. P≤0.05 is significant. WBCs: white blood cells; NS: non significant.

Table 2. Frequency of MN-RETs, MN-RBCs and reticulocytes in malnourished children with pneumonia, well nourished children with pneumonia and controls

Frequency	Malnourished children with pneumonia (35)	Well nourished children with pneumonia (20)	Control group (20)	*P*-value[1]	*P*-value[2]	*P*-value[3]
MN-RETs	2.13±0.78	1.45±0.37	0.41±0.21	<0.001	0.004	0.001
MN-RBCs	0.32±0.19	0.16±0.09	0.03±0.02	<0.001	0.424	0.001
RETs	1.95±0.73	5.17±2.51	2.08±0.40	0.949	<0.001	<0.001

[1]Malnourished children with pneumonia vs. controls. [2]Well nourished children with pneumonia vs. controls. [3]Malnourished children with pneumonia vs. well nourished children with pneumonia. Data are presented mean ± SD. P≤0.05 is significant. MN-RETs: micronucleated reticulocytes; MN-RBCs: micronucleated mature erythrocytes; RETs: reticulocytes.

Table 3. Frequency of MN-RETs, MN-RBCs and reticulocytes in children with SAM, MAM and kwashiorkor with pneumonia

Frequency	kwashiorkor (9)	SAM (12)	MAM (14)	*P*-value[1]	*P*-value[2]	*P*-value[3]
MN-RETs	2.66±0.82	1.92±0.46	1.49±0.38	0.019	0.001	0.306
MN-RBCs	0.44±0.20	0.25±0.08	0.19±0.14	0.011	0.003	0.730
RETs	1.39±0.51	2.11±0.41	2.74±0.57	0.003	<0.001	0.028

[1]kwashiorkor vs. SAM. [2]Kwashiorkor vs. MAM. [3]SAM vs. MAM. Data are presented mean ± SD. P≤0.05 is significant. MN-RETs: micronucleated reticulocytes; MN-RBCs: micronucleated mature erythrocytes; RETs: reticulocytes; MAM: moderate malnutrition; SAM: severe malnutrition.

monia and the controls. Within the malnourished group; the frequency of RETs was significantly lower in patients with kwashiorkor than patients with marasmus (MAM & SAM). Also RETs was significantly lower in patients with SAM than MAM (**Table 3**).

The frequency of MN-RETs was higher both in the malnourished children with pneumonia and well-nourished children with pneumonia than the controls. At the same time, the frequency of MN-RETs was also higher in malnourished children with pneumonia than well-nourished children with pneumonia. Malnourished children with pneumonia had higher frequencies of MN-RBCs compared to the well-nourished children with pneumonia and the controls. While the frequencies of MN-RBCs in well nourished children with pneumonia were higher than the controls, but the difference was not statistically significant (**Table 2**). Within the malnourished children with pneumonia, patients with kwashiorkor had more MN-RBCs and MN-RETs than patients with marasmus (MAM & SAM). The frequency of MN-RBCs and MN-RETs was not significantly different between patients with MAM & SAM (**Table 3**). No significant correlation was found between the frequency of MN-RBCs and MN-RETs with age.

Discussion

Protein energy malnutrition in children is a public health problem especially in developing countries. PEM is clearly related to a higher mortality of hospitalized children, with death being due mainly to overwhelming infections despite the fact that many of these children receive appropriate antimicrobial therapy [3, 24]. Pneumonia is a common and potentially serious infection that affects children throughout the world. The annual incidence of pneumonia in children younger than 5 years of age is 34 to 40 cases per 1000 in Europe and North America. In the developing countries, pneumonia is not only more common than it is in Europe and North America; it is also more severe and fatal [8, 18].

Previous studies in human beings and laboratory animals have assessed the effect of malnutrition and infections on genetic damage. Several studies have reported that PEM and respiratory tract infections exert a number of changes at the cytogenetic level; including: increased frequency of chromosomal aberrations, sister chromatid exchange, micronuclei and DNA damage [23-28]. But we noticed that studies in children were few in number, also the assessment of PEM depend on old parameters and primitive tools with very old classifications, in addition; the previous studies described respiratory tract infections as a broad term without specific diagnosis, which lacked the radiological and microbiological diagnosis. Also MN frequency in these studies was measured mainly in lymphocytes, buccal epithelium and bone marrow cell cultures and rarely in reticulocytes and mature RBCs [23-28]. In our study we adjusted these defects by using recent solid international rules in diagnosis & classifications of PEM and pneumonia [5, 7, 9, 19, 20]. We used flow cytometry to assess the effects of bacterial pneumonia and PEM on the frequency of chromosome breaks and abnormal chromosome segregation identified as MN in peripheral blood of pediatric patients.

