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EVALUATION OF NITRIC OXIDE LEVELS IN CHRONIC MYELOID LEUKEMIA

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ABSTRACT

Nitric oxide (NO) is a free radical and multifunctional molecule that is produced from L-arginine by NO synthases (NOS). It is required for many physiological functions and produces many reactive intermediates that account for its bioactivity. Sustained induction of the inducible form of NOS (iNOS) in chronic inflammation may be mutagenic, through NO-mediated DNA damage or hindrance to DNA repair, and thus potentially carcinogenic. Due to the short half-life of NO, usually its end products (nitrate or nitrite) are measured as an index of NO production. Nitric oxide (NO) is involved in different stages of malignancies. Increased levels of NO have been reported in different leukemias. There is evidence that expression of iNOS in tumor cells, including acute myeloid leukemia and chronic lymphocytic leukemia is increased. Chronic myeloid leukemia (CML) is a clonal myeloproliferative disease of primitive hematopoietic stem cells. Imatinib is the preferred drug for the treatment of chronic myeloid leukemia (CML). This study was conducted on 25 patients of CML receiving imatinib for six weeks and the levels of nitrite (nitric oxide product) in the serum of patients was determined before and after receiving therapy and were analyzed statistically. The results of serum nitrite of patients were compared with corresponding values obtained in 25 healthy volunteers. The results indicate that patients with chronic myeloid leukemia had a significant increase in the serum level of nitrite and the levels were found to be significantly decreased after receiving the treatment with imatinib. It was concluded from the present study that oxidative stress is a feature of chronic myeloid leukemia and the measurement of nitric oxide (NO) could be a diagnostic, as well as prognostic, tool during treatment of patients with chronic myeloid leukemia.

Keywords: Nitric Oxide, Chronic Myeloid Leukemia, Imatinib, Nitrite, Nitrate, Nitric Oxide Products

INTRODUCTION

Leukemia, a haemopoietic cell's neoplasm, is one of the bone marrow malignancies often characterized by abnormal increase in white blood cells. Leukemia constitutes the twelfth most common class of neoplastic disease, and the eleventh most common cause of cancer-related death. About 90% of all leukemia is diagnosed in adults (Mathers *et al.*, 2001). Although, the exact cause of leukemia is still unknown, scientists suspected that viral, genetic, environmental or immunological factors may be involved (Tanner *et al.*, 2001). There are two main categories of leukaemia: acute and chronic. Chronic leukemia is primarily the disease of adults, with the exception of chronic myelogenous leukemia which sparingly occur in children. In acute leukemia, about 80% of Acute Lymphoblastic Leukemia (ALL) occurs in children and Acute Myeloblastic Leukemia (AML) is far more common in adults. While acute leukemia is acutely fatal if untreated, the chronic leukemia is relentlessly insidious and may only be diagnosed in the early stage during routine medical check-up (Devi *et al.*, 2000).

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disease of primitive hematopoietic stem cells. Exposure to ionizing radiation is one of the risk factors for CML though exact etiology is not yet clear. The Philadelphia chromosome (translocation between chromosome 9 and chromosome 22.t (9;22) (q34; q11) is the hallmark cytogenetic abnormality of CML. It is present in virtually 95% patients with CML (Deininger *et al.*, 2000). Treatment of CML traditionally has palliative intent. Various modalities which have been tried in the treatment of CML are bone marrow transplantation, splenectomy, interferon, and chemotherapy (Manero *et al.*, 2003; Aziz *et al.*, 2007). Imatinib mesylate, a tyrosine kinase inhibitor, is now considered one of the most effective treatment for bcr- abl (breakpoint cluster region gene on

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chromosome 22 and a gene on chromosome 9 named after Abelson murine leukemia virus) positive CML patients. It blocks the binding of adenosine triphosphate (ATP) to bcr-abl tyrosine kinase inhibiting kinase activity, which in turn inhibits proliferation and induces apoptosis in bcr-abl positive cell lines. Imatinib is given orally and is well tolerated. Commonly observed mild to moderate grade side effects include nausea, vomiting, muscle cramps, diarrhoea, headache, rash, and oedema. More severe adverse effects (grade 3/4) are seen in more than 1% of patients including neutropenia, thrombocytopenia, anemia, elevated liver enzymes, and arthralgia (Aziz *et al.*, 2007; Williamson *et al.*, 2001).

