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Abstract
Nitric oxide (NO) is involved in different stages of malignancies. Increased levels of NO have been reported in different leukemias. Imatinib is the preferred drug for the treatment of chronic myeloid leukemia (CML). Turmeric powder contains curcumin which has anti-leukemic property and also decreases NO synthesis. This study was conducted on fifty patients of CML divided into two groups, group A receiving imatinib alone and group B receiving turmeric powder along with imatinib for six weeks. Nitric oxide levels were estimated in these patients before and after receiving therapy and were analyzed statistically. Nitric oxide levels were found to be significantly decreased in both the groups, but more significantly in group B after receiving the respective treatments. Thus, curcumin acts as an adjuvant to imatinib in decreasing the NO levels and may help in the treatment of CML patients.

Keywords
Chronic myeloid leukemia, nitric oxide, imatinib, turmeric powder, curcumin

Introduction

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.[1]

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.[1–3] Imatinib mesylate, a tyrosine kinase inhibitor, is now considered one of the most effective treatment

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for bcr-abl (breakpoint cluster region gene on 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, anaemia, elevated liver enzymes, and arthralgia.[3,4]

Turmeric is a spice derived from the rhizomes of Curcumin longa. Curcuminoids are polyphenolic compounds that give turmeric its yellow color. Curcuminoids are major active components (70%) of the spice turmeric and consists of pure curcumin (diferuloylmethane) (75%), demethoxycurcumin (10–20%), and bisdemethoxycurcumin (<5%). Curcumin is 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. Curcumin undergoes extensive metabolic conjugation and reduction in gastrointestinal tract.[5] It possesses anti-inflammatory, antioxidant, and anti-tumor properties. Preclinical studies have revealed the chemo-preventive potential of curcumin in several different animal tumor bioassay systems, including colon, duodenal, stomach, prostate, and breast carcinogenesis, both *in vitro* and *in vivo*. Curcumin is beneficial in all three stages of carcinogenesis (initiation, progression, and promotion). Much of its beneficial effect is found to be due to its inhibition of the transcription factor nuclear factor kappa B (NF-kappaB) and subsequent inhibition of proinflammatory pathways. It blocks the NF-kappaB signalling and inhibits activation of NF-kappaB mainly via I-kappaB kinase (IKK)-mediated phosphorylation of inhibitory molecules. The pathways activated by NF-kappaB upregulation are implicated not only in tumour growth and progression but also in development of resistance to anticancer drugs, radiation, and death cytokines.[5,6] Curcumin has also been reported to possess anti-leukemic property by inhibiting the cellular proliferation and the expression of signal transducers and activators of transcription 5 (STAT5) mRNA and also by down-regulating the activation of STAT5 in primary CML cells.[7]

Curcumin increases the expression of phase II conjugation enzymes such as sulfotransferase and glutathione-S-transferase, responsible for metabolism of carcinogens from dietary and environmental sources. It also induces apoptosis by activation of caspase 3 and 9 and suppressing various cell survival and cell proliferative genes, including bcl-2, bcl-X (L), cyclin D1, and IL-6 (interleukin 6).[8] It has been shown to decrease the proliferation of various cancer cells by downregulating cyclooxygenase-2 (COX-2), which acts as tumour promoter.[5,6] Curcumin reduces the invasion and subsequent metastasis of cancer cells by suppressing the matrix-metalloproteinase (MMP) expression, which plays a major role in mediating neovascularisation through their matrix-degrading capacity and is significantly increased during tumour progression. It inhibits tumour necrosis factor-alpha (TNF-alpha)-induced vascular cell adhesion molecule-1(VCAM-1) expression, which is related to the activation of the mitogen activated protein kinase (MAPK) NF-kappaB pathway. Curcumin reduces cell migration and invasion induced by an extracellular matrix protein, osteopontin (OPN) by blocking signals leading to IKK activation. It also blocks the expression of proangiogenic and prometastatic factors like vascular endothelial growth factor (VEGF) and interleukin IL-8. It supresses nuclear human β-catenin/ T-cell factor (TCF) target genes including cyclin D, MMP 7, OPN, IL-8, and matrilysin, which play a role in tumour promotion and progression.[5,6]

