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Original Article

Oncology Section

Alterations in the Reactive Oxygen Species in Peripheral Blood of Chronic Myeloid Leukaemia Patients from Northern India

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ABSTRACT

Introduction: There is a significant difference in the Reactive Oxygen Species (ROS) levels of Chronic Myeloid Leukaemia (CML) patients before and during treatment with Tyrosine Kinase Inhibitors (TKIs). This is because high ROS levels support oncogenic phenotype of CML by inducing proliferation pathway and accumulation of further genetic mutations. Often the measurement is done on WBC or serum for ascertaining one type of ROS species, but measurement of global ROS in fresh whole blood will give more accurate estimation of ROS.

Aim: To measure global ROS in peripheral blood of CML patients.

Materials and Methods: A case control study was undertaken to measure ROS in peripheral blood of CML patients from Northern India. CML patients on TKIs (n=40 on imatinib herein called treated) and untreated (n=17, who were not on any TKIs or alternative medicine, called as treatment naive) and 52 healthy controls were also enrolled. Chemiluminescent assay was carried out using luminol as signal enhancer in 400 µl of blood to measure ROS. The chemiluminescence was measured as Relative Light Units (RLU)/sec/10^4 WBC. Data was presented in terms of mean±SE or geometric mean (95% Confidence Interval) for continuous variables and percentage for categorical variables. Groups were compared using two sample t-test for continuous variables and chi-square test for categorical variables.

Results: The WBC profile and ROS levels of patients taking TKIs were quite similar and showed no significant difference ($p<0.999$) compared to healthy controls. In contrast, significant increase was observed in the ROS levels of CML patients not on TKIs (untreated) compared to patients under treatment ($p<0.029$) and healthy controls ($p<0.007$). We also observed that the absolute ROS values and WBC counts were higher in untreated patients compared to patients on TKIs and healthy controls, even though mean ROS value was less.

Conclusion: To ascertain the alterations in ROS levels of CML patients before and during treatment with TKIs, it is better to measure global ROS in fresh whole blood by chemiluminescent method using luminol. Luminol assay is a quick, easy and inexpensive method to measure global ROS. Patient under treatment with TKIs show significant decrease in ROS levels almost similar to the levels measured in healthy controls yet the mechanisms by which this decrease occurs needs to be elucidated.

Keywords: Luminol assay, Tyrosine kinase inhibitors, White blood cells

INTRODUCTION

Reactive Oxygen Species (ROS) are a heterogeneous group of free radicals generated naturally in cellular metabolism from diatomic oxygen. Physiologically, they have a diverse range of functions, ranging from antimicrobial defence in myeloid cells to cell cycle progression, cell motility and growth factor signalling in normal cells [1]. At low levels, ROS maintains the pool of Haematopoietic Stem Cells (HSC), therefore HSC are often located in deep hypoxic pockets of bone marrow [2]. Increased levels of ROS pull them out of their quiescent phase, switches them to decreased self-renewability and increased proliferation which may eventually lead to bone marrow failure [3,4]. Thus, role of ROS in normal cells is dose dependent [5,6]. If ROS levels increase beyond a threshold (a state called oxidative stress), it can damage cellular proteins. High levels of ROS can induce double strand DNA breaks and cause lipid peroxidation that may eventually lead to apoptosis and/or even necrosis of the normal haematopoietic cell [5-8]. This oxidative stress has been distinctly implicated in several pathological states like cardiovascular disorders, neurodegenerative diseases like Parkinson's disease, Alzheimer's disease [9] and in different types of cancers like melanoma [10], prostate carcinoma [11], acute lymphoblastic leukaemia [12], myelodysplastic syndrome [13] and chronic and acute myeloid leukaemias [14]. In CML ectopic expression alone of BCR-ABL fusion gene causes elevated ROS levels [15] and has the potential to transform myeloid cells [16]. The BCR-ABL fusion gene is a result of balanced translocation of ABL gene from chromosome 9 to BCR region of chromosome 22, t(9;22)(q34.1;q11.2). The resultant minute 22nd chromosome, called Philadelphia chromosome, codes for a fusion oncoprotein; a constitutively expressed tyrosine kinase which is diagnostic of CML [17,18]. The pathways modulated by this oncoprotein results in clonal myeloproliferative neoplasia which is found to be associated with elevated levels of ROS [19]. The exact mechanism of how ROS sustains a neoplastic phenotype is not clear however, several reports indicate that increase in ROS promotes cell survival [20,21], migration and metastasis [22,23], proliferation [24] and may contribute to drug-resistance [25]. Nevertheless, there is limited information about the association of ROS in CML patients both in treated and untreated patients. Hence, a systemic analysis of ROS levels using luminol as stimulator in whole blood of CML patients from Northern India was undertaken. Variations in ROS levels of untreated patients and patients under treatment (healthy subjects) were analysed to understand the impact of drug treatment in CML patients. To the best of our knowledge, this is the first case control study to measure global ROS in peripheral blood of CML patients from Northern India.

