

Neutrophils phagocytic function in chronic myelogenous leukemia after imatinib mesylate therapy

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Abstract:

Introduction: Chronic myelogenous leukemia (CML) is a pluripotential stem cell disease defined by the consistent presence of Philadelphia (Ph1) chromosome, in more than 95% of patients. In chronic phase of CML the functional abnormalities of neutrophils are mild, this functional abnormality possibly because these neutrophils produced from abnormal stem cell. Nitro blue tetrazolium (NBT) dye reduction test a semiquantitative method that test the defects related to oxidative burst and superoxide generation.

The aims of the study: To evaluate the phagocytic function of neutrophils by NBT test in normal control and compare it to that of CML patients after imatinib mesylate therapy, to correlate between the absolute number of neutrophils and the percentage of NBT positive neutrophils in CML patients after imatinib mesylate and to find out if there is any correlation between the percentage of NBT positive neutrophils and the percentage of (Ph1) positive cells.

Patients, material and methods: Fifty CML patients were included in this study all of them were on imatinib mesylate therapy. Group 1: thirty six patients were not in complete cytogenetic remission. Group 2: fourteen patients were in complete cytogenetic remission. Control group: Thirty apparently healthy, medication free volunteers matched for age and sex.

The Nitro blue tetrazolium dye reduction test was performed by slide method, blood film and complete blood count were also performed.

Results: The patient ages were ranged from 15 to 77 years with mean age of 45.3 ± 14.9 , with male to female ratio of 1.43:1. The NBT percentages in control group were ranged from 3 to 9 with mean percentage of 5.8 ± 1.6 , in group 1 patients were ranged from 0 to 4 with mean percentage of 1.28 ± 1.2 , while in group 2 were ranged from 3 to 6 with mean percentage of 4.46 ± 1 . There was a significant difference between the control group and group 1 patient ($p=0.000$) while there was no significant difference between control group and group 2 patients ($p=0.06$). There was no significant correlation between the absolute neutrophils count and the percentage of NBT positive neutrophils in both groups of patients (1 and 2) with p value of 0.23 and 0.1 respectively. In 26 patients the results of FISH showed different percentage of cells positive for (ph1) chromosome ranged from 4 to 90% with average of 48.3% and there was a significant inverse correlation between the percentage of NBT positive neutrophils and the percentage of cells containing (Ph1) chromosome at the 0.01 level (Pearson Correlation = -0.769).

In conclusion: the neutrophils function in CML patients after therapy varies according to (Ph1) chromosome conversion and complete cytogenetic remission is associated with normal neutrophils function while the presence of Ph1 chromosome positive cells associated with defective neutrophils phagocytic activity.

Keywords: chronic myeloid leukemia, CML, nitroblue tetrazolium test, NBT, imatinib mesylate.

Introduction:

Chronic myelogenous leukemia (CML) is a pluripotential stem cell disease characterized by anemia, marked granulocytosis and granulocytic immaturity, basophilia, of-

ten thrombocytosis, and splenomegaly. It is defined by the consistent presence of translocation t (9; 22) (q34; q11) in more than 95% of patients, also termed the Philadelphia chromosome (Ph1), a translocation which causes a BCR/ABL rearrangement. This translocation most commonly produces a fusion protein, termed p210, with increased tyrosine kinase activity.

Chronic myelogenous leukemia (CML) is the most common chronic myeloproliferative disorders (CMPD) accounts for approximately 15-20% of all cases of leukemia and ap-

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proximately 3 percent of all childhood leukemias. It can occur at any age, although most cases are seen between 50 and 60 years, rarely below the age of 20 years (1), less than 10 percent of all cases occur in subjects between 1 and 20 years old. The disease is slightly more predominant in male; the age-adjusted incidence rate is approximately 2.0 per 100,000 persons for men and approximately 1.1 per 100,000 persons for women. The incidence around the world varies by a factor of approximately twofold. (2, 3, 4, 5).

