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ORIGINAL ARTICLE

Assessment of circulating biochemical markers and antioxidative status in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients

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Abstract Various circulating biochemical markers are indicators of pathological state in leukemia and its subtypes. Increased oxidative stress and decreased antioxidant factors portray clear image associated with malignancies during subtypes of leukemia. In this research work we investigated the inter-relationship among the subtypes of leukemia with circulating biochemical markers and oxidative stress in the Pakistani population. This research work was conducted on a total number of 70 subjects in which 20 were control participants and 50 were suffering from leukemia and divided into two subtypes (ALL and AML). Various circulating biomarkers were investigated including hematological, hepatic and renal profiles as well as oxidative stress markers, electrolytes and vitamins C and E. Results show that vitamin E was found to be decreased in diseased sub-types ($P < 0.05$).

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Malondialdehyde (MDA) levels were very high in disease sub-types (ALL-B = 8.69 ± 1.59; ALL-T = 8.78 ± 0.97; AML = 8.50 ± 1.29) compared to controls (1.22 ± 0.10; $P < 0.05$) while the levels of antioxidants [superoxide dismutase (SOD), glutathione peroxidase (GPx), reduced glutathione (GSH), catalase (CAT)], platelets, as well as electrolytes (Ca and Mg) were reduced in patients suffering from leukemia (sub-types). Enhanced levels of oxidative stress (MDA) and decreased levels of enzymatic and non-enzymatic antioxidants reflect the pathological state and impaired cell control in patients suffering from leukemia (subtypes) and show a strong correlation with oxidative stress, indicating that patients' biological systems are under oxidative stress.

1. Introduction

Acute myeloid leukemia (AML) is a disorder of hematopoietic malignancy due to the accumulation of the blast and uncontrolled proliferation factors which really affects differentiation. On the other hand, acute lymphoblastic leukemia (ALL) is the most common childhood cancer, and the second most common cause of mortality in children aged 1–14 years.

Acute lymphoblastic leukemia ALL occurs approximately five times more frequently than AML and accounts for about 78% of all childhood leukemias diagnosed (Charalambous, 2012). Approximately 3250 children under the age of 20 years are diagnosed with leukemia each year, of about 2400 new cases of ALL are diagnosed (Jensen et al., 2004). ALL is an incurable disease due to the resistance toward treatment. There are many factors which are involved to cause resistance from chemotherapy which include oxidative stress due to the generation of reactive oxygen species (ROS) and presence of hypodiploid cells. Most common antioxidants include vitamins A, C, and E, glutathione (GSH), and the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx). Chemotherapy resistance is a basic hurdle in the treatment of ALL patients (Mendivil-Perez et al., 2012). The incidence of ALL in elderly patients is 1.0 to 1.6 per 100,000 patients, which is much higher than the patients 25–54 years old (0.6 to 0.7 per 100,000) according to the surveillance epidemiology and end results study. In ALL patients above 60 years of age the prognosis rate is very poor due to some other correlated factors like, decreased bone marrow function, and other comorbidities but in children prognosis is better due to the introduction of central nervous system treatment, hematopoietic stem cell transplant (HSCT), intensified post remission therapy and molecular targeted therapy such as imatinib mesylate (Shin et al., 2011).

Reactive oxygen species (ROS) are the heterogeneous group of compounds which are generated by the mature myeloid lines in an innate response. ROS have an important role in intracellular signaling process. An excessive production of ROS can lead to oxidative stress which is quite clear in a number of hematopoietic malignancies including acute and chronic leukemias but it is unclear that ROS are involved in the initiation, progression and maintenance of diseases (Hole et al., 2011). Oxidative stress which is caused by ROS is responsible to cause DNA damage, because in normal conditions DNA-repair mechanism is functional to repair the DNA. DNA damage is not repaired properly in the oxidative stress which leads to mutagenesis (Kryston et al., 2011). Moreover, MUC1-C is an oncoprotein which suppresses the activity of the ROS. Inhibition of MUC1-C is directly associated with the increase of ROS and depletion of glutathione (Yin et al., 2011).

