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Review Article

The Role of Adiponectin in Prostate Cancer: A Narrative Review

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ABSTRACT

Prostate cancer (PCa) is the most common type of cancer among men over 60 years old. The aggressiveness and mortality of PCa can be correlated with obesity. Adipose tissue-derived cytokines such as adiponectin may explain the correlation between PCa and obesity. Since the correlation between adiponectin and aggressive PCa is still not fully evaluated, we aimed to investigate the probable role of adiponectin in PCa. Adiponectin is considered as a link between obesity, insulin resistance and diabetes. On the other hand, adiponectin is a key mediator of systemic insulin sensitivity and glucose homeostasis. Moreover, low level of adiponectin is associated with inflammation and angiogenesis. These processes could promote tumor growth. Special effects of adiponectin are mediated via adenosine monophosphate-activated protein kinase (AMPK). AMPK activation inhibits growth of androgen-independent and androgen-sensitive PCa cell lines. Moreover, c-Jun N-terminal protein kinase (JNK) and Signal transducer and activator of transcription 3 (STAT3) signaling pathway are known as adiponectin's mediators on the metabolic syndrome and cancer. Furthermore, adiponectin acts as a tumor suppressor gene via inhibition of Epithelial-to-mesenchymal Transition (EMT) of PCa cells, but it is down regulated through hypermethylation of promoter gene in PCa cells. Therefore, according to the results of these studies, decreased concentration of adiponectin was associated with increased risk of PCa. It seems that hypoadiponectinemia may act as a promising biomarker for detection and diagnosis of PCa.

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Introduction

One of the most fatal diseases in human beings is cancer which leads to an annual death of 30000 people in Iran.[1,2] The most common type of cancer among men older than 60 years is prostate cancer (PCa).[3] PCa is the second leading cause of cancer death among American men. The highest incidence of PCa is in the United States, Canada, and northwestern Europe, but less common in Asian countries and South America so that 9% of all cancer-related deaths are among men within the European Union.[4] Since androgens play a main role in smooth muscle proliferation associated with the development of PCa, men with increased risk of PCa may show higher risk of cardiac disease.[4] Patients with PCa are often treated with androgen deprivation therapy (ADT).[5] Tumor biomarkers like prostate specific antigen (PSA), acid phosphatase (ACP) and prostatic acid phosphatase (PAP) can be used for early diagnosis, staging and monitoring of the disease.[3] Decreasing level of prostatic biomarkers in serum of PCa patients could serve as an indicator of response to the treatment.[3] Nowadays, several risk factors contributing to the development of PCa have been identified.[4] Moreover, numerous studies have shown that the aggressiveness and mortality of PCa could be correlated with obesity.[6] Adiponectin is considered as a potential biological link between obesity and PCa, but there is paucity of epidemiological data confirming this association.[7] Therefore, the aim of the current review was to assess the relationship between obesity, adiponectin, and PCa.

Adiponectin

Adiponectin is a product of the APM1 gene[8] located on

chromosome 3q27.[9] It is a 244 aminoacid polypeptide with 30 KDa molecular weight which is widely synthesized and secreted by adipose tissues.[9-15] Studies have shown a link between obesity, insulin resistance and diabetes which play an important role in diabetes and cardiovascular disease.[9]

Serum concentrations of adiponectin is in the range of 2 to 20 mg/L.[5,16,17] The mean level of adiponectin concentration is 1000 times higher than leptin and cortisol level and 1000000 times higher than cytokines like interleukin-6 and TNF-α.[18] The level of adiponectin in men is about 40% lower than in women.[16,19] It seems that it is due to androgens, since androgens have an inhibitory effect on adiponectin secretion.[17,18,20] Moreover, circulating levels of adiponectin are determined through several genetic, anthropometric, hormonal, inflammatory, dietary, and pharmacological factors.[21]

Adiponectin contains three domains including a signal peptide, a collagen-like motif and a globular domain. Moreover, circulating adiponectin exists in at least two forms; low molecular weight (LMW) oligomer that is a hexamer of two trimers and high molecular weight (HMW) oligomer containing four- six trimmers.[9] Plasma adiponectin concentrations are inversely correlated with fasting plasma insulin levels.[18] Expression of adiponectin mRNA takes place exclusively in adipose tissues;[21] however, bone, mammary glands, salivary glands and cardiac tissue may also express limited amounts of adiponectin.[10,22-24] Maturation and secretion of adiponectin is controlled through a mechanism called "thiol mediated retention",[25] so that endoplasmic reticulum chaperones such as endoplasmic reticulum protein with molecular weight of 44 kDa (ERp44) and endoplasmic reticulum oxidoreductin 1-like alpha (Ero1-La) are induced during adipogenesis.[25]

Adiponectin Receptors

Adiponectin acts via binding to main receptors; adiponectin receptor 1(AdipoR1) and adiponectin receptor 2 (AdipoR2).[26] AdipoR1 and AdipoR2 encoding genes are located on chromosomes 1p36.13-q41 and 12p13.3, respectively.[16] Expression of these receptors (AdipoR1 and AdipoR2) has been detected in monocytes, and megakaryocyte cell lines.[26]

Adiponectin and Signaling Pathway

Adiponectin inhibits inflammation by suppressing the phagocytic activity of mature macrophages and induces apoptosis in vitro.[7] Adiponectin works through many intracellular signaling pathways such as AMPK, mechanistic target of rapamycin (mTOR), Phosphatidylinositol 3-kinase (PI3K/Akt), mitogen activated protein kinase (MAPK), STAT3 and Nuclear factor-κB (NF-κB).[18] Adiponectin also inhibits the pro-inflammatory pathways via inhibition of NF-kB phosphorylation. Inhibition of NF-κB via adiponectin plays a main role in suppression of monocyte adhesion to endothelial cells.[20] Most of the effects of adiponectin are mediated via AMPK.[18] AMPK activation also inhibits growth of androgen-independent and androgen-sensitive PCa cell lines.[27] AMPK also inhibit FAS (a key lipogenic enzyme), which has been associated with colon, breast, prostate and ovarian cancer (Figure 1).[18]

The JNK and STAT3 signaling pathway are known as adiponectin's mediators in metabolic syndrome and cancer. STAT3 is activated through adipokine or cytokine-induced JAK phosphorylation, so that many cancer-related processes including cell survival and differentiation are connected to adipokine pathways. Therefore, JAK/STAT3 pathway dysregulation leads to carcinogenesis.

Another study is reported that endogenous level of adiponectin acts as a tumor suppressor via inhibition of epithelial-to-mesenchymal transition (EMT) of PCa cells, but it is down regulated through hyperrmethylation of promoter gene in PCa cell.[28]

Adiponectin and PCa

Comments of previous researchers about the role of adiponectin and obesity in PCa have been shown in Table 1.

Most of the studies showed the role of obesity in PCa except Baillargeon et al study.[34] They reported that due to small sample size they did not observe any relationship between obesity and PCa.

Obesity and Adiponectin in PCa

The relationship between obesity and aggressive PCa is still not fully evaluated. Obesity may be associated with

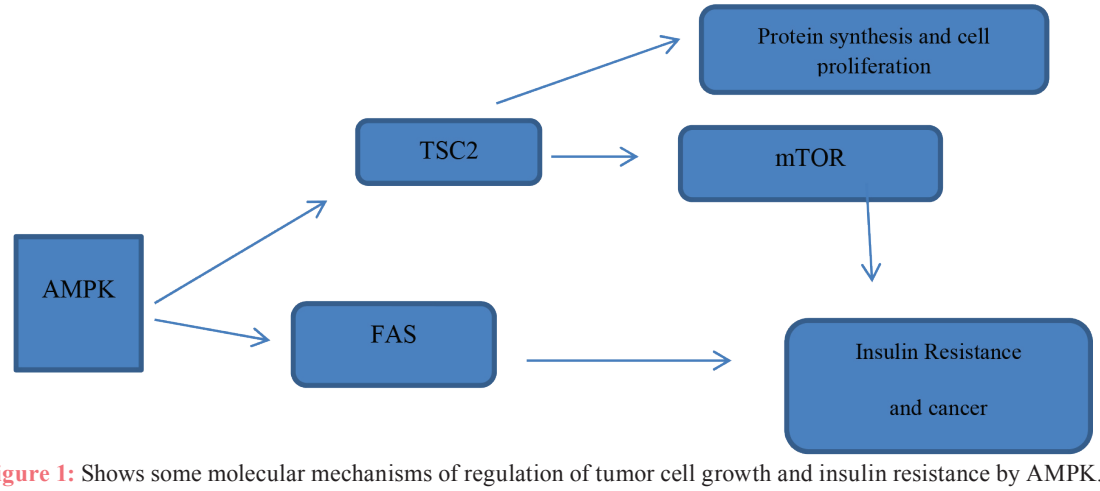

Figure 1: Shows some molecular mechanisms of regulation of tumor cell growth and insulin resistance by AMPK.[18]