MATERIALS AND METHODS

A case control (n=109) study was undertaken to measure ROS in peripheral blood of CML patients from Northern India. Patients (n=57) with confirmed CML diagnosis visiting haematology OPD of All India Institute of Medical Sciences, New Delhi, India (from August

2015 till September 2016) were considered after informed consent. Healthy controls with matched age and gender (n=52) were also enrolled. Since there is no preceding study we have assumed that 85% of patients visiting the clinician would show altered ROS levels in their blood. Therefore, sample size of 49 subjects will be needed after assuming prevalence of 85% (subjects who will report altered ROS) with 10% precision and 5% level of significance.

Out of 57 patients 17 were untreated (who were not on any TKIs or alternative medicine, called as treatment naïve) and 40 patients were taking 400 mg of imatinib (TKI) once daily (duration of treatment varied from one to nine years). The mean age of the patients was 38.04±1.37 with 39 males (68.42%) and 18 (31.58%) females. Detailed history of the patients was recorded including regional background, eating habits, family and past illness. A pedigree chart was also drawn to see if any family history of leukaemia was present.

Inclusion criteria: Patients with confirmed diagnosis of CML based on smear positivity (WHO guidelines) [26] and Real Time PCR for BCR-ABL were included.

Exclusion criteria: Patients suffering from any other haematological disorder or other malignancy or any previous history of systemic illness were excluded.

Inclusion criteria for controls: Healthy individuals without any systemic illness or chronic conditions were included.

Ethical Clearance

The study was approved by the Institutional Ethical Committee of All India Institute of Medical Sciences (letter No: IEC/NP-404/2013) and Dr BR Ambedkar Centre for Biomedical Research (letter No: F-50-2/Eth.com/ACBR/11/2106).

Collection of Blood Samples

A total of 3 ml blood was collected by venepuncture from CML patients and controls. Immediately, 0.5 ml blood was transferred into heparin vials (BD Biosciences, USA) for ROS estimation while 2.5 ml was transferred to EDTA vial for measurement of blood parameters – total erythrocyte count (RBC count) and Total Leukocyte Count (TLC). Peripheral blood samples were drawn in the morning between 10 am-12 noon. The patients (those under treatment) had consumed their daily dose of imatinib (400 mg once daily) before the sample was drawn.

ROS Estimation

ROS levels were measured in 400 µl of whole blood using luminol (Sigma-Aldrich) enhanced chemiluminescence method [27] with minor modifications. For each sample, initially readings of whole blood (400 µl) without luminol were taken for 10 minutes with one second pulse. This was followed by addition of 10 µl of luminol to the tube. The readings were again recorded in luminometer (Sirius, Berthold Detection systems GmbH, Pforzheim, Germany, Sirrus K software with single electrode luminometer) for 10 minute with one second pulse. The readings taken in whole blood without luminol (negative control) were subtracted from readings with luminol to remove background chemiluminescence if present. H_2O_2 (400 µl, Sigma-Aldrich) was used as a positive control. The average of 10 minutes could be taken for estimation of ROS but it was observed that in controls as well as in patients ROS values reached a peak between two to four minutes of addition of luminol and started to decline after five to six minutes. Therefore, for all experiments average ROS was calculated using readings from two to six minutes. The chemiluminescence so obtained was expressed as Relative Light Units (RLU)/sec/10^4 WBC. Since ROS was measured in 400 µl of blood, total WBC in 400 µl was also calculated from the TLC/µl readings for each patient. The final RLU values were normalized against 10^4 WBC instead of routine adjustment against an absolute neutrophil count [27]. Heparin vials were chosen to collect blood for ROS measurement as blood stored in EDTA vials show significantly less chemiluminescence presumably because EDTA sequesters many charged moieties [27].

STATISTICAL ANALYSIS

Demographics and disease characteristics were compared using descriptive statistics. Data was presented in terms of mean±SE or geometric mean (95% confidence interval) for continuous variables and percentage for categorical variables. Groups were compared using two sample t-test for continuous variables and chi-square test for categorical variables.