The natural history of the disease is biphasic or triphasic disease so the initial chronic phase, either changes to an acute blastic phase or, more commonly, evolves into an accelerated phase that later progress to a blastic phase which is refractory to therapy. (2, 5).

In chronic phase of CML the usual clinical presentation is with splenomegaly, hepatomegaly, symptoms of anemia, and systemic symptoms such as sweating and weight loss.

In 15-20% of patients the diagnosis is incidental, made when a blood count performed for an unrelated reason (6). Very rarely CML has developed in a patient with a pre-existing Ph1-negative myeloproliferative disorder. (7)

The peripheral blood usually shows anemia and leucocytosis with a very characteristic differential count with predominant myelocyte and the mature neutrophil. Earlier granulocyte precursors are also present but promyelocytes are fewer than myelocytes and blasts are fewer than promyelocytes. (8)

After a variable period in chronic phase, usually several years, CML undergoes further evolution. There may be an abrupt transformation to an acute leukemia, designated blast transformation, or there may be an intervening phase of accelerated disease (9).

According to WHO classification the criteria for accelerated phase are : (i) myeloblasts constituting 10–19% of peripheral blood white cells or bone marrow nucleated cells; (ii) peripheral blood basophils 20% or more of nucleated cells; (iii) persistent thrombocytopenia (platelet count $<100 \times 10^9/l$) that is not a result of treatment or persistent thrombocytosis (platelet count $>1000 \times 10^9/l$) that does not respond to treatment; (iv) increasing WBC and increasing spleen size that does not respond to treatment; (v) cytogenetic evolution; or (vi) marked granulocyte dysplasia or prominent proliferation of small dysplastic megakaryocytes in large clusters or sheets, while the criteria suggested for recognition of blast transformation are: (i) myeloblasts constituting at least 20% of peripheral blood white cells or bone marrow nucleated cells; (ii) extramedullary proliferation of blast cells; or (iii) large aggregates and clusters of blasts in bone marrow biopsy sections .(10)

The introduction of imatinib mesylate is a major advance in the management of patients with CML this drug decrease the cells bearing the translocation t(9;22) (leukemic cells) to the lowest level , under this conditions normal (polyclonal) hematopoiesis is restored, (11,12) ,the efficacy of therapy is judged by measuring hematologic response , cytogenetic response including Major cytogenetic and Complete cytogenetic response and molecular response including a Major

molecular and Complete molecular response. (12, 13, 14) Hematologic response achieved when white cell count $<10 \times 10^9/L$, platelet count $<450 \times 10^9/L$, no immature myeloid cells in the blood, and no signs and symptoms related to leukemia for at least 4 weeks. In major cytogenetic response less than 35% of cells containing the Ph chromosome by cytogenetic analysis of marrow cells ,while in Complete cytogenetic response no cells containing the Ph chromosome by cytogenetic analysis of marrow cells .In Major molecular response Blood cell BCR-ABL/ABL ratio $<0.05\%$ and in complete molecular response Blood cell BCR-ABL levels undetectable (5)

The use of classical cytogenetic technique in monitoring minimal residual disease is limited this is because it needs a satisfactory bone marrow biopsy to assess metaphases and also its poor sensitivity, but the application of fluorescence in situ hybridization (FISH) can increase sensitivity tenfold and considered the most sensitive, specific, and reliable test (15) that can be carried out on metaphase preparations or on cells in interphase so it is especially important for cells of patients with leukemia to monitor the response to therapy. (16)

By FISH the Chromosomes can be stained and visualized, the technique is dependent on the recognition of specific DNA sequences by means of a fluorescent probe that can anneal to a specific DNA sequence. (17)

The Functional abnormalities of neutrophils (adhesion, emigration, phagocytosis) in chronic phase of CML are mild and compensated by high neutrophils count, so do not predispose patients to infections by the usual or opportunistic organisms. (18, 19) This functional abnormality possibly because these neutrophils produced from abnormal stem cell containing abnormal chromosome (20, 21)

Since imatinib mesylate therapy decrease the cells bearing the translocation t (9; 22), therefore the study of neutrophil function after imatinib mesylate therapy could be of value in follow up CML patients after therapy.