Nitric oxide (NO) is a free radical molecule, that at physiological levels is associated with neurotransmission and vasodilatation and at higher levels has tumoricidal and bactericidal effects (Shmidt *et al.*, 1994). In the cell mediated immune responses, NO is produced in macrophages, neutrophils and lymphocytes. NO is produced through the oxidation of L-arginine to L-citrulline, by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS exist, namely the constitutive forms, endothelial NOS (eNOS/NOS2) and neuronal NOS (nNOS/bNOS/NOS1), and the cytokine inducible NOS (iNOS/NOS2) (Bredt, 1994). NOS are a unique family of P₄₅₀-type hemoproteins which use NADPH, FAD, FMN, heme and tetrahydrobiopterin as co-factors (Stamler, 1992). The constitutive NOS (cNOS) forms produces small amount of NO for short periods, in response to receptor stimulation such as acetylcholine or shear stress. The inducible NOS, enzyme releases high levels of NO for extended periods of time. Inducible NOS is a cytosolic enzyme of many cells, such as macrophages, endothelial cells, chondrocytes, hepatocytes, synoviocytes and smooth muscle cells. Inducible NOS is induced by inflammatory stimuli and NO produced by this enzyme is a vital component of tumouricidal and fungicidal apparatus of macrophages (Clancy *et al.*, 1998). There is evidence that increased amount of blood nitrate can be detected *in-vivo* during infections, following cytokine administration, sepsis, ulcerative colitis, arthritis, multiple sclerosis, type-I diabetes and a variety of rheumatic diseases, including systemic lupus erythematosus, Sjogren's syndrome, vasculitis, osteoarthritis and rheumatoid arthritis (Clancy *et al.*, 1995).

Raised levels of NO are involved in tumorigenesis by affecting actions of various protein kinases and transcription factors resulting in damage to DNA (deoxyribonucleic acid) structure by deamination of nucleotides and generation of free radicals (Moncada *et al.*, 1991). It has been observed that expression of inducible NO synthase (iNOS) is positively associated with p53 mutation in tumours of the colon, lung, and oropharynx. NO can stimulate tumour growth and metastasis by promoting migratory, invasive, and angiogenic abilities of tumour cells. There is also evidence indicating that tumour-derived NO promotes tumour angiogenesis as well as invasiveness of certain tumours in animals, including humans. It has also been suggested that tumour cells utilize certain NO-mediated mechanisms for promotion of growth, invasion, and metastasis and that NO-blocking drugs may be useful in treating certain human cancers (Lala, 1998; Meeta, 2001). NO has been shown to contribute to carcinogenesis in gastrointestinal tissues by causing DNA lesions, inhibiting DNA repair enzymes and blocking apoptosis via nitrosylation of caspases and functioning as an angiogenesis factor (Jadeski, 2002). Some authors have studied the characterization of cancer stem cells in chronic myeloid leukemia. It was observed by them that tyrosine kinase inhibitors resulted in inhibition of nuclear factor kappa B and iNOS (Jorgensen *et al.*, 2007). Significant activity of inducible NOS has been reported in tumor cells, including acute and chronic leukaemic cells (Brando *et al.*, 2001).

Thus, this study was planned to evaluate the levels of nitrite (nitric oxide product) in chronic myeloid leukemia (CML) patients before and after imatinib therapy so as to define their roles in diagnosis, improving treatment and appropriate care in remission of chronic myeloid leukemia patients.

MATERIALS AND METHODS

This study was conducted on twenty five patients of CML after obtaining the informed consent from the patients and approval from the institutional board of studies. The patients were attending the Haematology clinic in Department of Medicine in Pt. B.D. Sharma, PGIMS, Rohtak. Only newly diagnosed CML cases (diagnosis made by history, clinical examination, complete hemogram, and bone marrow examination) were taken for study. Cytogenetic study was done in beginning of the study by real

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time polymerase chain reaction (RT-PCR using Stratagene, Germany) and only bcr-abl positive patients were taken for study irrespective of the age and phase of the disease. Newly diagnosed bcr-abl negative CML patients were excluded from the study. The CML patients were given imatinib therapy (400mg twice a day for 6 weeks) only. Twenty five age-matched healthy subjects were taken as control group. All participants consented to the study.

Biochemical Measurement

Five milliliters (5 ml) of venous blood was collected from each subject into EDTA vacutainer to obtain plasma after centrifugation for 5 minutes at 4000 x g. The plasma was separated and analysed on the same day. The serum concentration of nitric oxide metabolite (nitrite) was estimated in fasting plasma samples before and after the imatinib therapy by Greiss reaction. Greiss Reaction measures nitrite formed from NO which is a stable and nonvolatile end product of NO which, itself has a short half life of 6–10 s. Nitrite reacts with Greiss reagent to form a purple colored complex, whose absorbance was measured at 546nm using colorimeter (Dimitrios, 2007). The details of the patient’s clinical and haematological data were entered in a specialized performa made for the purpose of the study. After a period of 6 weeks of treatment, patients were assessed for hematological response (cell counts and bone marrow examination) and clinically for side effects (secondary outcome) with change in NO levels being the primary outcome.