ROS and WBC parameters were non-normally distributed; therefore, log-transformed data were used for the comparison. Non normality of data was confirmed using Shapiro-Wilks test. Log transformed data were presented as geometric mean (95% CI) and two sample t-test for two group comparisons and Bonferroni test for the multiple group comparison were used. The correlation between level of significance was set at 0.05. All statistical analyses were conducted using STATA (Version 10.0; STATA Corp).

RESULTS

Out of 109 subjects enrolled for ROS estimation, fifty seven were patients with confirmed diagnosis of CML and fifty two were healthy controls. Amongst these, 98.16% (n=107) subjects were from urban background while 1.83% (n=2) were from rural background. Fifty four (54) out of 57 patients reported in chronic phase, two in accelerated phase (one relapsed case) and one in blast crisis. Pedigree chart analysis and family background showed none of the subjects had a family history of leukaemia. Out of fifty-seven patients, seventeen were untreated and forty were treated with TKIs (imatinib) with minimum dose of 400 mg given once daily as prescribed [28]. The baseline characteristics of patients and controls are given in [Table/Fig-1].

Characteristics	Patient (n = 57)	Control (n = 52)	p-value
Age in years*	38.04 ± 1.37 (14-65)	34.23 ± 1.40 (18-68)	0.055
Gender †			
Male	39 (68.42 %)	22 (42.31 %)	0.006
Female	18 (31.58%)	30 (57.69%)	
WBC /µl (range) ‡	17045.86 (11394.37-25500.43)	7586.75 (7155.38-8044.13)	0.0002
Phase of CML			
Chronic Phase	54(94.74%)	NA	NA
Accelerated phase	2(3.51%)		
Blast Crisis	1(1.75%)		

[Table/Fig-1]: Baseline characteristics of patients and controls evaluated for ROS.
*Data presented as mean ± SE (Range) and two sample t test used for the comparisons.
†: Data presented as n (%) and chi-square test used for the comparison.
‡: Data presented as geometric mean (95% Confidence Interval) and two sample t test used for the comparison after log transformation of the data,
NA: not applicable.

The WBC count of the patients not on TKIs (untreated) was considerably high and showed significant difference (p<0.001) as compared to patients under treatment of TKIs (treated) as well as healthy controls. On the other hand, there was no significant difference (p<0.999) in WBC count of treated and healthy controls. Analogous to WBC profile the mean ROS production of treated and healthy controls was found to be almost similar, 14.04 and 16.15 RLU/sec /10^4 WBC respectively while it was low for untreated patients (5.41 RLU/sec/10^4 WBC) as is evident in [Table/Fig-2]. The mean ROS was found to be less even though WBC counts and ROS levels of individual patient who were not on TKIs was considerably high. However, the ROS values in 400 µl of blood showed higher mean ROS (23140.78 RLU/sec, [Table/Fig-2]) in untreated patients

Parameters	Under treatment (A) (n = 40)	Untreated (B) (n = 17)	Healthy controls (C) (n = 52)	p-value A Vs B	p-value B Vs C	p-value A Vs C
WBC *	7813 (6262.09–9748.32)	106852 (53632.54–212881)	7586.75 (7155.38–8044.13)	<0.001	<0.001	0.999
ROS/sec/10^4 WBC*	14.04 (9.59–20.56)	5.41 (2.36–12.43)	16.15 (11.71–22.27)	0.029	0.007	0.999
ROS /sec /400µl of blood *	4389.14 (3175.37–6066.85)	23140.78 (12746.51–42011.19)	4900.91 (3591.56–6687.60)	<0.001	<0.001	0.999

[Table/Fig-2]: Comparison of ROS levels between healthy subjects, treated patients and untreated patients with respect to WBC counts, ROS/sec/10^4WBC, ROS /sec /400 µl of blood *Data presented as geometric mean (95%Confidence Interval) and Bonferroni test used for the comparison on log transformed data.

as compared to patients under treatment or healthy controls.

Parallel to the above profile as evident in [Table/Fig-2] treatment naïve patients characteristically showed significant difference in ROS production in comparison to CML patients taking TKI's (p<0.029). Nonetheless, there was no significant difference between the ROS values of patients taking imatinib and healthy controls (p<0.999).