In this study an evaluation of phagocytic function of neutrophils was by Nitro blue tetrazolium dye reduction test a semiquantitative method that test the defects related to oxidative (respiratory burst) and superoxide generation .The oxidative burst in the neutrophils is deficient due to a defect in one of the components of the nicotinamide adenine dinucleotides phosphate oxidase (NADPH) system, reflecting the inability to generate superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hypochlorite (OCL^-),so the neutrophils are unable to kill microorganisms.(18)

The aims of the study are:

1. To evaluate the phagocytic function of neutrophils by NBT test in normal control and compare it to that of CML patients after imatinib meyselate therapy, to find out if there is any change in the neutrophil phagocytic function in CML patients after imatinib mesylate therapy (in patients not in complete cytogenetic remission and in CML patients in complete cytogenetic remission)
2. To correlate between the absolute number of neutrophils and the percentage of NBT positive neutrophils in CML pa-

tients after imatinib mesylate therapy (in patients not in complete cytogenetic remission and in CML patients in complete cytogenetic remission) .

3. To find out if there is any correlation between the percentage of NBT positive neutrophils and the percentage of (Ph1) positive cells that measured by fluorescence insitu hybridization (FISH) technique in CML patients not in complete cytogenetic remission

Patients, Material and Methods:

Patients groups:

Fifty CML patients were randomly selected during the year 2012; all of them were on imatinib mesylate therapy and were diagnosed by hematology specialist in the national center of hematology (NCH), on the bases of clinical status, peripheral blood, bone marrow findings and cytogenetic analysis in some patients.

According to the results of FISH which had been done in a private laboratory, the patients were classified into 2 groups: Group 1: thirty six patients were not in complete cytogenetic remission (26 patients were in hematological remission, 8 patients were in chronic phase, 2 patients were in accelerated phase)

Group 2: fourteen patients were in complete cytogenetic remission

Control group:

Thirty apparently healthy volunteers matched for age and sex were studied as control

Sampling

Four milliliter of peripheral blood samples were obtained, 3ml in ethylene diamine tetra-acetic acid (EDTA) anticoagulant tubes were used for complete blood count and blood film, and 1ml of blood was obtained in glass tubes using heparin as anticoagulant in a concentration of 75-100 units/ml for nitroblue tetrazolium test.

Nitroblue tetrazolium test:

In this test neutrophils ingest the dye, nitroblue tetrazolium, and in the presence of reactive oxygen species, the yellow colored NBT compound is converted to the purple-blue formazan compound (22). The NBT test was performed within one hour after specimen collection, according to method of Park et al (23) with some modification (24).

Reagents:

1. Phosphate buffered saline solution pH 7.2,

2. NBT (US Biologics) 0.2 % NBT solution was prepared by dissolving 200 mg of NBT in 100 cc of phosphate buffered saline solution. (25, 26, 27, 28)

NBT solution then filtered into dark bottle .This solution is stable at room temperature for several weeks (28)

3. Counter stain (Leishman's stain)

Methods:

1. Approximately 0.1 of heparinized blood was transferred into a second plastic tube and mixed with equal amount of NBT solution.(29).

2. The mixture was incubated at 37 °C for 10 - 15 minutes and subsequently kept at room temperature for additional 10 - 15 minutes

3. At the end of this period, the solution mixed gently and blood smear was done . When the smears were dried they were stained with Leishman's stain as a counter stain.(25)

Method of cells count:

Smears were examined under the microscope with oil immersion and a total of 100 neutrophils for controls group and 500 neutrophils for patients group were counted. Only those with large blue black deposits were classified as NBT positive neutrophils. The results were reported as the percentage of NBT positive neutrophils according to the method of Park et al. (23)

Statistical analysis:

All values were expressed as mean \pm SD. Comparison between control and each group were performed using two-tailed Student's t-test and were considered significant if the corresponding P value was lower than 0.05. Correlations were assessed by calculation of Pearson's correlation coefficient.