2. Materials and methods

The study was conducted at the Institute of Molecular Biology and Biotechnology (IMBB), the University of Lahore, and all samples were collected from the Inmol Hospital, Lahore and stored at −20 °C. In this study a total number of 70 subjects were selected in which 20 were normal healthy individuals and 50 were leukemic patients which were divided into three subgroups (ALL-B = 25; ALL-T = 11; AML = 14). Informed consent was taken from all the persons participating in this study. The study was also approved by the local ethics committee of the University of Lahore.

2.1. Blood collection and laboratory investigation for hematological parameters

First skin was cleaned thoroughly and sterilized with a 70% alcohol swab (Kandall HealthCare, USA) and dried before withdrawing 2 ml of peripheral blood by a 3 cc disposable syringe (Becton Dickinson Pak) from enrolled subjects. For hematological variables (white blood cells, hemoglobin and platelets), blood was transferred to an ethylenediamine tetra acetic acid (EDTA) coated purple-top test tube. Hematological parameters including WBCs, Hb and platelets were estimated by a Hematological Analyzer.

2.2. Processing of blood sample for other all clinical parameters

6 ml of venous blood was taken from each subject and quickly transferred into a tube. The tubes were marked with the code assigned to the subject. Samples were quickly transported to the laboratory for further processing. After blood sample collection, sample tubes were centrifuged for fifteen minutes at 4000 rpm to obtain a clear serum. Thus overwhelmingly tests were carried out within six hours after the collection.

ALT, AST, ALP, creatinine, phosphate, uric acid, K, Ca, Na, Mg and BUN were estimated by direct kit method using precipitants of Spinreact Co, Spain and estimation of vitamins (C and E) was carried out through commercially available, Human Diagnostic Kit by Spinlab - Spinreact clinical analyzer.

Superoxide dismutase (SOD) and catalase activities were measured by Kakkar et al. (1984), and Aebi and Bergmeyer (1983) respectively. Reduced glutathione and glutathione peroxidase were evaluated by the methods of Moron et al.

Table 1 Levels of different variables (bio-chemicals and antioxidative markers) in subtypes of leukemia.

Variables	Control	Disease sub-types			*P* value
	(n = 20)	ALL-B (n = 25)	ALL-T (n = 11)	AML (n = 14)	
ALT	20.00 ± 5.17	86.00 ± 36.07*	89.54 ± 44.80*	83.71 ± 24.61*	0.001
AST	24.20 ± 5.40	56.08 ± 18.63*	59.00 ± 15.17*	56.71 ± 17.62*	0.001
ALP	194.97 ± 32.45	377.95 ± 34.76*	367.59 ± 41.14*	375.97 ± 31.86*	0.001
MDA	1.22 ± 0.10	8.69 ± 1.59*	8.78 ± 0.97*	8.50 ± 1.29*	0.001
SOD	0.51 ± 0.18	0.14 ± 0.12*	0.17 ± 0.11*	0.15 ± 0.11*	0.001
GSH	9.23 ± 1.44	2.15 ± 0.74*	2.38 ± 0.66*	2.33 ± 0.91*	0.001
Catalase	4.07 ± 0.84	0.73 ± 0.67*	0.83 ± 0.32*	0.77 ± 0.58*	0.001
NO	10.64 ± 1.83	19.71 ± 1.75*	20.83 ± 2.32*	20.26 ± 1.77*	0.001
Creatinine	1.04 ± 0.18	3.03 ± 0.43*	3.13 ± 0.27*	3.10 ± 0.41*	0.001
BUN	13.65 ± 2.34	33.88 ± 5.55*	34.36 ± 7.18*	33.78 ± 4.49*	0.001
K	3.86 ± 0.55	6.02 ± 0.93*	5.91 ± 0.90*	6.40 ± 1.04*	0.001
Ca	8.79 ± 0.90	7.49 ± 1.06*	7.49 ± 1.06*	7.00 ± 1.25*	0.001
Na	139 ± 5.22	167 ± 12.06*	161 ± 6.03*	163 ± 9.17*	0.001
Mg	1.76 ± 0.21	1.41 ± 0.21*	1.40 ± 0.19*	1.40 ± 0.27*	0.001
Phosphate	3.36 ± 0.89	5.75 ± 0.50*	5.77 ± 0.77*	5.95 ± 0.49*	0.001
Uric acid	3.77 ± 0.78	10.35 ± 1.49*	10.71 ± 1.13*	10.48 ± 1.76*	0.001
WBC	7.60 ± 1.39	50.94 ± 28.20*	63.48 ± 33.90*	53.41 ± 27.24*	0.001
Hb	13.96 ± 0.94	9.64 ± 1.71*	9.16 ± 1.44*	9.43 ± 2.24*	0.001
Platelets	217 ± 20.45	83.40 ± 21.03*	85.27 ± 24.37*	77.21 ± 19.12*	0.001
GPx	0.60 ± 0.32	0.29 ± 0.13*	0.33 ± 0.14*	0.33 ± 0.19*	0.001
Vit.C	0.38 ± 0.19	0.49 ± 0.13ns	0.46 ± 0.10ns	0.48 ± 0.90ns	0.145
Vit.E	0.46 ± 0.11	0.22 ± 0.09*	0.24 ± 0.13*	0.20 ± 0.10*	0.001

