BioChemMed

Technium BioChemMed Vol. 3, Issue 1 pp.22-33 (2022)
ISSN: 2734-7990
www.techniumscience.com

Evaluation of Tumor Necrosis factor-alpha level in acute leukemic Sudanese patients with pancytopenia

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ABSTRACT

Objectives: To evaluate intracellular and extracellular TNF-alpha in pancytopenia 'patients with acute leukemia Sudanese patients. Also, to calculate the sensitivity of flow cytometry compared to ELISA technique for extracellular level.
Methods: This was a prospective case-control laboratory-based study. The technical work was carried out at the Khartoum Centre of Flowcytometry and University of Khartoum, University of Medical Laboratory Sciences. The sample size was 150; 50 leukemia, 50 leukemia with pancytopenia, and 50 were control groups. Complete blood count (CBC) to confirm selection was evaluated by flow cytometry for CD3, CD4, and CD34, and intracellular TNF-alpha was measured by flow cytometry and extracellular TNF-alpha ELISA. All the participants gave informed consent, approval from Khartoum Flowcytometry Centre, and clearance from the University of Khartoum, Faculty of Medical Laboratory Sciences. Data management and analysis using SPSS.
Results: The factor significantly associated with leukemia and leukemia with pancytopenia was age (p-value 0.00) and presence of disease, either AML or ALL (p-value 0.00). The flow cytometer significantly detected intracellular TNF-alpha (p-value 0.012). However, it mainly detected extracellular TNF-alpha in leukemic patients, leukemia patients with pancytopenia, and the healthy control group (p-value 0.01). The sensitivity of flow cytometry (97.5%) is significant (p-value 0.00) for the detection of TNF-alpha in leukemia patients and thus more sensitive than ELISA (79.9%).
Conclusion: Flow cytometry is an effective method for detecting TNF-alpha in patients with leukemia and leukemia with pancytopenia. We recommend that flow cytometry be offered in public hospitals and made accessible to patients.

Keywords: Leukaemia, pancytopenia, fowcytometry, EELISA, TNF-alpha.

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1.INTRODUCTION

Lymphomas, leukemias, myeloproliferative neoplasms, plasma cell dyscrasias, histiocytic tumors, and dendritic cell neoplasms are the hematopoietic malignancies with significant morbidity and mortality. (1). Worldwide acute leukemia is among the top diagnosed malignancies (2). Leukemia is the second most common cancer (3). Cytokines play a pathological role in developing and progressing hematologic malignancies. Tumor necrosis factor-alpha (TNF-α), one of the best-characterized cytokines, was discovered in mouse serum during endotoxemia and is known for its anti-tumor activity (4).

Tumor necrosis factor-alpha (TNF-α), one of the best-characterized cytokines, was discovered in mouse serum during endotoxemia and was known for its anti-tumor activity (5). In 1984, the TNF gene was identified (6), ushering in the era of clinical trials. The location of TNF is on chromosome 6p21.3. It is expressed primarily by activated macrophages, NK cells, and T lymphocytes, but other cell types (e.g., fibroblasts, astrocytes, Kupffer cells, smooth muscle cells, keratinocytes, and tumor cells) have also been shown to express it (7).

Pancytopenia develops because of a decrease in hematopoietic cell production as in aplastic anemia, normal cells in the hypertrophied and overactive reticuloendothelial system, and hypersplenism ineffective hematopoiesis in megaloblastic, or displacement by abnormal or malignant tissue in the bone marrow (8). Acute myeloid leukemia is the second most common cause of pancytopenia after megaloblastic anemia (9). In addition, pancytopenia has been reported in approximately 8-12% of patients from ALL, and 2% of patients had hypocellular bone marrow, as noted by Makhija et al. (10).

Flow cytometry allows rapid analysis of numerous characteristics of individual cells, and the information obtained is qualitative and quantitative. Flow cytometers are powerful tools for analyzing the influx of bioparticles (11). Due to the success of conventional flow cytometers and advances in microfluidics, microflow cytometers have attracted considerable attention from researchers (12) and are widely used for bioanalysis (13).