Our results showed higher frequencies of MN-RETs and MN-RBCs in the malnourished children with pneumonia than well-nourished children with pneumonia and the controls. In addition the frequencies of MN-RETs and MN-RBCs were significantly higher in patients with kwashiorkor than patients with marasmus (SAM and MAM). This may indicate that malnutrition can cause DNA damage and this damage is correlated with the severity of malnutrition. The increased frequency of MN-RETs in well-nourished children with pneumonia than the controls in our study may indicate that bacterial pneumonia also leads to DNA damage and MN release, and this damage is aggravated in the presence of malnutrition. Our data are consistent with Cervantes-Ríos et al [23]; who reported that the MN-RETs and MN-RBCs frequencies in malnourished infected patients were higher than well-nourished infected and well-nourished uninfected groups and the frequencies were increased in well-nourished infected than well-nourished uninfected. Padula et al [27]; studied the structural chromosomal aberrations in peripheral blood lymphocytes of 25 PEM children (mean age, 22 months) and found that the chromosomal aberration frequency was significantly higher in malnourished than in well-nourished children (7 times higher in malnourished infants than in controls. Padula and Seoane [28]; studied the effects of bacterial infections on structural chromosomal aberrations in malnourished and well-nourished children and found that structural chromosomal aberration was higher in infected malnourished than infected well-nourished children and the infected well-nourished children showed higher, but insignificant differences than non infected well-nourished. Gonzalez et al [24]; observed significant increments of DNA damage assessed by comet assay in malnourished infected versus healthy infected children under medical treatment, suggesting that malnutrition enhance drug susceptibility inducing DNA damage. The same result was observed by Betancourt et al [26]. In addition, Ortiz et al [25]; showed that there was an increase in the frequency of mitomycin C induced MN in cells from malnourished infected children as compared to well-nourished children. Ortiz et al [29]; evaluated the effect of malnutrition on the frequency of spontaneous and mitomycin C-induced micronuclei in the peripheral blood of malnourished rats and well-nourished uninfected rats and showed that malnourished rats have a high frequency of MN-RETs.

The DNA damage in the cells of malnourished children may be due to the severe deficiency of essential micronutrients as zinc and iron which required for the synthesis of DNA. The role of folate and vitamin B12 deficiency as a risk fac-

tor for increased MN frequency has been well established. Also the deficiency of the essential amino acids in malnutrition; can cause a decrease in protein synthesis (which is found among our malnourished children); and reduce the production of enzymes required for DNA repair and may alter DNA duplication [23, 24, 30]. González et al [31], found a reduction in the ability to repair DNA damage in lymphocytes of malnourished children over the controls.

DNA damage that associated with pneumonia may be caused by reactive oxygen species and free radicals formed during infection which increased oxidative stress and cause oxidative damage and DNA breakage. Leukocytes and other phagocytic cells combat bacteria by destroying them with a powerful oxidant mixture as NO, O_2^-, H_2O_2, and OC_1^-. These oxidants protect humans from immediate death from infection but cause oxidative damage to DNA and mutation. Also some bacterial enzymes can act as DNases, and thus cleave the host cell DNA. The oxidative damage is more obvious in malnourished patients [32-34]. Moreover, it has been proposed that cells from malnourished children would be more susceptible to environmental damage [25]. So; micronuclei release is more marked in malnourished children with pneumonia than in well-nourished children with pneumonia.

In our study; the increased frequency of RETs in well-nourished children with pneumonia than in the controls may suggest that the infection could increase the number of RETs in the peripheral blood. Our result is in accordance with Cervantes-Ríos et al [23]. Jackson et al [35], detected an increased number of RETs in the spleen during salmonella infection. The frequency of RETs in malnourished children with pneumonia was significantly lower than in well-nourished children with pneumonia. This frequency was also lower in malnourished children with pneumonia than in the controls, but the difference was not statistically different. This could indicate that PEM is associated with bone marrow suppression which reduces RETs production. The infection enhances RETs production in malnourished children which compensate for reduced RETs production induced by PEM. The frequencies of RETs in patients with kwashiorkor were lower than in patients with marasmus (SAM & MAM), and the frequency in patients with SAM was lower than in patients with MAM. This result was in consistent with el-Nawawy et al [36] who reported that the RETs index was significantly lower in infants with PEM than controls. Borelli et al [37], evaluated the effect of PEM on some aspects of hemopoiesis in Swiss mice and reported that malnourished animals presented anemia, reticulocytopenia and leucopenia. The hemopoietic tissue, present a high rate of renewal and cellular proliferation and has a high demand for nutrients. The need for protein by the process of hemopoiesis justifies the occurrence of anemia and leucopenia and decrease in the number of RETs occurred in malnourished patients [38].

In conclusion; pneumonia is associated with an increased frequency of MN and this increment is more pronounced in children with severe malnutrition especially kwashiorkor group. Data presented herein support the hypothesis that the frequency of MN in human peripheral blood circulation can be used to index recent chromosomal damage induced by bacterial pneumonia and malnutrition.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Khaled Saad, Department of Pediatrics, Faculty of Medicine, Assiut University, Assiut 71516, Egypt. Tel: +20-100-608-0182; Fax: +20-88-236-8371; E-mail: ksaad8@yahoo.com

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