The results:

The patients were 29 male and 21 female with a male to female ratio of 1.43:1.

The patient ages were ranged from 15 to 77 years with mean age of 45.3 ± 14.9 .

The NBT percentages in control group were ranged from 3 to 9 with mean percentage of 5.8 ± 1.6 .

The NBT percentage in group 1 patients were ranged from 0 to 4 with mean percentage of 1.28 ± 1.2 , while in group 2 were ranged from 3 to 6 with mean percentage of 4.46 ± 1 .

There was a significant difference between the control group and group 1 patient ($p=0.000$) while there was no significant difference between control group and group 2 patients ($p=0.06$) as shown in table 1.

Table 1: Comparison of mean percentage of nitroblue tetrazolium test (NBT) in control group, group 1 and group 2 patients

groups	Number of cases	Mean percentage of NBT test	SD	(Significance (p value
control	30	5.8	1.6	
Group 1	36	1.28	1.2	0.000
Group 2	14	4.46	1	0.06

The absolute neutrophil count in group 1 patients was ranged from 1.17 to 46.8 ×10⁹/L with an average count 6.16 ×10⁹/L, while in group 2 patients was ranged from 1.12 to 5.04×10⁹/L with an average count 3.55 ×10⁹/L

There was no significant correlation between the absolute neutrophils count and the percentage of NBT positive neutrophils in both groups of patients (1and2) with Pearson Correlation of - 0.23 and 0.1 respectively as shown in table 2

Table 2: The correlation between the absolute neutrophils count and the percentage of nitroblue tetrazolium positive neutrophils in group 1 and group 2 patients.

groups	Number of cases	Absolute neutrophils count ((mean×10 ⁹ /L	Absolute neutrophils count ((range×10 ⁹ /L	Pearson Correlation
Group 1	36	6.16	46.8 - 1.17	0.23 -
Group2	14	3.55	5.04 - 1.12	0.1

In group 1 patient there were 26 patients had results of FISH showed different percentage of cells positive for (ph1) chromosome ranged from 4 to 90 % with an average of 48.3% and there was a significant inverse correlation between the

percentage of NBT positive neutrophils and the percentage of cells containing (Ph1) chromosome by using FISH technique at the 0.01 level (Pearson Correlation =-0.769) as shown in table 3.

Table 3: The Correlation between The percentage of nitroblue tetrazolium test (NBT) positive neutrophils and the percentage of cells containing Philadelphia (Ph1) chromosome by using fluorescence insitu hybridization (FISH) technique

		NBT	FISH
% NBT	Pearson Correlation	1	**769-.-
	(Sig. (2-tailed		001.
		N	26

** Correlation is significant at the 0.01 level (2-tailed).

Discussion:

In this study the CML cases occur most frequently between the ages of 15 to 77 years with mean age of 45.3 ± 14.9, only 3 patients (6%) were below the age of 20, which is relatively similar to other reports. (1, 2, 3, 4, 5, 24, 30).

The disease occurs slightly more often in men than women with a male to female ratio of 1.43:1 and this ratio was almost comparable to the ratio reported by other studies. (1,2,5,24,30,31,32,33) in which the disease affect males more than females in a ratios ranged from 1.3: 1 (3) up to 2:1(30) such differences in ratio could be attributed to different numbers of studied cases in different studies.

The phagocytic function of neutrophils was investigated by means of nitroblue tetrazolium dye reduction test and the normal range of NBT positive neutrophils were ranged from 3 to 9% with mean percentage of 5.8 ±1.6 in normal controls, similar results were reported by applying the method of Park etal (23), other studies mentioned a range from 3-10%(24), 1-11%(34), and 2-8%(35).