The most popular method for determining cytokine levels is enzymatic immunoassay (ELISA). However, flow cytometry can also be used to determine intracellular cytokine levels (14).

In practice, knowledge of tumor marker data should contribute to a decision that leads to a better outcome, including prolongation of overall survival, prolongation of disease-free survival, and improvement of quality of life.

This study will evaluate intracellular and extracellular TNF-alpha in patients with acute leukemia in Sudan. It will also calculate the sensitivity of flow cytometry compared to the ELISA technique for extracellular levels.

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2.Methodology

2.1 Study design and study settings: This was a prospective case-control study laboratory-based study. Technical was done in Khartoum Centre of Flowcytometry and University of Khartoum, College of Medical Laboratory Sciences.

2.2 Study population: The study population of this research was:

2.2.1 Patients: Patients diagnosed of acute leukemia with pancytopenia regardless of its type and before treatment.

2.2.2 Controls: include acute leukemic patients without pancytopenia and before treatment.

2.2.3 Healthy volunteers Are appearantly healthy individuals without leukemia or other chronic diseases.

2.3 **Sample Size: The following** formula was used for sample size calculation, the confidence level of 95% and degree of precision 0.04.

$$n= (t2 \times p(1-p)) / m2$$

Using the equation mentioned above the sample size was calculated. Since the flow cytometry technique has high sensitivity and specificity and high expenses, we will enroll 50 cases of acute leukemia, 50 patients with acute leukemia who develop pancytopenia, and 50 healthy volunteers.

2.4 **Inclusion criteria:**

2.4.1 Patients diagnosed with acute leukemia with pancytopenia were included as cases, and those who did not develop pancytopenia were included as controls.

2.4.2 Only patients or healthy controls who agreed to sign the informed consent were included in the study.

2.5 Exclusion criteria:

The patients having the following features were excluded from the study.

2.5.1 Chronic leukemia.

2.5.2 Patients started a treatment program.

2.5.3 Patients refuse to sign the informed consent.

2.5.4 Pattients with chronic diseases, especially liver and renal disease

2.6 Samples collection: The investigator collected venous blood and bone marrow samples from each patient.

2.7 Techniques: The collected peripheral blood and Bone marrow samples, following the steps mentioned below:

2.7.1 Complete Haemogram (mainly Leukocyte analysis)

2.7.2 Lymphocytes separation

2.7.3 The expression of the markers CD3, CD4, tumor necrosis factor-alpha (TNF-alpha with levels were measured with four-color flow cytometry

2.8 Complete Haemogram: Each sample was analyzed for complete haemogram, using Automated Hematology Analyzer Sysmex XK-21N, according to standards of manufacturer protocol.

2.9 ELISA principles: ELISA technique was employed to assess the extracellular TNF- alpha level in serum. The method followed the manufacture protocol (Quantikine Human TNF-alpha Immunoassay). ELISA compined the specificity of the antibody with the sensitivity of the sample enzyme assay by antibodies or antigens. ELISA can provide a valuable measurement of antigene or antibody concentration.

2.10 Data analysis: Data was analyzed by SPSS after coding and data management. Descriptive analysis, frequency, correlation, cross-tabulation, and Odds ratio were applied.

2.11 Ethical consideration: The ethical consideration of this research includes each of the following processes:

2.11.1 A signed written or oral consent was obtained the patient.

2.11.2 Khartoum Flowcytometry Centre approval and acceptance.

2.11.3 Ethical clearance from University of Khartoum, Faculty of Medical Laboratory Sciences.

3. RESULTS

In this research total number of participants was 150. They were 50; 50 leukemia, 50 leukemia with pancytopenia, and 50 were control groups. Table 1 showed that most of the participants were female 84 (56%). The majority of the participants were in the age group 65 (43.3%). However, less than 18 years were 50 (33.3%), and more than 60 years old were 14 (9.3%). The factor significantly associated with Leukaemia and Leukaemia with pancytopenia was age (p-value 0.00) and the presence of the disease either AML or ALL (p-value 0.00).