Statistical Analysis

Results were expressed as mean ± SD and paired and unpaired student t-test was used for comparison of NO levels in the two groups.

RESULTS

The levels of NO in CML patients at the time of presentation was 42.43 ± 5.79 mmol/L and after 6 weeks of therapy with imatinib was found to be 14.26 ± 2.76 mmol/L ($p < 0.01$). The levels of NO in healthy controls were found to be 10.67 ± 0.34 mmol/L. Thus, a significant decrease ($p < 0.01$) was observed in the NO levels in CML patients after imatinib therapy (Table 1).

Table 1: Comparison of nitric oxide metabolite (nitrite) levels in the serum of chronic myeloid leukemia patients with healthy controls

Group	Number	Nitrite ($\mu\text{mol/l}$) Mean S.D
Control	25	10.67 ± 0.34
CML patients before imatinib therapy	25	41.48 ± 5.12
CML patients after imatinib therapy	25	14.26 ± 2.76

DISCUSSION

In the present study the serum concentration of nitric oxide product (nitrite) of patients with chronic myeloid leukemia was analyzed. All CML patients had higher levels of nitrite, compared to the control group. A significant decrease ($p < 0.01$) was observed in NO levels in CML patients after imatinib therapy.

Table 2: Comparison of nitric oxide metabolites (nitrite) levels in the serum of acute leukemia patients with healthy controls in different studies

Study	Group	Number	Nitrite ($\mu\text{mol/l}$) Mean S.D
Ghaffari MA <i>et al.</i> ,	Control	40	7.52 ± 0.44
	Acute leukemia patients	40	12.36 ± 0.18
Olaniyi J A <i>et al.</i> ,	Control	25	15.96 ± 33.64
	Acute leukemia patients	25	32.12 ± 19.61

Ghaffari *et al.*, in their study monitored serum nitric oxide levels in 40 patients of acute leukemia and compared the levels of serum nitrite of acute leukemia patients with the levels of serum nitrite found in 40 healthy controls. It was observed by them that serum nitrite levels of healthy controls was 7.52 ± 0.44 $\mu\text{mol/l}$, whereas this level of nitrite in acute leukemia patients was 12.36 ± 0.18 $\mu\text{mol/l}$ (Ghaffari *et al.*, 2005). In study conducted by Olaniyi J A *et al.*, serum nitrite levels in 25 healthy controls was observed

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as $15.96 \pm 33.64 \mu\text{mol/l}$ and the level of nitrite in 25 acute leukemia patients was found $32.12 \pm 19.61 \mu\text{mol/l}$ (Olaniyi *et al.*, 2011) (Table 2).

NO has been found to be involved in angiogenesis and iNOS to be expressed in vitreoretinal disorders and cancer (Jadeski, 2002). NO is associated with all the stages of carcinogenesis, that is, initiation, progression and metastasis and the mechanisms put forward are mainly DNA damage and neovascularisation and it was also suggested that NO blocking drugs may be helpful in the treatment of malignancies (Li, 2001).

A study showed that, the combination of phenolic antioxidant compounds (such as curcumin, carnosol, or quercetin) in conjunction with NOS inhibitors may be particularly valuable as a novel strategy for treating acute leukaemia (Kellner, 2004). Another study demonstrated that sodium nitroprusside (SNP), a NO donor, had cytotoxic effects on cells of some patients with Malignant Lymphoma (ML), Acute Myeloblastic Leukaemia (AML) or Chronic Myelomonocytic Leukaemia (CMMoL), but not with multiple myeloma (Tsumori *et al.*, 2002). Also, elevated production of NO has been observed in B-cell chronic lymphocytic leukaemia, and appears to play a survival role in these types of cells (Zhao *et al.*, 1998; Kolb, 2000). Tetrahydrobiopterin, a cofactor for NO synthase, is produced when the cells involved in cellular immunity are activated. Furthermore, it has been reported that urine concentrations of neopterin, an intermediate product of tetrahydrobiopterin, changes according to immunological conditions of the host. Urine neopterin levels were remarkably elevated at patients with malignant lymphoma, acute myelocytic leukemia and multiple myeloma. Thus, the serum NO levels were also elevated in these patients (Tanaka *et al.*, 2002)

Therefore, we have observed that NO levels were raised significantly in CML patients as compared to healthy controls and it was also observed that NO levels decreased significantly after imatinib therapy. Though it being a small scale research, further studies with longer follow up evaluation are needed to confirm the observed results.

Conclusion

The results of present study suggest that oxidative stress is a feature of chronic myeloid leukemia and indicate that the measurement of nitric oxide (NO) could be a diagnostic, as well as prognostic, tool during the treatment of patients with chronic myeloid leukemia.

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