Many studies were conducted to test the neutrophils phagocytic function by NBT test in patients with CML and these studies revealed significant lower percentages of NBT positive neutrophils in CML patients. (36, 37, 38, 39)

Only few studies were conducted on CML patients following treatment. This study regarded the first one in Iraq to study

the phagocytic function of neutrophils in CML patients after imatinib mesylate therapy.

In CML patients not in complete cytogenetic remission ,the NBT test was significantly lower than control (p=0.000) , such a result could be explained by the fact that morphologically mature neutrophils that are originated from cytogenetically abnormal stem cells (carrying ph1 chromosome) are functionally defective, while in patients in complete cytogenetic remission there was no significant difference in NBT test than control (p=0.06) because neutrophils originated from normal stem cells had a normal phagocytic function comparable to normal control (13,14). Similarly Ajdary S. etal study on 9 CML patients in remission show normal values of NBT test in remission group and impaired phagocytic function of neutrophils in 21 CML patients in chronic phase and 7 in blastic phase. (40)

Kasimir –Bauer etal study on neutrophil phagocytic activity in CML patients treated with alpha interferon revealed that there was no significant difference between the phagocytic activity of alpha interferon treated CML patients with (Ph1) chromosome positive compared to untreated CML , in contrast to neutrophils of all three patients with (Ph1) chromosome conversion exhibited phagocytic activity within the range of healthy control. (41)

Enhanced some of neutrophils functional defect in CML patients after alpha interferon therapy was also reported (42),

but Bdad-Alpdgan et al showed that all CML patients treated with alpha interferon had an increased skin reaction to needle prick, and this reaction may reflect altered neutrophil activities. (43)

In this study there was no significant correlation between the absolute neutrophils count and the percentage of NBT positive neutrophils in both groups of patients (1and2) with p value of 0.23 and 0.1 respectively. Some studies revealed a poor correlation between the absolute number of neutrophils in patients with CML treated by INF- α and the percentage of NBT positive neutrophils (24),also after busulphan treatment the change in neutrophil count did not correlate with the raise in NBT positive neutrophils.(44)

Other studies revealed inversed correlation between phagocytic activity and leucocyte count or the percentage of immature cells.(40,45)

A significant inverse correlation was reported between the percentage of NBT positive neutrophils and the percentage of cells containing (Ph1) chromosome by using FISH technique

at the 0.01 level (Pearson Correlation =-0.769), such correlation may be explained by the fact that the lower percentage of cells containing Ph1 chromosome mean an increased normal progenitor cells that produce morphologically mature and functionally normal neutrophils and thus a higher NBT positive neutrophils.

In conclusion, the data presented in this study demonstrate that neutrophils function in CML patients after therapy varies according to (Ph1) chromosome conversion and complete cytogenetic remission is associated with normal neutrophils function while the presence of Ph chromosome positive cells associated with more neutrophils with defective phagocytic activity. So NBT test could be of value as indirect test to assess the response to therapy and it is recommended to assess the phagocytic activity of neutrophils before starting treatment and follow up the patients after hematological, partial and complete cytogenetic and molecular remission, and using flowcytometry to obtain more precise results.

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الوظيفة البلعمية لخلايا العدلات في مرضى ابيضاض الدم النقوي المزمن بعد العلاج بعقار (امانتب مسليت)

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كلية الطب – الجامعة المستنصرية – فرع الامراض و الطب العدلي

الخلاصة:

المقدمة: ينشأ ابيضاض الدم النقوي المزمن من خلايا جذعية متعددة المكامن ويتميز بوجود كروموسوم فلادلفيا في اكثر من 95% من المرضى ويعد هذا المرض الاكثر شيوعا من بين اضطرابات النقوي المزمن اذ يشكل نسبة 15-20% من حالات ابيضاض الدم. يكون التاريخ الطبيعي للمرض تنائي الطور او ثلاثي الطور. في الطور المزمن يحصل خلل بسيط في وظائف العدلات قد يعزى الى كونها ناشئة من خلايا جذعية غير طبيعية. يعتبر فحص نايترو بلو تترازوليوم فحص شبه كمي لوظائف العدلات البلعمية المتعلقة بالاكسدة.