DISCUSSION

To the best of our knowledge, this is the first study that measures overall ROS species in the peripheral blood of CML patients from Northern India. ROS moieties are involved in important normal physiological process like cell signalling and homeostasis. Acting as signalling mediators in an oncogene milieu, the elevated steady state level of ROS helps in malignant transformation, pro-survival pathway upregulation and DNA damage induced mutations in tumours as well as leukaemia [14,21,29]. A study was therefore undertaken to systematically analyse CML patients under treatment of TKIs (treated) showing complete compliance and those not on any TKIs (untreated/treatment naive) to understand the role of ROS in pathophysiology of CML. Classically, ROS is quantified in plasma because of ease of collection and the belief that it reflects the redox status of the patient [30]. However, it negates the role played by circulating cells which are laden with catalases and other enzymes and also ignores oxidant scavenging abilities of whole blood [31]. ROS are significantly higher in human capillary blood versus venous blood versus plasma as determined by electron paramagnetic resonance together with spin trapper [32]. In addition, RBCs also act as an effective sink for these free radicals because it houses several antioxidants especially haemoglobin. Paradoxically, RBCs have a pro-oxidant role by producing deoxygenated or partially oxygenated haemoglobin which acts as a superoxide radical [30,33]. On the other hand, WBCs being one of the sources of ROS in blood, there has been a bias to use isolated Peripheral Blood Mononuclear Cells (PBMC) to measure ROS rather than whole blood. Keeping the above facts in mind, PBMCs alone would not give a fair picture of the effective ROS levels in the blood of a CML patient. Other workers have also implicated platelets together with WBCs in ROS production in congestive heart failure [33]. Furthermore, the process of isolation of WBC is time consuming, complex and sample handling might affect the results. Hence, instead of isolating WBC, whole blood was chosen for measurement of ROS to obtain a global ROS assessment. In addition, the use of fresh blood sample rather than frozen tissue adds to precision and validity of the studies.

ROS are highly reactive moieties and have low steady state concentration, therefore, it is difficult to measure ROS production directly [34]. Even though electron paramagnetic resonance [35] is a priori direct method of instantaneous detection, it has limited clinical application because of its complexity and technical difficulties [32]. Thus, measurement of the free radicals is done by indirect methods such as; change in the absorbance levels of cytochrome reductase/ nitro blue tetrazolium salt or measuring intensity of luminescence emitted by intact cells stimulated by lucigenin, luminol etc., or fluorescence given by probes like hydroethidium/dihydroethidium (HE/DHE) upon reaction with the free radical [36,37]. In the present study, luminol was added as a probe which served as signal enhancer. This produces an excited reaction intermediate, which emits a photon of light when it returns to its ground state. This emitted photon is measured by the luminometer and expressed as relative light units per second. This is a very sensitive technique and detects all the species of ROS (hydroxyl, superoxide and hydrogen peroxide free radical) that is global ROS. Simple and inexpensive, luminol method measures both intracellular and extracellular ROS unlike commonly used lucigen probe [38].

ROS values were normalised against 10^4 TLC/WBC as against only neutrophil counts due to several reasons. A leukaemic blood sample is a mixture of mature, blasts and several precursors of the myeloid lineage. Every type of these cells harbours BCR-ABL translocation which is instrumental in production of ROS. An adjustment against a single cell type like neutrophil [27] would not correctly and completely reflect the ROS in comparison to normalization against absolute leucocyte count. Moreover, the number of such blasts, precursor and mature WBC show patient specific variations and no two patients with identical WBC values showed same differential counts of leukocytes in our study.

Even though the WBC counts and ROS values of each untreated/ treatment naive patient as compared to treated patients and healthy controls is high, puzzlingly, the mean or average ROS (5.41 ROS/ sec/10^4 WBC) of these untreated patients is low as compared to that of treated and healthy subjects [Table/Fig-2]. This may be because in our study the increase in the number of WBC far exceeds the increase in ROS levels as seen in each untreated patient. This seemingly low average ROS is due to simple divisional mathematical decrease where the denominator WBC far exceeds the numerator that is ROS values. The significant increase in ROS levels in untreated patients [Table/Fig-2] supports the role of ROS in maintaining the leukaemic state in these patients. Fusion oncogene BCR-ABL alone is enough to induce high ROS levels in CML patients as an increase has been demonstrated by transforming normal hematopoietic cell lines like Ba/F3, 32Dcl3, and MO7e with BCR-ABL alone [19]. Activation of PI3K/mTOR pathway resulting in increased ROS levels could transform hematopoietic cells into leukaemic state that can utilize this oxidative stress for its own survival and maintenance [15,16,39]. When such BCR-ABL transformed cells are blocked by the mitochondrial complex I inhibitor rotenone as well as glucose transport inhibitor like phloretin, the levels of ROS decrease, suggesting that the source of increased ROS might be related to increased glucose metabolism [15]. Further oxidative stress potentially induces genetic alterations that would aid in maintenance of leukaemic phenotype by creating genetic heterogeneity which is essential for progression of any cancer. The DNA damaging effect of ROS could play a central role in placing sporadic leukaemia as the most frequent type amongst all the categories of leukaemia [40]. In addition, ROS also induces mutations in kinase domain of the BCR-ABL fusion protein which leads to drug resistance [41]. The cytokines and bone marrow stromal factors mediated increased resistance to TKIs in the BCR-ABL positive cells is also associated with elevated ROS levels [42,43]. Estimation of ROS levels has therefore prognostic significance and hence should be measured in both leukaemic status before and after therapeutic intervention. This prognostic importance can further be extrapolated to different phases of CML as oxidative stress seems to also increase in accelerated phase [44] with concomitant depletion of non-enzymatic antioxidants [45].