Table 1: Comments of researchers about the role of adiponectin and obesity in PCa

Study	Explanations	Population	References
Stephan (2005)	Obesity was associated with altered level of adiponectin.	Maryland	29
Goktas (2005)	Plasma adiponectin level was significantly lower in the PCa group in comparison to the group with benign prostate disorders or controls.	Turkey	9
Housa (2006)	Circulating level of adiponectin is inversely associated with the risk of malignancies.	Prague	30
Bub (2006)	Adiponectin at physiological concentrations inhibited both androgen-dependent and androgen-independent PCa cell growth in vitro.	USA	31
Baillargeon (2006)	There was no significant association between obesity with PCa risk due to small sample size.	USA	32
Michalakis (2007)	Lower concentration of plasma adiponectin was found among PCa patients compared to healthy men.	Greece	33
Buschemeyer (2007)	Obesity and lower level of adiponectin increased the risk of PCa.	USA	34
Sher (2008)	Lower serum adiponectin was independently associated with high-grade PCa.	USA	35
Housa (2008)	Increased level of adiponectin has a protective effect against tumor progression in cancer patients.	Prague	36
Mistry (2008)	Low level of adiponectin may be important in driving obesity-related PCa progression.	United kingdom	37
Fontana (2009)	Adiponectin may acts as an anti-prostatic factor. Obesity may promote progression of PCa.	Argentina	38
Freedland (2010)	Higher adiponectin concentration was associated with a lower risk of PCa in comparison to controls.	North Carolina	39
Li (2010)	Men with higher adiponectin concentrations presented lower risk for developing high-grade or metastatic cancer.	USA	7
Dimmer (2010)	There was no association between Single Nucleotide Polymorphism (SNP) and PCa risk.	USA	40
Dhillon (2011)	SNP of ADIPOR1/R2 genes was not significantly associated with PCa risk.	USA	41
Alokail (2011)	There was inverse association between adiponectin level and total PSA.	Saudi Arabia	42
Lu (2012)	Lower level of adiponectin in obese people was associated with prostatic oxidative stress.	Canada	43
Izadi (2012)	There was negative correlation between adiponectin level and PCa.	Iran	44
Tewari (2013)	Higher BMI and obesity was significantly correlated to PCa.	India	45
Gao (2014)	Adiponectin suppresses proliferation but doesn't affect apoptosis of human prostate adenocarcinoma cell line.	China	46
Ikeda (2015)	Inverse correlation was seen between adiponectin and BMI.	Japan	47
Baslan (2015)	Globular adiponectin and full-length adiponectin was decreased in obese and insulin-resistant rats.	Brazil	25

increased risk of advanced disease and death in PCa patients.[27] Several factors can increase the risk of tumor initiation and progression. Environmental factors such as diet with higher content of saturated fat and obesity itself can be connected to PCa. The consumption of fat is concordantly correlated to PCa mortality rate.[36] It seems that high level of dietary fat stimulates PCa cells proliferation. Obesity and diabetes together lead to hyperinsulinemia and may promote the risk of PCa development.[27] Insulin resistance can alter the risk of PCa via diverse biologic pathways such as obesity-sex hormone pathway and non-obesity-related pathways.[9,20] Insulin resistance can increase the risk of PCa via non-obesity-linked mechanisms like inflammation, oxidative stress, and apoptosis. Therefore, it seems that many factors such as insulin resistance and obesity may be related to PCa.[9]

Obesity causes increased level of free Insulin-like growth factor (IGF-1) which stimulates growth of prostate cell lines in vitro. Moreover, obesity is related to advanced stage of PCa in humans and leads to elevated level of serum interleukin-6 in adipose tissues.[27] PCa cell line and PCa patients have been shown to be capable of increased production of IL-6 and IL-6 receptor. Circulating level of IL-6 is associated with progression of metastatic disease.[47] In addition, human PCas expressed high amounts of leptin receptor.[27,48] Moreover, studies have shown that the relationship between leptin and PCa is contradictory. Some studies have found a positive correlation between serum leptin level and PCa risk,[49,50] but others have found no association.[39] Adipose tissue-derived cytokines like adiponectin could explain the association between PCa and obesity.[50] Adipokines may provide a molecular mechanism whereby obesity exerts its effects on prostate tumor biology.[38] It seems that obesity affects prostate tumor biology via exposing of prostate cells to circulating adipokines. Adiponectin plays a main role as a molecular basis for the association between obesity and PCa.[37]

Adiponectin also increased sensitivity of insulin in various stromal and epithelial cells.[9] It seems that adiponectin has potent insulin-sensitizing actions.[27] On the other hand, adiponectin is a key mediator of systemic insulin sensitivity and glucose homeostasis. The main metabolic effects of adiponectin are suppression of hepatic glucose production and modulation of suppressing inflammatory responses in other cell types including macrophages.[25] Another study reported that the anti-neoplastic activity of adiponectin may be explained possibly by decreasing insulin resistance and hyperinsulinemia.[51]

Low levels of adiponectin could result in higher inflammatory states and angiogenesis. These processes promoted tumor growth.[39] Another study reported that the effect of adiponectin on neovascularization remains contradictory, so that it has both pro and anti-angiogenic effects.[27] Moreover, adiponectin mRNA expression is decreased in adipose tissues. This change is related to a higher risk of diabetes evolution.[39] Therefore, it is hypothesized that decreased level of adiponectin may underlie the association between PCa and obesity/ insulin resistance.[39]

Another study showed that there is inverse relationship between metformin and serum prostate-specific antigen in PCa, independent of other anti-hyperglycemic medications.[51] Metformin improves PCa recurrence and survival rate.[52] Metformin as an anti-diabetic drug caused activation of AMPK and inhibited growth of PCa cells.[7] Antineoplastic mechanism of metformin can be due to inhibition of mTOR in the PI3K/AKT/mTOR pathway.[53,54] Moreover, metformin has a main role in decreasing level of gene expression involved in mitosis.[55] Therefore, it seems that metformin therapy leads to a better prognosis in patients with PCa.[56]

Conclusion

According to the ample studies in the literature, decreased concentration of adiponectin was associated with increased risk of PCa. Therefore, it seems that hypoadiponectinemia may be a promising biomarker for early detection of PCa.

Conflict of Interest: None declared.

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Brain MRI Findings in Children with Acute Lymphoblastic Leukemia

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ABSTRACT

Background: Patients with leukemia are facing more complications in order to achieve longer survival. We aimed to evaluate the frequency of central nervous system abnormalities (CNS) on MRI of children with acute lymphoblastic leukemia (ALL).

Methods: Sixty-six children with diagnosis of ALL aged 2-18 years were recruited. Non-contrast sequences of brain MRI in addition to diffusion weighted imaging of brain were obtained with 1.5 T (Siemens medical system) scanners in their maintenance phase of treatment. The age of onset, type of leukemia, protocol of treatment, and elapsed time from diagnosis were recorded. Chi-square test was used to compare the groups and t-test was used to evaluate the effect of not normally distributed variables.

Results: 19 (28.8%) had abnormal CNS findings identified on MRI images including: nonspecific white matter high signal intensity in flair images with normal DWI, white matter ischemia proved on DWI, generalized brain atrophy, isolated mild enlargement of lateral ventricle and extracerebral complications including sinus thrombosis and sinusitis. Brain abnormalities were correlated with leukemia type, chemotherapy protocol and radiotherapy (P=0.006, 0.036, and 0.01, respectively).