Hb = g/dl, ALT = IU/L, AST = IU/L, ALP = IU/L, Creatinine = mg/dL, MDA = nM/ml, SOD = ng/ml, GSH = mg/dl, CAT = μM/mol of protein, Na^+ = mEq/L, K^+ = mEq/L, Ca^{++} = mEq/L, Mg^{++} = 1.8–3 mg/dL, Phosphate = mg/dL, Uric acid = mg/dL, WBCs = $\times 10^9$/L, platelets = $\times 10^9$/L, GPx = μmol/ml, Vit. C = μg/ml, Vit. E = μg/ml, BUN = mg/dL, NO = μM/L,
* Significant $P < 0.05$).

(1979) and Aebi and Bergmeyer (1983) respectively. Malondialdehyde (MDA) and nitric oxide (NO) were measured by the methods of Ohkawa et al. (1979) and Moshage et al. (1995) respectively.

2.3. Data analysis

SPSS version18 was used for statistical analysis. Results were expressed by mean and standard deviation and the correlations between various parameters and different subgroups were also determined. $P < 0.05$ was considered as statistical significance.

3. Results

The leukemic subjects were sub-divided into different sub types and showed a highly significant difference among themselves and with controls. All the results are summarized in Table 1. The ALT level in ALL-T sub-type (89.54 IU/L) was surpassed as compared to ALL-B (86.00 IU/L) and AML (83.71 IU/L) respectively. In case of AML involvement of the liver is rarely reported. In a number of previous research studies it is reflected that cholestasis and obstructive jaundice are involved in elevated hepatic profile. In reported cases it was analyzed that the patients receiving traditional combined drug therapy of anthracyclines and cytarabine have an elevated hepatic profile in AML. In case of ALL elevated levels of ALT and AST are associated with completion of chemotherapy as compared to the values of ALT and AST before chemotherapy. Elevated levels of transaminases were common at the initial stages of ALL and caused due to the injury by the leukemic cell infiltration. Elevated levels of AST were observed in ALL-T (59.00 IU/L), AML (56.71 IU/L) and ALL-B, (56.08 IU/L) respectively.

From ALL group in the case of ALL-T elevated levels of MDA were observed (8.78 μmol/ml) than other sub-groups [AML and (ALL-B)] ALL-B (8.69 μmol/ml) and in AML (8.50 μmol/ml). Decreased levels of anti-oxidant enzyme highlighted the impaired anti-oxidant system and ROS accumulation. SOD levels were assessed on the basis of disease sub-type, ALL-T had more accelerated values (0.17 ng/ml) than the values of the other groups (AML 0.15 ng/ml and ALL-B 0.14 ng/ml) respectively. In the case of ALL-T the levels of GSH were 2.38 mg/dl and slightly greater than AML levels of GSH (2.33 mg/dl), while the value of ALL-B type was less than the rest of the others (2.15 mg/dl) respectively. Decreased plasma levels of reduced GSH were due to the over-production of ROS. The non-enzymatic antioxidant may take up by the hematopoietic cells to minimize the oxidative stress. Reduced GSH expression is common in acute leukemia patients such as in the case of ALL. The levels of catalase of ALL-T were more elevated than other sub-types (0.83 μmol/mol of protein) while the other groups have ALL-B (0.73 μmol/mol of protein) and AML (0.77 μmol/mol of protein). Similarly in the case of NO, ALL-T the values were more elevated (20.83 μmol/L) than other groups AML (20.26 μmol/L) and ALL-B (19.71 μmol/L) respectively.