It has been observed in different studies that curcumin prevents phosphorylation and degradation of inhibitor kappa B-alpha, thereby blocking NF-kappa B activation, which results in downregulation of iNOS gene transcription.[6,9] Li and Lin-Shia reported that in CML patients resistant to imatinib therapy, cancer cells were characterized by over-expression of multi drug resistance 1 (MDRI) gene, which encodes P-glycoprotein. Curcumin exhibits characteristics of a MDR modulator and decreases MDRI gene expression in leukemic cells by decreasing MDRI mRNA level in leukemic cells having high level of MDRI gene groups.[10] It has been shown in many studies that Wilm's tumour 1 (WTI) gene was highly expressed in leukemic blast cells. Curcumin, an excellent curcuminoid derivative, decreased WTI gene expression in both transcriptional and translational levels. Thus, curcumin is one of a potent potential chemotherapeutic agent that can be used for treatment for CML.[9] It has been observed that curcumin strongly inhibits cell proliferation and affects cell viability by inducing apoptotic symptoms and causes G2M cell cycle arrest and induces mitotic catastrophe (mode of death caused by aberrant mitosis) in the bcr-abl expressing cells. This type of cell death overcame the apoptosis resistance that was typical of high bcr-abl expressing cells leading to conclusion that curcumin can be used as an agent for new combination regimens for drug resistant CML.[11]

Nitric oxide (NO), at physiological levels, is associated with neurotransmission and vasodilatation. 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.[12] 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.[13,14]

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.[15] 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.[16] Serum concentration of NO products (nitrite or nitrate) in leukemia has been reported to be higher as compared to controls[17] but not many studies are available to comment on the effect of treatment on NO levels in CML patients.

So, the present study was planned to determine the NO levels in the CML patients before and after imatinib therapy with and without turmeric powder.

Materials and methods

This study was conducted on 50 patients of CML after obtaining the informed consent from the patients and approval from the institutional board of studies. 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 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 divided randomly into two groups having 25 patients each. Group A patients were given imatinib therapy (400 mg twice a day for 6 weeks) only and Group B patients were given imatinib therapy (400 mg twice a day) along with turmeric powder (5 g three times/day dissolved in 150 mL of milk to improve its palatability and absorption) for 6 weeks. Patients in both groups were age, sex, hemoglobin, total leukocyte count (TLC), and platelet count matched.

NO levels were estimated in fasting plasma samples of both the groups before and after the above mentioned 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 546 nm.[18]

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. Complete hematological response was defined as platelet count $<450 \times 10^9$/L; white blood cell count $<10 \times 10^9$/L; differential count showing absence of immature granulocytes and basophils <5%; nonpalpable spleen. Criteria for partial hematological response consisted of above findings except for presence of immature cells, platelet count <50% of pretreatment but $>450 \times 10^9$ L, persistent splenomegaly but <50% pretreatment size.[19] Patients were monitored clinically for occurrence of adverse effects like skin rash, arthralgia, gastritis, nausea, vomiting, dirrhoea, oedema, signs of myelosuppression (anemia, neutropenia and thrombocytopenia), and elevated levels of serum aspartate transaminase and alanine transaminase.

Statistical analysis

Statistical analysis was done by applying paired and unpaired student t-test for comparison of NO levels in the two groups. The difference in respose and adverse effects in the two groups was analyzed statistically using Chi square test. Alpha level and power was also calculated for the sample size beforehand. Z value at α level was found to be 1.96 and 1.64 at β level, with a power of 90% at a predicted difference of 5 units.