Conclusion: The wide spectrum of CNS abnormalities that were observed in children with ALL showed correlation with treatment methods and type of leukemia in this study. Combination of radiation therapy and chemotherapy increased CNS complications. Among extracerebral complications, dural sinus thrombosis proved by MRV was seen more frequently in T-cell leukemia patients treated with multiple high doses of the chemotherapy agent "L-asparaginase". Since some neurological complications of leukemia are treatable, early diagnosis sounds essential.

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Introduction

Leukemia is the most common cancer in children and has increased in frequency in the twentieth century.[1] The most common signs and symptoms include fever, anemia, thrombocytopenia, hepatosplenomegaly, and lymphadenopathy.[2] Acute lymphocytic leukemia (ALL) comprises about two-third of cases, while acute myeloblastic leukemia (AML), chronic myelogenous leukemia (CML), and juvenile myelomonocytic leukemia (JMML) are less prevalent subtypes in children. Despite the current improvements in diagnosis and treatment, induction failure and relapse has been reported in up to

10% of the cases.[3] The improved cure rate and survival of the patients has caused the complications to become more noticeable.[4] Chemotherapy, radiotherapy and stem cell transplantation in specific cases are the main therapeutic options for childhood leukemia,[5] which in turn have their own complications and adverse effects that might have some impact on patients' survival.[6,7] Among the most important complications are central nervous system (CNS)-related, as they mainly affect child's cognition and neuropsychological function.[8] Damage to normal brain tissue is hypothesized to be mainly due to treatments such as irradiation.[9] In addition, neurotoxicity and leukoencephalopathy is proposed to be induced by some chemotherapy drugs especially systemic and intrathecal methotrexate (MTX) injections.[10-13] Studies have confirmed beneficial role for imaging modalities such as magnetic resonance imaging (MRI) and computed tomography (CT) in diagnosing CNS abnormalities.[14,15] Evaluating these complications thoroughly, in terms of incidence, risk factors, diagnosis, and treatment reveals the priority of each treatment modality for physicians and researchers.

We aimed to evaluate the frequency of CNS abnormalities on MRI of patients with ALL which may help diagnosis and management of their neurological side effects in a timely manner.

Materials and Methods

In this cross-sectional study, 66 children of 2-18 years with diagnosis of ALL based on flowcytometry analysis of bone marrow aspirate were recruited. They were visited regularly in an outpatient pediatric oncology clinic affiliated to Shiraz University of Medical Sciences in Shiraz, south of Iran. The patients were at different time courses of their chemotherapy treatments and had been treated for at least 6 months. The treatment strategy was determined based on the type of leukemia and the risk category. Some patients had received cranial irradiation if they had CNS involvement or prophylactically in cases of T-cell ALL. Those with underlying neurological and metabolic diseases were excluded from the study.

Non-contrast sequences of the brain MRI, obtained with 1.5 T (Siemens medical system) scanners, in addition to diffusion weighted imaging of brain were obtained in the maintenance phase of treatment and the results were recorded by a single radiologist.

The frequency of abnormalities on the patients' brain MRI were determined according to the age at diagnosis, type of leukemia, treatment protocol and elapsed time from treatment. Moreover, it was assessed whether radiotherapy had an additional effect on brain MRI including intra and extracerebral findings.

Data were analyzed by SPSS software, version 21. Descriptive data were presented as mean, standard deviation, frequency and percentage. Qualitative and quantitative variables were compared by Chi-square test and Student t-test, respectively between two groups of patients. Level of significance less than 0.05 was considered statistically significant.

Results

The study group included 66 patients (42 boys, M/F ratio 1.75), with the mean age of 5.74±3.65 years, (range 15-192 months). The mean time elapsed from treatment until MRI assessment was 27.85±14.62 months. The clinical characteristics as well as the treatment protocols of the studied population are demonstrated in table 1.

As identified on MRI (Figure 1), sinusitis with or without retention cyst was the most common finding. Air-fluid level and mucosal thickening more than 2 mm was considered as criteria for diagnosis of sinusitis on MRI (30.2%, n=20).[16] Other extracerebral findings included mastoiditis (n=6) and otitis media (n=1). Nineteen patients (28.8%) had CNS complications including: nonspecific white matter high signal intensity in flair images with no associated DWI abnormalities (n=7), brain atrophy (n=6), white matter acute ischemia approved with DWI (n=5), sinus thrombosis on MRV (n=4), and mild enlargement of lateral ventricles without any dilatation of extra-axial fluid (n=3). Some patients showed more than one abnormal MRI finding. There was also one case with incidentally found a parietal arachnoid cyst most probable having no relation to the patient's treatment.

The patients were treated according to ALL-BFM protocols for pre-B and T-cell leukemia.[17,18] They were all in their maintenance phase of treatment which included monthly injections of vincristine, oral mercaptopurine, weekly methotrexate and boosts of prednisolone for 5

Table 1: Clinical characteristics of the study population

	Number	%
Leukemia type		
Pre B ALL	58	87.9
T cell ALL	8	12.1
Seizure		
Yes	8	12.1
No	58	87.9
Chemotherapy protocol		
Standard risk pre-B ALL	50	75.8
High risk pre-B ALL	9	13.6
T cell ALL	7*	10.6
Radiotherapy treatment		
Yes	11	16.7
No	55	83.3

*One T cell ALL patient was treated with high risk pre-B ALL protocol

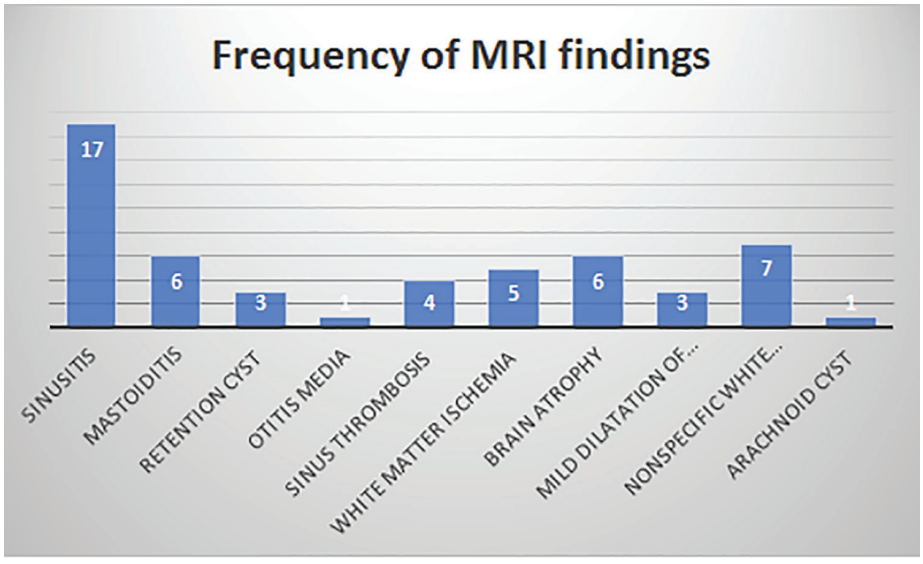

Figure 1: Frequency of MRI findings in the study population

days after each vincristine injection. They also received intrathecal injection of methotrexate every two months. Eleven cases were also given craniospinal irradiation; including 8 cases with T-cell ALL prophylactically, and 3 patients with pre-B cell ALL who had CNS involvement.

The patients were classified into two groups based on their MRI finding. Those with normal brain imaging and extracerebral findings were classified as group 1, while those with CNS abnormalities were classified as group 2.

Table 2 demonstrates the association between MRI findings and different variables including age at diagnosis, age at the time of imaging, gender, type of leukemia, treatment protocols, time elapsed since diagnosis/treatment, and radiation therapy between the two treatment groups.

There was a significant correlation between leukemia type, chemotherapy protocol and brain abnormalities on MRI (P=0.006 and 0.036, respectively), indicating more abnormalities in T-cell leukemia. Radiotherapy was also associated with more brain abnormalities (P=0.01). Among extracerebral findings, thrombosis of dural sinus was seen more frequently in T-cell ALL patients (P=0.009), while other MRI findings were not correlated with the type of leukemia (table 3). Moreover, there was no significant correlation between gender, age, treatment duration and seizure (seen in 8 cases) with brain abnormalities on MRI (table 2).