Table 2 Correlation among various circulating biomarkers in subtypes of leukemia.

Group	Correlation coefficient (r)	P value
MDA vs SOD	0.457*	0.001
MDA vs CAT	0.251	0.079
MDA vs GSH	0.021	0.886
MDA vs ALT	0.456*	0.001
SOD vs CAT	0.383*	0.006
SOD vs GSH	−0.061	0.672
GSH vs Vit. E	−0.285*	0.045
CAT vs Phosp	0.310*	0.028
ALT vs AST	0.631*	0.000
ALT vs Vit. E	−0.329*	0.02
ALT vs Ca	−0.344	0.015

* Significant $P < 0.05$).

4. Discussion

The present research work is based on two acute sub-types of leukemia; ALL (ALL-T & ALL-B) and AML. Oxidative stress is caused due to the imbalance, which is present among the ROS generation and antioxidant activity (Ott et al., 2007). Antioxidants are working in the human body in the form of antioxidant defense system against the ROS generation. Enzymes, which include GPx, SOD and catalase, have the ability to degrade the superoxide into H_2O_2 (Paes et al., 2001). Lipid peroxidation is accelerated by the oxidation of lipid; as a result the production of MDA is accelerated. The elevated levels of MDA act like a marker of oxidative stress in the human body in the form of lipid peroxidation (Termini, 2000). The plasma MDA levels act as an important biomarker of leukemia having diagnostic and prognostic role indicating disease progression (Ahmad et al., 2008). Serum protein carbonylation and MDA levels are higher which indicates that the ALL patients have impaired plasma antioxidant status. In case of AML increased amount of ROS and decreased concentration of antioxidant enzymes are an important feature of AML relapse (Zhou et al., 2010).

In the present study results shows that MDA level increases as compared to the control/normal which is a marker of oxidative stress. The antioxidant enzyme impairment in leukemia and in other cancers is a prominent feature. The coefficient of correlation in MDA vs SOD, $r = -0.476^*$, which shows that O_2^- may be produced in this disease/condition, and resultantly the H_2O_2 is the end product of this cascade; So, H_2O_2 must be neutralized and catalase is the main player for this entity as reflected by the correlation coefficient (SOD vs CAT $r = 0.383^*$), increases to minimize the oxidative stress. It is clear that the correlation coefficient of MDA vs CAT, $r = 0.251$ which is not as much higher. It reflects that some other mechanism/pathways are also involved to minimize the oxidative stress possibly GPx is involved to minimize the oxidative stress. MDA vs GSH, $r = 0.021$ GSH levels were also decreased as compared to the MDA, have an insignificant correlation between MDA and GSH (0.86) (Table 2).

The present study is in agreement with the previous studies that in the case of leukemias including ALL and AML the levels of SOD are lower than the normal patients. In case of disease the impaired antioxidant system is the primary cause behind the accumulation of ROS. The accelerated amount of ROS further affects a number of growth and proliferation pathways. SOD vs GSH, $r = -0.061$ have inverse as well as weak correlation, due to the impairment of antioxidant system. There was an insignificant correlation (0.672) observed between SOD and GSH. And SOD vs CAT $r = .383^*$ have weak correlation coefficient.

The antioxidant enzymes and essential micronutrients are in minute amount in leukemics that is a first line of defense especially in CML patients (Pande et al., 2012). GSH deficiency may indeed be responsible for the immunological non-responsiveness under the excessive antigenic stimulation by non-professional stimulator cells. Reduced GSH is an important key factor and has an important role as scavenger of ROS and an important constituent of the thiol pool. The major key role of GSH is to work as a non-enzymatic reducing agent to support the key cysteine thiol side in the reduced state on the surface of the protein. GSH is involved in DNA synthesis and repair. In the case of leukemia reduced levels of GSH reflect the depletion of non-enzymatic antioxidant reserve. It reflects that the GSH depletion, normally acts as an antioxidant factor, while decreased plasma levels of GSH may be due to the over-production of ROS in hematopoietic cells. ROS play critical role in tumor metastasis. ROS are capable of oxidizing different target molecules such as protein kinase C (PKC) and protein tyrosine phosphatase (PTPs) which have an important role in tumor invasion. Mitogen activated protein kinase (MAPK) and p21 regulated by ROS and Philadelphia chromosome which is produced by the fusion proteins. All these factors reflect that these signaling pathways interrelate with the up-regulated expression of growth and proliferation (Ahmad et al., 2008).