Table 1 and figure 1 showed the distribution according to the clinical presentation at the time of sample taken for investigations by flow cytometry. The study population's main presentation was fever as the primary clinical symptom 64 (64%). Other presentations were fatigabilities 47 47) %), lymphadenopathy 36 (36%), organomegaly 34 (34%), and bleeding disorders and tendency 18 (18%). Other's presentation was 14 (14%), including; abdominal distension, proptosis, dizziness, loss of appetite, and cough. There was a significant statistical association between the clinical presentation and leukemia and leukemia with pancytopenia (p-value > 0.05).

Table 2 shows the flow cytometer measurements of intracellular and extracellular TNF-alpha. The Intracellular TNF-alpha among the leukemic patients 3.245±14.08 (min 0- max 91.8), in leukemia with pancytopenia 0.194±0.44 (min 0- max 0.194), compared with the control group 0.194±50 (min 0- max 0.194). Regarding the extracellular TNF-alpha in the leukemic patients, it was 189.43±127.8 (min 14- max 389), in leukemia with pancytopenia 125±139.7 (min 1- max 125), compared with the control group 260.9±322 (min 64- max 2331).

The flow cytometer was significantly detecting intracellular TNF-alpha (p-value 0.012). However, it mainly detected the extracellular TNF-alpha in leukemic patients, leukemic with pancytopenia, and the healthy control group (p-value 0.01).

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Flow cytometer measurements of CD3, CD4, CD34, and TNF details shown in table 4 and figure 2. Flow cytometer was significantly detected CD3 (p-value 0.00) and CD4 (p-value 0.04). While not significantly detected CD34 (0.34) and TNF (0.12) (figure 2).

Table 3 showed the detailed D-dote measurements of CD34/3, CD4/TNF, CD3/4, and CD3/TNF among the study and control groups. The flow cytometer significantly detected CD4/TNF (p-value 0.01) and CD3/4 (p-value 0.01). However, no significant association in the measurement of CD34/3 (p-value 0.47) and CD3/TNF (p-value 0.15) of the control and study groups.

Figure 3 shows the comparison between the flow cytometry and ELISA in detecting intracellular and extracellular TNF-alpha. Flowcytometry is more sensitive and stable, while the ELISA measurement varied and ranged from one sample to another.

The sensitivity of flow cytometry (97.5%) is significantly (p-value 0.00) detect the TNF-alpha among leukemic patients, more sensitive than ELISA (79.9%) (Table 4).

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Table 1sociodemographic data and existence of the disease among the study group.

Characteristics	**Variables**	**Leukemia** **N (%)**	**Leukemia with Pancytopenia** **N (%)**	**Control** **N (%)**	**Total** **N (%)**	**p-value**
Gender	Male	18 (12)	22 (14.7)	26 (17.3)	66 (44)	0.27*
	Female	32 (21.3)	28 (18.7)	24 (16)	84 (56)	
Age	Less 18	23 (15.3)	27 (18)	0 (0)	50 (33.3)	0.00*
	19-30	7 (4.7)	5 (3.3)	9 (6)	21 (14)	
	31-60	15 (10)	11 (7.3)	39 (26)	65 (43.3)	
	> 60	5 (3.3)	7 (4.7)	2 (1.3)	14 (9.3)	
Disease	AML	17 (11.3)	23 (15.3)	0 (0)	40 (26.7)	0.00*
	ALL	33 (22)	27 (18)	0 (0)	60 (40)	
	Normal	0 (0)	0 (0)	50(33.3)	50 (33.3)	

* Qui square test

AML acute myeloid Leukaemia

ALL acute lymphoid Leukaemia

Table 2: The flow cytometer measurements of Intracellular and extracellular TNF-alpha

Group		**Intracellular TNF-alpha By flowcytometry**	**Extracellular TNF-alpha By ELISA**
Leukemia	Mean	3.245	189.430
	N	49.000	44.000
	Std. D	14.086	127.871
	Min	0.000	14.000
	Max	91.800	389.000
Leukemia with Pancytopenia	Mean	0.194	125.950
	N	50.000	44.000
	Std. D	0.444	139.737
	Min	0.000	1.000
	Max	0.194	125.950
Control	Mean	0.476	260.920