Table 2: Comparison of clinical and demographic characteristics between the two groups of leukemia patients with and without CNS MRI findings

Variables	Without MRI findings N=47	With MRI findings N=19	P value
Gender			
Male	29	13	0.779
Female	18	6	
Leukemia type			
Pre B cell ALL	45	13	0.006*
T cell ALL	2	6	
Seizure			
Yes	4	4	0.099
No	43	15	
Chemotherapy protocols			
Standard risk pre-B ALL	38	12	0.036*
High risk pre-B ALL	7	2	
T cell ALL	2	5¶	
Radiotherapy			
Yes	4	7	0.01*
No	43	12	
Age (month) (mean±SD)	95.1±45.9	112.3± 49.8	0.183
Age at diagnosis (month) (mean±SD)	63.1±41.8	83.2±47.6	0.094
Treatment duration (month) (mean±SD)	28.1±13	27.4±18.7	0.866

¶ One patient with T-cell ALL was treated with high risk pre-B –cell ALL protocol

Table 3: MRI findings in the study population with regards to their diagnosis

	Pre-B ALL N=58 Number (%)	T cell ALL N=8 Number (%)	P value
Thrombosis	1 (1.7)	3 (37.5)	0.005*
White matter disease	10 (17.2)	2 (25)	0.611
Brain atrophy & CSF enlargement	6 (10.3)	3 (37.5)	0.326
Extra CNS	23 (39.6)	4 (50)	0.702

Extra CNS: sinusitis, mastoiditis, otitis media, retention cyst

Discussion

The male to female ratio in our study was 1.75, which was similar to another Iranian study, assessing 368 cases that reported a male/female ratio of 1.5.[2] Among 66 cases who were assessed by MRI, 40 patients (60.6%) had MRI abnormalities, but 19 cases showed MRI findings which might be attributed to the treatment. Chen and colleagues have also reported 12 cases of brain abnormalities among 19 cases on MR and CT scans.[19]

Ulu and colleagues have retrospectively evaluated CNS imaging findings of 15 patients with acute leukemia. They found that most of abnormalities (17/22) were attributed to the disease itself, and a minority (4/22) were treatment related. The most common complications in decreasing order of frequency were meningeal leukemia, orbital chloroma, posterior reversible encephalopathy syndrome (PRES), retinal and intracranial hemorrhage.[20] While we did not observe any documented case of leukemic meningitis, 34.3% of our patients had extracerebral abnormalities, with sinusitis being the most prevalent finding.

Some authors have attributed the cerebrovascular complications such as sinus thrombosis to the chemotherapy agent L-asparaginase.[19,21-23] Others have also ascribed white matter brain changes to MTX, which was not associated with neuropsychological deficit.[24] There was not any case of periventricular complication probably due to MTX among our patients.

Our results did not show any association between the type of leukemia and brain abnormalities, except dural sinus thrombosis which was seen more frequently in T-cell ALL patients who had received multiple doses of high-dose L-asparaginase in their treatment regimen. Cerebral venous thrombosis is a unique adverse effect of "L-asparaginase" which has been reported in up to 3% of cases.[21,25] It has been ascribed to the depletion of anticoagulant proteins mainly antithrombin III. The dose, frequency and preparation of the drug have been proposed to play some role in its coagulant activity.[21,25,26] Nevertheless, as our patients had not been treated with high-dose MTX, and they were all receiving low-dose oral weekly MTX, no conclusion could be reached on the association of MTX and brain abnormalities.

It was noted previously that leukemic infiltration of the meninges and orbits were the most common CNS findings in Ulu's patients, although their patients had a wide age range (1-22 years) and the sample size was too small (15 patients). Meanwhile, Chen and colleagues have reported the most common early CNS complications to be cerebrovascular and infections.[19] They also concluded in a case report that leukemic infiltration of paranasal sinuses should be considered in cases with sinusitis.[27] Thus, infection should be highly considered in patients with leukemia undergoing treatment in a timely manner which may prevent functional brain sequels in these children.

Chan et al. have reported cerebral hemorrhage as the most common finding in ALL cases, 15% of which were detected by T2 MRI and was mainly evident only on gradient echo imaging.[28] Chu and colleagues have also used MR spectroscopy and have concluded that it demonstrates metabolic changes in brain after high-dose MTX, which was not obvious by normal MRI.[29] Regarding different imaging methods used in different centers, which may not completely reveal the associated pathology, periodic neurocognitive tests are recommended for such children to prevent cognition deficits.[15] We found no case of intracerebral hemorrhage in our study.

Moreover, combination therapy (radiation plus chemotherapy), performed for patients with T-cell ALL or patients with CNS leukemia gave rise significantly to more brain abnormalities on MRI. Chan et. al used radiation for 58% of their cases and established the significant relationship between hemorrhage and radiation.[28] Although they had used different imaging methods which might cause them to reach to a different conclusion in terms of complications, we have also found a significant association between radiation and brain abnormalities. Vazquez et. al have also demonstrated that radiation leads to white matter disease, microangiopathy, vascular malformations, telangiectasia and secondary neoplasms.[30] Thus, it is suggested to pay more attention to patients undergoing radiation in order to diagnose and treat the complications earlier. Furthermore, some experts recommend to replace radiation with safer alternatives such as high-dose MTX to avoid its late side effects particularly in growing children.[31,32]

In the current study, 8 out of 66 studied patients reported to have experienced seizure at least once, but only half of them occurred during their present illness. Seizure was observed in two patients with T cell leukemia with sagittal sinus thrombosis. Another patient who had seizure was a boy with pre-B cell leukemia who developed decreased level of consciousness, headache, visual symptoms and convulsion in the induction phase of chemotherapy, which was attributed to PRES confirmed by MRI. He was found to have hypersignal images in deep white matter of left parietal and occipital lobes about 18 months after the incident. The last one was a case of pre-B cell ALL who presented with fever, convulsion and hemiparesis in the induction phase of treatment. MRI showed a large brain

abscess at that time which was successfully treated with drainage and broad-spectrum antibiotics. His MRI at the time of our study which was a few years later showed severe cortical atrophy.

This study investigated the detailed information of children with leukemia, and aimed to give physicians and researchers a broader view on CNS complications or abnormalities detected by MRI. In the current study sinusitis was the most common extracerebral pathology. This finding is clinically important due to devastating ophthalmological and neurological complications in the immunocompromised children.[33] Moreover, MRI abnormalities especially dural sinus thrombosis were observed in T-cell leukemia which emphasizes the necessity of more frequent CNS monitoring in this group of leukemia patients. Additionally, patients receiving adjuvant radiotherapy had higher brain abnormalities. Therefore, families should be informed about the long-term sequels of treatments and radiotherapy. The long-term sequels in particular, might adversely affect children's quality of life including academic performance. Taking into account the high frequency of adverse effects associated with anti-leukemic treatments, some investigators have recommended to consider some refinements in treatment modalities.[34,35]

To our knowledge, this is the first local study investigating CNS findings detected by MRI in pediatric patients with ALL. As a result, in parallel to concerns regarding the intensity of the treatment and quality of life of the patients, safety of the treatments and the long-term consequences are noteworthy as well.

Our study had also some flaws and limitations in some aspects. We did not follow the patients to study the outcome of each of these complications in long-term. Moreover, we did not assess the psychosocial and cognitive consequences of CNS abnormalities. In addition, the patients were not studied by the imaging modalities before starting any kind of chemotherapy at the beginning of thir disease and hence, the abnormalities detected on MRI could not be attributable exactly to the treatment. Larger cohort studies with longer periods of follow up and doing imaging studies at baseline are needed to disclose the outcome of these lesions and their impact on the quality of life and cognitive function and neurologic status of the survivors of leukemia.

The choice of treatment in each patient was also based on the physician's experience; as there are different local treatment strategies, which make the comparison between study groups difficult. Multicenter clinical trials with similar treatment protocols are advised to overcome these shortcomings.

In addition, our study included pediatric ALL patients, and patients with AML were not investigated. As patients with AML receive different chemotherapy drugs from ALL patients, and their treatment usually lack radiotherapy and intrathecal or high-dose systemic MTX, investigating CNS related side effects in this subgroup of patients may reveal diverse MRI abnormalities.

In conclusion, the wide spectrum of CNS abnormalities occurring during or after treatment of leukemia could be related to the treatment protocol or leukemia subtype. Additional radiotherapy also increases cerebral and exrtacerebral complications. Dural sinus thrombosis was associated with multiple doses of "L-asparaginase".