GSH and vitamin E have a coefficient correlation, GSH vs Vit. E, $r = -0.285^*$ is weak and inverse correlation between them, and are statistically significant ($P = 0.045$). So, it reflects that GSH and vitamin E are involved in antioxidant activity and impairment in physiological system causes disease. ROS are involved to cause the tissue injuries. An increased amount of the ROS causes cell death by apoptosis or by necrosis (Xavier et al., 2012). Research suggests that a small amount of the catalase is normally present in tissues (Marklund et al., 1982). The levels of anti-oxidant enzymes are significantly decreased in the case of acute leukemic subject erythrocytes. In simple words it is described as antioxidant level decreases and accumulation of oxidative stress in the form of ROS is evident (Demir et al., 2010). The levels of antioxidant enzymes, which include catalase, GPx and SOD, are lower in lymphocytes of ALL patients than control/normal (Mates and Sanchez-Jimenez, 2000). The reduced activity of the selenium dependent enzyme GPx in blood is associated with the increased risk of poor prognosis of cancer. The phosphorylation cascade leads to the activation of MAPKs and NF-κB (Mates and Sanchez-Jimenez, 2000). SOD and catalase activity reduced in the case of chronic leukemias as compared to the GPx activity. Zhou et al. (2010) reported that that the activity of catalase and GPx is reduced in the case of acute leukemias as compared to the control/normal individuals due to the impaired antioxidant status in serum.

Blood urea nitrogen (BUN) behaves as an important marker in disease state. BUN levels were elevated in leukemia patients compared to the control group ($P < 0.005$). Research

studies describe that not only in acute leukemias but also in chronic leukemias like in case of CLL (chronic lymphocytic leukemia) pneumonia, BUN was a major pulmonary complication. Patients have higher levels of BUN and severe neutropenia which were correlated with the mortality of leukemia patients (Ahmed et al., 2003).

Antioxidant enzymes interact with the free radicals in different ways, by controlling the chain of reactions. The example of the antioxidant includes SOD, CAT, GPx, vitamin A, E, and uric acid. In the case of leukemic cells higher levels of ROS are produced than normal cells. Deepti et al. (2012) reported that the vitamin E and A along with SOD, CAT, GPx, are significantly reduced in leukemia. So, these finding suggest that antioxidant enzymes along with vitamin E and A protects the biological system against ROS. In leukemia, levels of vitamin E are lower than normal control persons, not only in the case of ALL and AML but also in CLL and CML. One main reason behind this is the malnutrition because the under-nourished persons have impaired immune and hematopoietic systems. Oxidative stress in leukemic patients is another basic causative factor for the deficiency of vitamin E correlated with the elevated accumulation of ROS in the biological system. Segal et al. (2010) reported elevated levels of transaminases and the causative factors behind that accelerated expression are, high WBC count at diagnosis, bulky disease, older age and T-cell leukemia. Like in the case of ALL elevated transaminases are due to hepatic injury from leukemic infiltrates (Segal et al., 2010).

5. Conclusion

It is concluded from this study that enhanced levels of oxidative stress (MDA) and decreased levels of enzymatic (SOD, GPx and CAT) and non-enzymatic antioxidants (vitamin E and GSH) reflect pathological state and impaired cell control in subtypes (ALL and AML) of leukemia depending upon the alterations in genetic makeup as well as have a strong correlation with oxidative stress, showing that patients' biological systems are under oxidative stress. Levels of antioxidants must be regularly evaluated during the treatment of patients with leukemia.

Conflict of interest

The authors declared that they have no conflict of interest.

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