الاهداف: تقييم وظائف العدلات باستخدام فحص نايترو بلو تترازوليوم في العينة الضابطة و مقارنتها مع وظائف العدلات في مرضى ابيضاض الدم النقوي المزمن بعد العلاج بعقار (امانتب مسليت) الذين حصلوا على شفاء خلوي وراثي تام والذين لم يتم شفائهم الخلوي الوراثي و للربط بين العدد المطلق للعدلات و نسبة العدلات الايجابية لفحص نايترو بلو تترازوليوم وكذلك اثبات ان كان هناك ارتباط بين نسبة الخلايا الايجابية لكروموسوم فلادلفيا و نسبة الخلايا الايجابية لفحص نايترو بلو تترازوليوم.

المرضى وطرائق العمل: شملت العينة 50 مريضه من مرضى ابيضاض الدم النقوي المزمن جميعهم تمت معالجتهم بعقار (امانتب مسليت) وقد تم تقسيمهم وفقاً لنتائج تقنية التهجين الموضعي المشع الى مجموعتين هما : المجموعة 1: لم يتم شفائهم الخلوي الوراثي والمجموعة 2 : حصلوا على شفاء خلوي وراثي تام مع عينة ضابطة من 30 متطوعا من الاصحاء المطابقة للعمر و الجنس . تم اجراء فحص نايترو بلو تترازوليوم بطريقة شبة كمية بطريقة الشريحة للمجموعات اعلاه مع صورة دم و تعداد دم كامل .

النتائج: تراوحت اعمار المرضى 15 حتي 77 سنة مع متوسط العمر 45.3 ± 14.9 . كانت نسبة الذكور الى الإناث 1: 1.43. تراوحت النسبة المئوية لفحص نايترو بلو تترازوليوم في المجموعة الضابطة 3 حتي 9% مع نسبة متوسط قدرها 5.8 ± 1.6 . وفي مجموعة المرضى 1 تراوحت من 0-4% مع نسبة متوسط قدرها 1.28 ± 1.2 . وفي مجموعة المرضى 2 تراوحت من 3-6% مع نسبة متوسط قدرها 4.46 ± 1 . هناك فارق معنوي كبير بين المجموعة الضابطة ومجموعة المرضى 1 في حين لم يكن هناك فارقا معنويا بين المجموعة الضابطة ومجموعة المرضى 2. لم يكن هناك ارتباط كبير بين عدد العدلات المطلق و نسبة العدلات الايجابية لفحص النايترو بلو تترازوليوم في كلتا المجموعتين من المرضى . أظهرت نتائج تقنية التهجين الموضعي المشع في 26 مريضاً نسب مختلفة من الخلايا الايجابية للكروموسوم فلادلفيا تراوحت من 4 إلى 90% مع معدل 48.3% وكان هناك علاقة عكسية بالغة الدلالة بين نسبة العدلات الايجابية لفحص نايترو بلو تترازوليوم و نسبة الخلايا الايجابية لفحص كروموسوم فلادلفيا (ارتباط = -0.769).

الاستنتاج: ترتبط الوظيفة البلعمية للعدلات في مرضى سرطان الدم النقوي المزمن بعد العلاج بنسبة الخلايا الايجابية لكروموسوم فلادلفيا حيث كانت الوظائف البلعمية للعدلات طبيعية في المرضى الذين حصلوا على شفاء خلوي وراثي تام و بذلك قد يكون فحص وظائف العدلات طريقة غير مباشرة لتقويم استجابة المرضى للعلاج وخاصة اذا تم استخدام تقنية مقياس جريان الخلايا (فلوسايتوميتر) و التي تعد الطريقة الاسرع و الأكثر دقة لمتابعة المرضى قبل البدء بالعلاج و بعده.