As long as most of the neurologic complications can be debilitating while treatable, early diagnosis by imaging methods and cognitive tests is essential.

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ORIGINAL ARTICLE

The Difference in Initial Leukocyte Count, Bone Marrow Blast Cell Count and CD 34 Expression in Patients with Acute Myeloid Leukemia with and without NPM1 gene Mutation

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ABSTRACT

Background: Mutation in NPM1 gene has been reported to be the most common genetic mutation in de novo acute myeloid leukemia (AML). AML with NPM1 gene mutation usually presents with higher initial leukocyte and blast cell counts and negative CD34 expression. We aimed to investigate the difference of initial leukocyte counts, bone marrow blast cell counts and expression of CD34 among patients with AML with and without NPM1 mutation.
Methods: In this study, 25 de novo patients with AML were investigated for NPM1 exon 12 gene mutation using ASO-RT-PCR. Initial leukocyte counts, bone marrow blast cell counts and expression of CD34 on blasts were examined in all patients.
Results: 13 of 25 de novo patients with AML (52%) had NPM1 gene mutation. Initial leukocyte counts in AML patients with NPM1 gene mutation was not significantly higher than patients without this mutation (23.400 /µL versus 16.000 /µL, P=0.53). Blast cell counts were not significantly higher in AML patients with NPM1 gene mutation than patients without mutation. (41% versus 19%, P=0,18). Expression of CD34 was not significantly different between AML patients with and without NPM1 gene mutation (P=0.48).
Conclusion: There were no difference in initial leukocyte count, blast cell count and CD34 expression among patients with AML with and without NPM1 exon 12 type A gene mutation.

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Introduction

Acute myeloid leukemia (AML) is a heterogenous disease with various clinical features and several genetic abnormalities. The incidence of AML is reported to be 3.7 per 100.000 persons in the world.[1] It has proposed that age of the patients, leukocyte count at diagnosis, bone marrow blast cell count and expression of CD34 are important prognostic factors in AML patients.[2,3] Based on cytogenetic and molecular abnormalities, AML can be classified into low, intermediate and high risk groups. Low risk AML is defined as patients with normal karyotype with mutated NPM1 and no FLT3 gene mutation. Intermediate risk group is defined as abnormal karyotype, such as +8 and all other combinations of NPM1 and FLT3 gene mutation. High risk group of AML patients have high risk cytogenetic features such as inv (3)(q21q26), t(3;3) (q21;q26), monosomy 7, monosomy 5, 5q-, 7q-, 11q23, t(9;11) and complex karyotype with >3 abnormalities.[4] In 40-50% of patients with AML with normal karyotype, various clinical and prognostic factors have been reported. It can be related to some genetic mutations such as NPM1, FLT3, and CEBPA

gene mutations that also contribute to the pathogenesis of AML. The exon 12 NPM1 gene mutation is a common genetic mutation in AML (35% in de novo AML and 45% in the case of AML with normal cytogenetic).[5] There is evidence that mutation in exon 12 NPM1 gene in AML is correlated with leukocyte count at diagnosis, bone marrow blast cell count and CD34 expression on blast cells. Patients with NPM1 gene mutation have reported to have higher leukocyte counts and bone marrow blast cell counts than patients without this mutation. Patients with NPM1 gene mutation have also down regulated CD34 expression.[2,3,6] In this study, we investigated the difference of leukocyte count, bone marrow blast cell count and CD34 expression among AML patients with and without exon 12 NPM1 gene mutation.

Materials and Methods

This study was approved with ethical clearance by the Ethical Committee, Faculty of Medicine, Airlangga University. Inform consent was taken from the patients. Bone marrow aspirates of 25 newly diagnosed patients with AML were collected from January 2015 to August 2015 from hospitals in Surabaya and Indonesia. The study was done in the department of biochemistry and clinical pathology, faculty of medicine, Airlangga University, Dr Soetomo general hospital Surabaya. Diagnosis of AML was made on the bone marrow aspirate cytology, considering cut off point of 20% blast cell count to establish the diagnosis. AML subtypes were defined based on FAB (French American British) criteria. Immunophenotyping by flowcytometry was used to confirm the diagnosis and determine the subtype of AML. The specimen was also studied for detection of exon 12 NPM1 gene mutation.

We extracted mRNA with RNA extraction kit (Trizol® LS reagent Invitrogen Cat: 10296-010) according to manufacturer's instruction. Exon 12 NPM1 gene mutation was detected by allele specific (ASO) RT-PCR (Reverse Transcriptase Allele Specific Polymerase Chain Reaction). Extract of mRNA was incubated in 55°C for 30 minutes with reverse transcriptase enzyme (Superscript III One Step RT-PCR with Platinum Taq Polymerase "Invitrogen" Cat No: 12574-026) to produce cDNA. We amplified cDNA of NPM1 mutant with RT-AS PCR technique. Amplification (resulting 319 bp amplicon) was achieved after 40 cycles of the following steps: hot start (95°C for 3 minutes), denaturation (94°C for 50 seconds), annealing (60°C for 50 seconds) and extension (68°C for 1 minute). We used forward primer NPM1-AN: 5'CAA-GAG-GCT-ATT-CAA-GAT-CTC-TGT-CTG-3' and reverse primer NPM-Rev6: 5'-ACC-ATT-TCC-ATG-TCT-GAG-CAC-C-3' to detect exon 12 NPM1 gene mutation.[7] Normal primer set was not used because all NPM1 mutations in patients with AML were heterozygote and homozygote states for NPM1 mutation were lethal.

Analysis of leukocyte count at diagnosis was done by ADVIA 2120i hematology analyzer system (Siemens®) as a routine procedure. Bone marrow aspirate blast cell counts were counted among 500 nucleated cells.

CD34 expression was examined in BD (Becton Dickinson®) Facs Calibur Flowcytometry, using anti CD34 monoclonal antibody. We examined the expression of CD34 in population of cells in blast gate with blastic gating strategy (low side scatter, moderate CD45 expression). Isotypic control was used to determine positive marker expression. Statistical analysis was done with SPSS version 22. Mann-whitney U-test was used to determine the difference in leukocyte count and bone marrow blast cell count and Fisher exact X2-test to indicate the difference between expression of CD34 in AML patients with and without exon 12 NPM1 gene mutation.

Results

Among 25 de novo patients with AML, 13 (52%) patients had exon 12 NPM1 gene mutation based on the positive 319 bp fragment in RT-AS PCR result; whereas 12 patients did not show any mutation in this exon. Figures 1 and 2 depict the positive and negative results of RT-AS PCR from de novo AML patients. Positive result was determined based on the positivity of 319bp fragment.

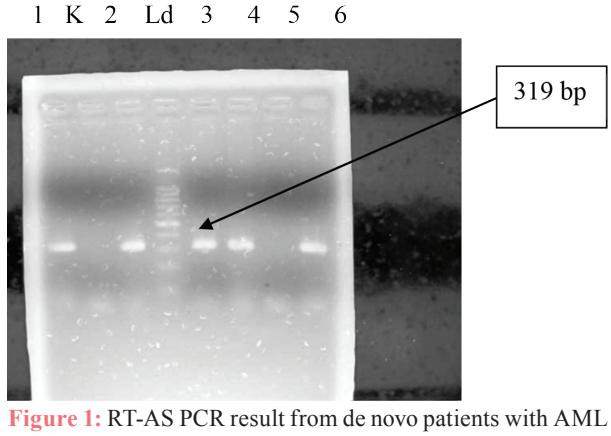

Figure 1: RT-AS PCR result from de novo patients with AML with positive exon 12 NPM1 gene mutation (positive 319 bp fragment in patients number 1,2,3,4,6), negative result in patient number 5, K=Negative control, Ld=DNA Ladder 100bp.