	N	50.000	50.000
	Std. D	2.122	322.205
	Min	0.000	64.000
	Max	13.700	2331.000
Total	Mean	1.292	195.090
	N	149.000	138.000
	Std. D	8.234	226.978
	Min	0.000	1.000
	Max	91.800	2331.000
p-value		**0.012**	**0.01**

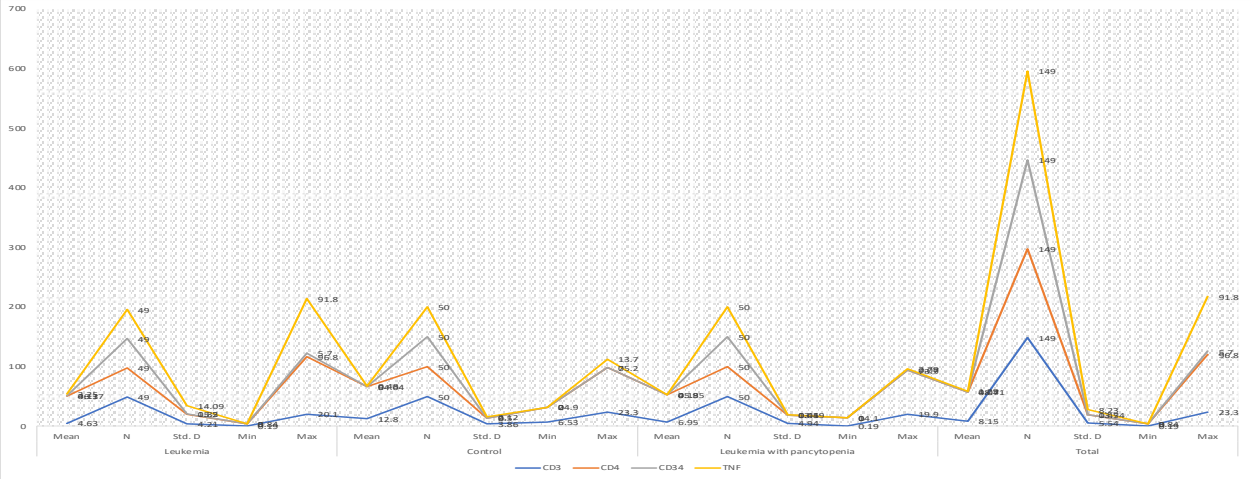

Figure 1: Flowcytometry measurements CD3, CD4, CD34 and TF among the leukemic, normal and Leukemia with pancytopenia patients

Table 3: The flow cytometer D-dote for the measurements CD34/3, CD4/TNF, CD3/4 and CD3/TNF among the study and control groups

Study group		**CD34/3**	**CD4/TNF**	**CD3/4**	**CD3/TNF**
Leukemia	Mean	0.00	60.35	2.61	6.14
	N	47.00	50.00	50.00	50.00
	Std. D	0.00	151.01	4.80	18.78
	Min	0.00	0.00	0.02	0.00
	Max	0.01	908.50	32.00	116.00
Control	Mean	0.01	182.90	16.13	1.90
	N	50.00	50.00	50.00	50.00
	Std. D	0.00	308.97	31.74	1.06
	Min	0.00	0.00	0.74	0.40
	Max	0.03	998.50	218.00	5.90

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Leukaemia with Pancytopenia	Mean	0.02	82.35	26.95	2.92
	N	50.00	50.00	50.00	50.00
	Std. D	0.13	189.29	65.39	5.76
	Min	0.00	0.00	0.14	0.17
	Max	0.90	982.50	305.00	30.00
Total	Mean	0.01	108.53	15.23	3.65
	N	147.00	150.00	150.00	150.00
	Std. D	0.07	231.39	42.95	11.42
	Min	0.00	0.00	0.02	0.00
	Max	0.90	998.50	305.00	116.00
P-value		0.47	0.01	0.01	0.15