The patients on daily dose of imatinib showed ROS values and WBC counts similar to those of healthy controls and there was no significant difference in the mean ROS (p<0.999), [Table/Fig-2]). In our study, the duration of TKIs therapy varied from one to nine

years in patients, irrespective of that, constant low levels of ROS were observed in each treated CML patient. TKIs are known to cause molecular remission due to caspase independent necrosis like programmed cell death of BCR-ABL positive cells [46]. This reduction in number of malignant cells effectually decreases ROS levels. Significant reduction in ROS levels has been reported without cell death in K562 CML cell lines treated by imatinib or new generation TKIs like nilotinb, dasatinib and imatinib through degradation of NADPH oxidase subunit reduces the ROS levels in CML patients [42]. High levels of ROS so observed in the untreated patients gives further credence to the role of TKIs in decreasing ROS levels through mechanisms still to be clearly understood. It is considered that this decrease in ROS levels is primarily due to decline of transformed cells. However, the decrease in ROS may also be due to increase in efficient scavenging mechanisms in the whole blood resulting in quenching of ROS production. In an oncogenic phenotype, already increased amount of antioxidant proteins are already present [47] to counterbalance the increased ROS in order to maintain altered steady state of redox homeostasis, which in turn ensures that both increased ROS and antioxidants do not reach toxic levels that causes harm to the malignant cell itself [48]. What needs to be studied is whether during decrease in the load of BCR-ABL cells the, antioxidant levels simultaneously increases to quench the ROS. Thus, overall antioxidant capability of the body, the effectiveness of RBC acting as sink, the scavenging capabilities of the increasing normal WBCs, all may contribute to decreased ROS levels in blood of patient on TKIs.

The current therapeutic regime has reached its limit of effectiveness. Increase in ROS levels of leukaemic cell can be an additional property which can be exploited for therapeutic purpose. Treatment of the patient with a drug that decreases ROS together with TKIs can significantly alleviate the symptoms and may perhaps increase the mean life span. A futuristic perspective would be therefore to ascertain the exact source of ROS production that is mitochondrial or NOX (NADPH oxidase) and species of ROS involved in oxidative stress. Comparison of the levels of ROS production by the blast and the mature myeloid cells would comment upon the patho-biological transition seen from a chronic phase to a more lethal blast phase. Therefore, modulations of ROS levels can serve as novel adjuvant or therapeutic target to develop strategies which would selectively kill malignant cells.

LIMITATION

This study gives a snap shot view of ROS in chronic phase but remains silent about the oxidative status in accelerated or blast phase. The actual status of ROS and antioxidants in the accelerated and blast phase is yet to be clearly elucidated. This study also does not delineate whether ROS is being produced by mitochondria or is produced by cytoplasmic NOX. A large sample size together with a structured study is required to establish the precise role of oxidative stress in pathobiology of CML.

CONCLUSION

To ascertain the alterations in ROS levels of CML patients before and during treatment with TKIs it is better to measure global ROS in fresh whole blood by chemiluminescent method using luminol. Luminol assay is a quick, easy and inexpensive method to measure global ROS which does not require elaborate instrumentation and expertise. Luminol assay in whole blood removes the artefacts involved in isolation and estimation of ROS based on only WBC pellets. Since ROS levels are increased in many cancers including haematological malignancies, a simple rise in levels cannot be used as biomarkers. Thus, a thorough understanding of the role of ROS as key mediators in leukaemogenesis is likely to provide opportunities for improved pharmacological intervention. We think that our study bolsters a role for ROS in the pathogenesis of leukaemic phenotype and outlines innovative approaches in the implementation of redox therapies for myeloid malignancies.

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