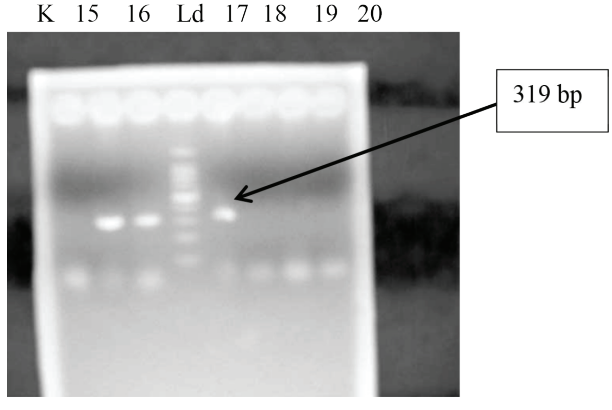

Figure 2: RT-AS PCR result from de novo AML patients with positive exon 12 NPM1 gene mutation (positive 319 bp fragment in patients number 15,16,17), negative result in patient number 18,19,20, K=Negative control, Ld=DNA Ladder 100bp.

The patients' characteristic (sex, age and FAB classification for AML subtype) is shown in table 1.

The initial leukocyte count and bone marrow blast cell

Table 1: Characteristic of AML patients

		Mutated NPM1	Non Mutated NPM1
Sex	Male	9	9
	Female	4	3
Age	Mean	50.6 years	46 years
	Range	17–78 yearrs	36–72 yearrs
FAB classification	M0	0	0
	M1	2	0
	M2	2	2
	M3	3	2
	M4	0	3
	M5	6	4
	M6	0	1
	M7	0	0

count is described in table 2. The median initial leukocyte count in AML patients with mutated NPM1 is 23,400 /µL, and in non mutated NPM1 group is 16,000 /µL (P=0.53). The median bone marrow blast cell count is 41% in AML patients with mutated NPM1 and 19% in non mutated NPM1 group (P=0.18).

The distribution of CD34 expression is shown in table 3. In AML patients with mutated NPM1, the CD34 expression can be seen in 6 patients (from total 8 mutated NPM1 patients) and in non mutated NPM1 group, CD34 expression can be found in 5 patients (from total 5 patients) (P=0.48) .

Discussion

The median initial leukocyte count in AML patients with exon 12 NPM1 mutation was 23,400 /µL which was higher than median leukocyte count (16,000/µL) in patients without this mutation, but not statistically significant. Previous studies reported that leukocyte counts were significantly higher in AML patients with mutated NPM1 gene than non-mutated group. This difference might be explained by other genetic mutations such as mutation in the FLT3-ITD and CEBPA mutation that could have some correlation with the leukocyte count at diagnosis. The other explanations for such dissimilarities were infectious complications of the patients and different frequency of mutations in geographic areas where the study was performed.[2,3,6,8]

The median bone marrow blast cell count in AML patients with exon 12 NPM1 mutation was 41% which was higher than in patients without this mutation (19%), but statistically was not significant. This result was different from previous studies reporting bone marrow blast cell counts were significantly higher in patients with AML with mutated NPM1 gene. Again, this difference might be interpreted by existence of other genetic mutations or difference in geographic area. Studies have reported that AML patients with higher bone marrow blast cell counts had a worse prognosis.[8,9] Based on French-American-British classification for AML, the majority of AML patients with exon 12 NPM1 gene mutation in this study were AML-M5 (46,2%). Other studies reported the same results they stated that AML patients with mutated NPM1 had a trend of differentiation to myelomonocytic series.[2,3]

The CD34 expression was not statistically different in AML patients with and without exon 12 NPM1 gene mutation. This result was different from the previous studies. Cauhan et al. reported that NPM1 gene mutation was significantly correlated with negative expression of CD34 and HLA-DR. Falini et. al described that 90-95% of their AML patients with mutated NPM1 gene were negative for CD34 expression. The possible causes proposed were other genetic mutations. Another possibility for lack of correlation between CD34 expression and NPM1 gene

Table 2: Leukocyte count and bone marrow blast cell count in patients with and without exon 12 NPM1 gene mutation

Parameter	Mutated NPM1	Non-mutated NPM1	P value
Leukocyte count ($\times 10^3/\mu L$)			
mean±SD	28.1±19.6	23.2±17.2	0.53
median	23.4	16	
range	6.3–65	5.9–42	
Bone marrow blast cell count (%)			
mean±SD	43.8±25.1	30.2±26.6	0.18
median	41	19	
range	9-75	4-72	

Table 3: The CD34 expression in patients with and without mutated NPM1 gene

	Mutated NPM1	Non mutated NPM1	P value
$CD34^+$	6 patients	5 patients	0.48
$CD34^-$	2 patients	0 patients	

mutational status in our study was the number of patients with acute promyelocytic leukemia that comprised almost 25% of the studied patients. In normal myeloid progenitor cell development, CD34 is mainly expressed on myeloblasts. This expression will be decreased in later maturation stages such as promyelocytes. In acute promyelocytic leukemia, most cells in bone marrow consist of promyelocytes that do not express CD34.[2,6] Martelli et. al reported that lack of expression of CD34 in AML with mutated NPM1 is caused by down regulation of CD34 gene expression. It is also related with the increment of HOX gene expression. In AML patients with mutated NPM1 gene, nucleolus and ribosomal stress cause down regulation of CD34 expression. Meanwhile, the positivity of CD34 expression has inverse correlation with the rate of complete remission. AML patients with CD34 expression show longer time to achieve complete remission than patients who lack its expression.[10]

Limitations of this study were small sample size and lack of data of other genetic mutations and cytogenetic abnormalities that could affect initial leukocyte counts, bone marrow blast cell counts and CD34 expression. In addition, infection status, ethnicity and genetic polymorphism of the patients are also required in order to determine all contributing factors.

Conclusion

Initial leukocyte counts, bone marrow blast cell counts and CD34 expression were not statistically different in the patients with and without exon 12 NPM1 gene mutation.

Conflict of Interest: None declared.

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Assessment of Cytotoxicity of Dimethyl Sulfoxide in Human Hematopoietic Tumor Cell Lines

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ABSTRACT

Background: Dimethyl Sulfoxide (DMSO) is a solvent most broadly used as a cryopreservative agent. Antitumor effects of DMSO is a recently recognized phenomenon. In this study, cytotoxic effects of DMSO on human monocytes and T leukemic cell lines has been investigated in vitro.
Methods: Human leukemic T cells (Molt-4 and Jurkat) and monocytes (U937 and THP1) were cultured in complete RPMI mediums. The cells at different logarithmic growth phases were incubated with different concentrations of DMSO (0.1, 0.2, 0.5, 1, 2 and 5%). Then viability and proliferative response of leukemic cell lines was assessed by trypan blue dye exclusion (TB test) and MTT assays, respectively.
Results: DMSO has a cytotoxic effect on the leukemic cells used in this study; dose and time-dependently. This cytotoxicity for all of these leukemic cells was shown at ≥ 2% concentrations of the DMSO after 24, 48 and 72 hours' incubation time. Moreover, there was not any significant difference between DMSO cytotoxicity in these different leukemic cell lines.
Conclusion: All of the used leukemic cells showed sensitivity to DMSO at ≥2% concentrations time dependently. This sensitivity significantly increased with time. DMSO might be a cytotoxic agent for leukemic cells. It might be a useful candidate in design of chemotherapeutic protocols for leukemia as well as other cancers.

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Introduction

Dimethyl sulfoxide (DMSO) is a solvent broadly used as a cryopreservative agent.[1,2] DMSO with various chemical characteristics is an appropriate pharmacological carrier for several drugs and materials.[3,4] Moreover, antioxidant and anti-inflammatory effects of DMSO have been determined.[5,6] The inhibitory effects of DMSO on inflammatory cytokine production in autoimmune arthritis have been proposed.[7] Meanwhile, antitumor effects of DMSO have been shown in different studies.[8-10] There are also some roles for DMSO in management of pain in cancers.[11] There are numerous reports of induction of antitumor immunotherapy by DMSO-treatment.[12]

On the other hand, pretreatment by DMSO has been reported to potentiate toxic effect of cisplatin on sensory hair cells.[13] Furthermore, antitumor effects of two ruthenium (II)-DMSO-chalcone complexes has been described.[10] Anticancer activities of two DMSO-complexes have also been demonstrated.[14] Tumor suppressor gene activation, induction of apoptosis and inhibition of the several cancer cells' proliferation by DMSO have also been shown.[8,15,16] Treatment of mouse hepatocellular carcinoma cell line with 2% solution of DMSO depressed proliferation and induced cell cycle arrest with no notable apoptosis or reduced viability.[12] It is clear that enhancement of anti-inflammatory, antioxidant and antineoplastic effects of several drugs and medicinal plants is dependent on their delivery vehicles. DMSO as

a polar solvent can dissolve numerous nonpolar and polar tiny ingredients, enhances cell membrane permeability, avoids free radical development and increases the penetration of pharmaceutical mediators in antitumor drugs through the cells.[17]

Moreover, epigenetic modifications by DMSO have also been reported,[18,19] and the role of epigenetic variations in cancer progress have been discovered.[20,21] In the above mentioned studies, different mechanisms of anti-cancer properties of DMSO such as induction of leukemic cell differentiation, tumor suppressor genes activation, apoptosis induction, inhibition of the various neoplasms proliferation and increase in penetration of pharmaceutical agents have been explored.[15-17] Considering the modulatory epigenetic anti-tumor properties of DMSO and its complexes, in the current study the cytotoxic effects of DMSO on human monocytic and T leukemic cells has been investigated in vitro to find out if anti-cancer effects of DMSO might be somewhat due to its direct cell cytotoxicity.