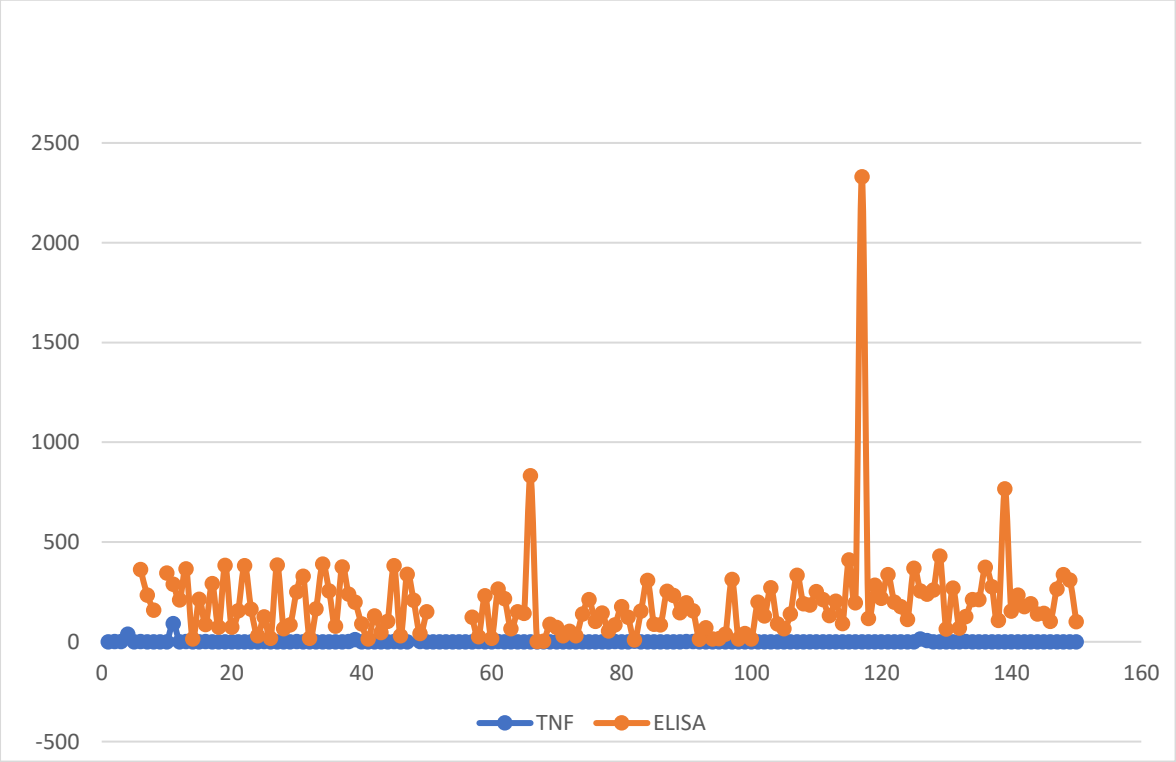

Figure 2: sensitivity and specificity of Flow Cytometry and ELISA in detecting intracellular and extracellular TNF-alpha

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Table 4: The sensitivity of the Flow Cytometry and ELISA in detecting the TNF-alpha

ANOVA					Sensitivity
		Sum of Squares	Mean Square	Sig.	
ELISA	Between Groups	3498525.0	38445.3	0.998	79.9%
	Within Groups	3559608.8	77382.8		
	Total	7058133.8			
TNF-Flow cytometer	Between Groups	9882.1	105.1	0.000	97.5%
	Within Groups	152.6	2.8		
	Total	10034.6			

4.Discussion

This research was carried out to evaluate the level of Tumor Necrosis factor-alpha in patients in acute Leukaemia with pancytopenia among Sudanese patients by using a flow cytometer, ELISA, and complete blood count. And the effectiveness of these techniques in detecting intracellular and extracellular TNF-alpha and determining the sensitivity and specificity of flow cytometry in detecting intracellular TNF-alpha, compared to ELISA technique for the extracellular level.