Results

All the patients were in the chronic phase of CML. The mean Hb at presentation in these patients was 8.84 g% ± 2.50 (range 4.5–16.2 g%). Mean TLC count at time of presentation was 80,700 ± 56,650/mm^3. The age of all the patients and controls ranged between 14 and 82 years while the median age of CML patients being 53 years. At the time of presentation, 73% of the patients had normal platelet count, 20% had thrombocytosis while 7% had thrombocytopenia. In both the groups, most of the patients (73%) had platelet count between 100 and 350×10^9 /L. The bone marrow was hypercellular with increased myeloid:erythroid ratio and varying number of immature cells of myeloid series in all the patients. The levels of NO at the time

Table 1. Comparison of nitric oxide levels in chronic myeloid leukemia patients before and after imatinib therapy with and without turmeric powder

	Group A (imatinib therapy only)			Group B (imatinib and turmeric powder therapy)		
	Before treatment	After treatment	p value	Before treatment	After treatment	p value
Number of patients	25	25		25	25	
Nitric oxide levels (μmol/L) Mean ± SD	41.48 ± 5.12	14.26 ± 2.76	<0.01	42.85 ± 5.67	4.06 ± 1.79*	<0.01

*p-Value <0.001 when compared with patients of group A after treatment.

of presentation was 42.43 ± 5.79 μmol/L and after 6 weeks of therapy was found to be 14.26 ± 2.76 μmol/L in group A ($p < 0.01$) and 4.06 ± 1.79 μmol/L in group B ($p < 0.01$) (Table 1).

In group A, 73% achieved complete hematological response, 20% showed incomplete hematological response, and 7% showed no response to imatinib therapy. In group B, 94% achieved complete hematological response and 6% showed incomplete hematological response. Though a larger percentage of group B patients achieved complete remission, difference was not significant statistically ($p > 0.05$). Patients were observed for any side effects of chemotherapy during each visit. In group A, most common side effects which were observed during study were of mild to moderate grade and included gastritis in 40%, skin pigmentation in 33%, arthralgia in 20%, and oedema in 13% patients. In group B, most common side effects observed were mild to moderate gastritis in 40% and skin pigmentation in 20% patients. No patient presented with arthralgia and oedema as a side effect of imatinib in group B. The difference in occurrence of adverse effects in two groups is not significant statistically with a $p > 0.05$ for each adverse effect.

Discussion

A significant decrease ($p < 0.01$) was observed in the NO levels in CML patients after imatinib therapy. Mean serum nitrite levels in acute leukemia patients have been reported to be higher as compared to controls.[13] Significant activity of inducible NO synthase (iNOS) has been reported by other authors in tumour cells, including acute and chronic leukemic cells.[20] NO has been found to be involved in angiogenesis and iNOS to be expressed in vitreoretinal disorders and cancer.[15]

NO levels in CML patients after 6 weeks of imatinib and turmeric powder therapy showed a statistically significant decrease ($p < 0.001$) as compared to NO levels with imatinib therapy alone as curcumin has been reported to downregulate the gene for iNOS.[18] No studies are available in literature to report the effect of imatinib or other tyrosine kinase inhibitors (e.g., dasatinib, nalotinib) on NO levels in CML. 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.[10]

It has also been observed that the treatment of CML with a combination of imatinib and curcumin was more effective than imatinib alone and this is mainly brought about by the induction of apoptosis.[21] We have also observed the similar results as complete hematological response was achieved in greater percentage of patients with fewer side effects with combination therapy as compared to imatinib therapy alone though the difference was not significant statistically ($p > 0.05$).

Therefore, we have observed that NO levels decreased significantly after combination of imatinib and turmeric powder therapy as compared to imatinib therapy alone. The hematological response and tolerance were found to be better with 6 weeks of combination therapy as compared to imatinib alone. Though it being a small scale research, further studies with longer follow up evaluation are needed to confirm these findings. Thus, we conclude that NO levels may be used as prognostic indicator in CML patients and turmeric powder can also be used as an adjuvant to imatinib therapy due to its prominent anti-neoplastic activity.

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Declaration of conflict of interest

No conflict of interest exists among authors and with anybody else.

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