Materials and Methods

Reagents

RPMI-1640 medium, penicillin, streptomycin, dimethyl sulfoxide (DMSO) and trypan blue (TB) were purchased from Sigma (USA). Fetal calf serum (FCS) was obtained from Gibco (USA) and MTT (3-[4, 5-dimethyl thiazol-2, 5-diphenyltetrazoliumbromide]) kit was purchased from Invitrogen (USA). Microtiter plates, flasks and tubes were purchased from Nunc (Falcon, USA).

Preparation of DMSO

DMSO was dissolved in RPMI-1640 medium and stored at -20°C until use. DMSO was diluted in culture medium to prepare appropriate concentrations before use.

Cell Lines

Human leukemic T cells [Molt-4 (NCBI C149) and Jurkat (NCBI C121)] and monocytes [U937 (NCBI C130)] and THP1 (NCBI C563) were obtained from NCBI (National Cell Bank of Iran, Pasteur Inst. of Iran, Tehran). The cells were maintained in RPMI-1640 medium supplemented with 10% FCS in 5% CO2 at 37°C.

Cell Culture and Treatment

Human leukemic and mouse fibrosarcoma cells were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO2. The cells were seeded at a density of 2×10^{4} cell/well and then incubated with different concentrations of DMSO (0.1, 0.2, 0.5, 1, 2 and 5%) for 24, 48 and 72 hours. All experiments were done in triplicate.

Cell Proliferation Assay

To evaluate the effect of different concentrations of DMSO on viability of leukemic cell lines, we used trypan blue dye exclusion (TB test)[22] and MTT assay.[23]

Trypan Blue Dye Exclusion Test

Principle of trypan blue dye exclusion test is exclusion of dye by viable cells and taking it up by dead cells. Viability is evaluated by direct counting of viable and dead cells. Percentage of the number of viable cells to the total number of cells is considered as viability percentage.

MTT Assay

In MTT test, the conversion of yellow water soluble MTT to a blue-insoluble formazon was assessed according to the method developed by Mosmann.[23] At the end of incubation time, the medium was replaced with 100 µl of fresh medium. The amount of 10 µl of MTT solution (5 mg/ ml in PBS) was then added to each well and incubated at 37°C for 4 hours. Then, 100 µl of the SDS-HCl solution (100 mg SDS was dissolved in 1 ml HCl) was added to each well and incubated at 37°C for 4 hours. The insoluble formazon derivative was dissolved and absorbance at 570 nm was measured using a microplate reader (Awarness Technology INC). The results were expressed as cell numbers per control.

Statistical Analysis

Effect of the DMSO on each cell line was performed in three independent experiments (n=3) and the results were expressed as mean±SD. Statistical comparisons between groups were made by analysis of variance (ANOVA). $P<0.05$ was considered significant. Test of multiple comparison of Tukey was applied (5%) for statistically significant differences. For statistical analysis and graph making the software SPSS-16.0 and Excel 2003 were used respectively.

Results

Cytotoxic effect of DMSO on human leukemic THP1, U937, Jurkat and Molt-4 cells and mouse fibrosarcoma Wehi 164 cells in different concentrations and time intervals are illustrated in figures 1 to 4. In every figure, "A and B" indicate the results of trypan blue dye exclusion and MTT tests, respectively.

Cytotoxic Effect of DMSO on Human Leukemic THP1 Cells

DMSO significantly decreased proliferative response of human leukemic THP1 cells in both staining techniques in all time intervals dose-dependently ($P<0.05$, figures 1A and B). As shown, DMSO significantly diminished the proliferation of THP-1 cells at ≥2% after 24 hours incubation compared with untreated control cells ($P<0.05$). DMSO cytotoxicity at ≥2 % concentration, significantly increased with time in this order: 72h >48>24 h in THP-1 cells ($P<0.05$, figure 1).

Cytotoxic Effect of DMSO on Human Leukemic U937 Cells

According to the results depicted in figure 2A and 2B, DMSO significantly reduced proliferation of human leukemic U937 cells in both staining methods in all time intervals in a dose dependent manner ($P<0.05$). The results displayed in figure 2(A and B) showed that DMSO significantly decreased the proliferation of U937 cells at ≥2 % after 24 hours' incubation in comparison

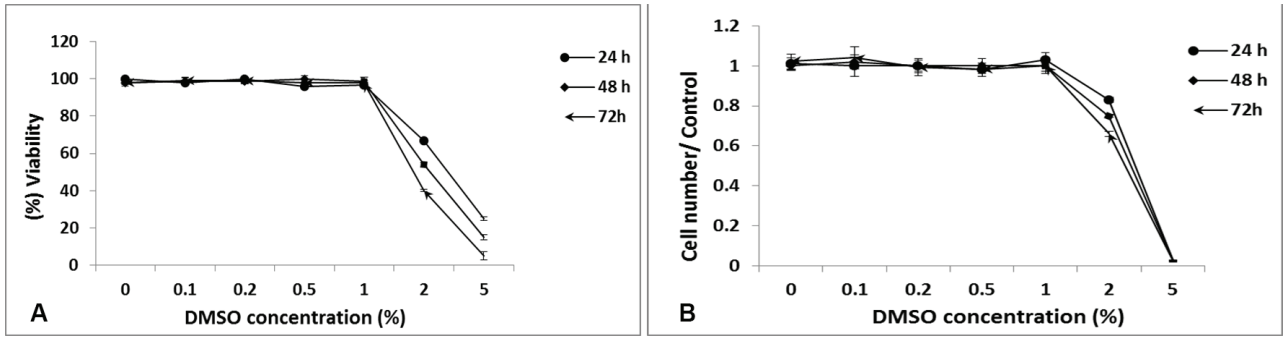

Figure 1: Effect of DMSO on proliferation of human leukemic THP1 cells (A) and (B). The THP1 cells (2×10^4 cell/well) were treated with different concentrations of DMSO (0.1, 0.2, 0.5, 1, 2 and 5%) for 24, 48 and 72 hours. The results are presented as % of viability demonstrated by trypan blue dye exclusion (TB) test (A) and cell number/control demonstrated by MTT assay (B). Data are mean±SEM of three independent experiments. *P<0.05 was considered significant

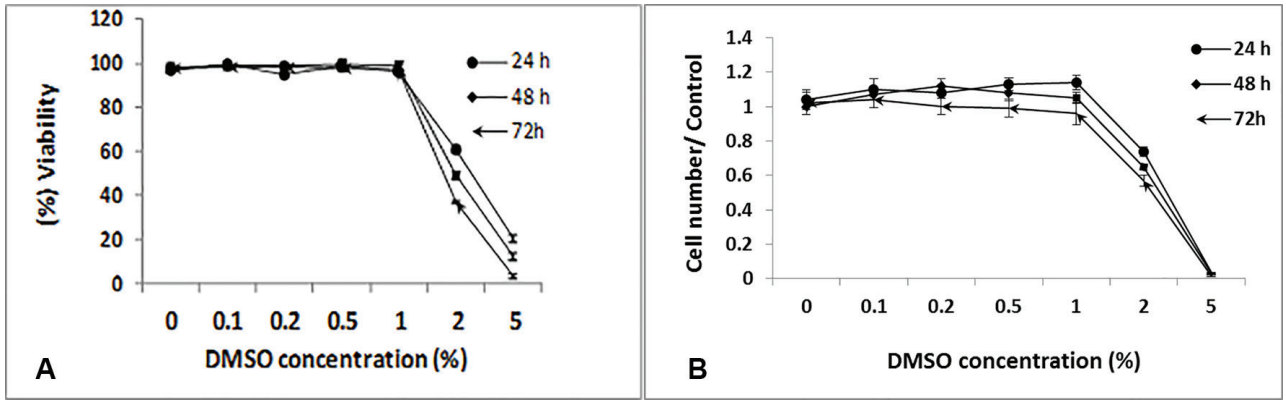

Figure 2: Effect of DMSO on proliferation of human leukemic U937 cells (A) and (B). The U937 cells (2×10^4 cell/well) were treated with different concentrations of DMSO (0.1, 0.2, 0.5, 1, 2 and 5%) for 24, 48 and 72 hours. The results are presented as % of viability demonstrated by trypan blue dye exclusion (TB) test (A) and cell number/control demonstrated by MTT assay (B). Data are mean±SEM of three independent experiments. *P<0.05 was considered significant

with untreated control cells (P<0.05).