Intracellular and extracellular TNF-alpha were evaluated by the flow cytometer and ELISA in this study. The measurement of intracellular TNF-alpha (0.194) versus extracellular TNF-alpha (125.950) in Leukemia with Pancytopenia group, compared with (3.245) intracellular TNF-alpha and (189.430) extracellular TNF-alpha in the leukemic group. While in the control group was 0.476 and 260.920, respectively. The flow cytometry and the ELISA were significantly detected the TNF-alpha either intracellular or extracellular (p-value <0.05). The values of extracellular TNF-alpha By ELISA were higher than the value of intracellular TNF-alpha by flow cytometry. This difference in reading is possible because there are other sources of TNF-alpha in extracellular. However, flow cytometry only detects the intracellular TNF-alpha.

Sun and colleagues (15). Pathologically elevated TNF-α levels have a negative effect on the efficiency of muscle cell differentiation. These findings agree with those of Meyer (16), who suggested that TNF-α levels are elevated in muscle wasting and chronic muscle degeneration and regeneration processes characteristic of primary muscle diseases. Explain the relationship between TNF-α and the immunopathophysiology of aplastic anemia (AA) and other syndromes of bone marrow failure (BM).

All the cutoff levels of TNF-alpha among the Leukemia, Leukemia with Pancytopenia, and control groups showed significant associations with the age and existence of the disease (p-value 0.00). Also, there was a significant statistical association between the clinical presentation and leukemia and leukemia with pancytopenia (p-value > 0.05).

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This result consisted of the study done among Sudanese patients (17). This also reflects the role of age, and its well-established immune status is not clear and fluctuates with age and brain region (18).
As potent producers of TNF-alpha could be identified, thus implicating the malignant population in the pathogenesis of hematopoietic failure due to inappropriate secretion of this cytokine.
These findings can be justified within different age groups of the study population, and even the case group includes leukemia and Leukaemia with pancytopenia.
In this study, flow cytometer significantly detected CD3 (p-value 0.00) and CD4 (p-value 0.04), CD34 (0.03), TNF (0.01) and CD4/TNF (p-value 0.01) and CD3/4 (p-value 0.01). These results are consistent with the hypothesis that AA CD34+ cells, which likely include hematopoietic progenitor cells, express high levels of the Fas receptor due to in vivo exposure to IFN-γ and TNF-α and are suitable targets for T cell-mediated killing (19). Many studies have confirmed these associations (20,21,22).

The same study groups (Leukaemia, leukemia with pancytopenia and control) their completed blood count was assessed with a focus on the white blood cell and differential cells. There was a significant association between the study groups among all the group regarding all cells parameters except for monocytes. It has been proven that CBC is an important predicting factor for detecting leukemia and its complications (23,24).
In this study, the sensitivity of flow cytometry (97.5%) significantly (p-value 0.00) detect the TNF-alpha among leukemic patients, which is more sensitive than ELISA. The sensitivity of flow cytometry in this study is comparable with that of others, which indicated comparable sensitivity of (25,26). The possible difference may be due to differences in reagents, gating, and staining techniques, and thresholds for positivity may also account for the discrepancy in the relevant studies.

5. Conclusion

Flow cytometry is an effective method for detecting TNF-alpha in patients with leukemia and leukemia with pancytopenia. We recommend that flow cytometry be offered in public hospitals and made accessible to patients.

Limitations

The limitations of this study were; the study was expensive, and reagents were challenging to obtain, especially under the current circumstances in Sudan and the flow cytometry is not widely available in Sudan. In addition, to that the availability of the sample (leukemia with pancytopenia), which was not on treatment, and was difficult.

Acknowledgement

The author would like to thank the Khartoum Flow Cytometry Center and Al Rayan Center for their valuable contribution to this work. I extended our thanks to Dr. Elhadi Miskeen for his technical assistant.

Fund: This research was not funded

Conflict of interest: Not reported

Author contribution:

Both authors made important contributions. They were equally involved in the conception, study design, implementation, data collection, analysis, and interpretation. Both reviewed the final draft, approved it, agreed on the journal, and agreed to be responsible for all aspects of the work.

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