DMSO cytotoxic effect at ≥2 % concentration, significantly augmented with time in this order: 72h>48h>24 h in U937 cells (P<0.05, figure 2).

Cytotoxic Effect of DMSO on Human Leukemic Jurkat Cells

DMSO significantly decreased proliferation of human leukemic Jurkat cells in every staining method in all time intervals dose-dependently (P<0.05, figure 3A and 3B). As shown, DMSO considerably diminished the proliferation of Jurkat cells at ≥2% after 24 hours incubation compared with untreated control cells (P<0.05). DMSO cell cytotoxicity at ≥2% concentration, significantly increased with time in this arrangement: 72h>48h>24 h in Jurkat cells (P<0.05, figure 3).

Cytotoxic Effect of DMSO on Human Leukemic Molt-4 Cells

DMSO significantly diminished proliferative response of human leukemic Molt-4 cells by both staining methods in all time intervals in a concentration-dependent manner (P<0.05, figure 4A and 4B). The results exhibited that DMSO significantly decreased the proliferation of Molt-4 cells at ≥2% after 24 hours' incubation compared with untreated control cells (P<0.05). DMSO cytotoxic effect at ≥2% concentration, significantly increased with time in this order: 72h>48h>24 h in Molt-4 cells (P<0.05, figure 4).

Discussion

In the present study, the effects of DMSO on proliferation of four human leukemic cell lines were evaluated. The results of this study showed that DMSO has a cytotoxic effect on the leukemic cells used in this study; dose and time-dependently. This cytotoxicity for all of these leukemic cells was shown at ≥2% concentrations of the DMSO after 24, 48 and 72 hours' incubation time. Moreover, there was not any significant difference between DMSO cytotoxicity in these different leukemic cell lines.

Justo and colleagues reported that DMSO had not any significant effect on proliferation of mouse peritoneal macrophages at ≥ 0.1%.[17] Similarly; in our study, DMSO did not show any cytotoxicity at 0.1% concentration on proliferation of leukemic cells. We also demonstrated that DMSO has no significant cytotoxicity on human leukemic cell lines at <2% concentration; however, Justo and co-workers had not studied the DMSO effect at >0.1% concentrations.[17] Furthermore, the decrease of clonogenic ability in some normal and leukemic cells by DMSO at >2% concentration has been shown in vitro.[24] Elisia and co-workers showed that DMSO significantly reduced monocyte viability at 2% concentration.[7] However, Elisia and colleagues used normal cells, but we assessed DMSO cytotoxicity effect on leukemic cell lines. DMSO cytotoxicity at less than 10% concentration has also been reported in vivo.[25]

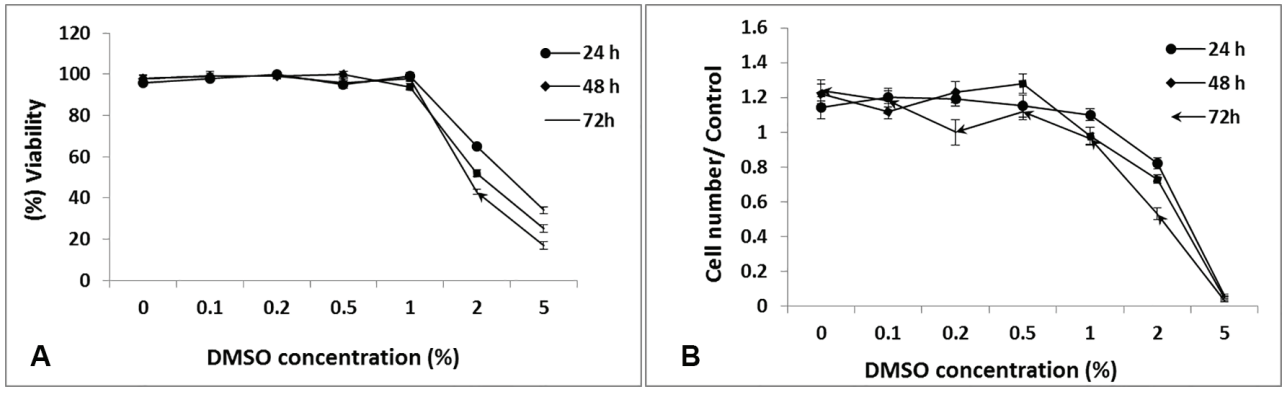

Figure 3: Effect of DMSO on proliferation of human leukemic Jurkat cells (A) and (B). The Jurkat cells (2×10^4 cell/well) were treated with different concentrations of DMSO (0.1, 0.2, 0.5, 1, 2 and 5%) for 24, 48 and 72 hours. The results are presented as % of viability demonstrated by trypan blue dye exclusion (TB) test (A) and cell number/control demonstrated by MTT assay (B). Data are presented as mean±SEM of three independent experiments. $P<0.05$ was considered significant

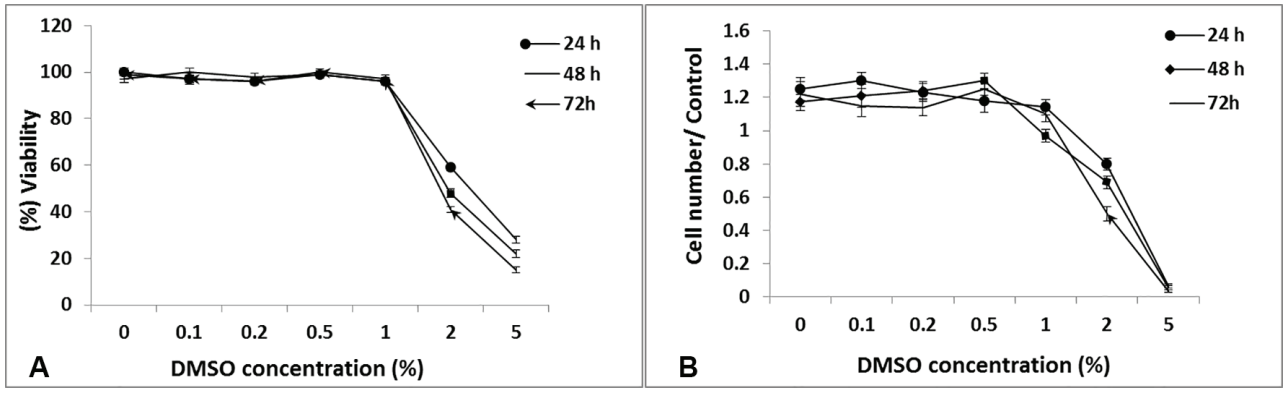

Figure 4: Effect of DMSO on proliferation of human leukemic Molt-4 cells (A) and (B). The Molt-4 cells (2×10^4 cell/well) were treated with different concentrations of DMSO (0.1, 0.2, 0.5, 1, 2 and 5%) for 24, 48 and 72 hours. The results are presented as % of viability demonstrated by trypan blue dye exclusion (TB) test (A) and cell number/control demonstrated by MTT assay (B). Data are presented as mean±SEM of three independent experiments. $P<0.05$ was considered significant

Antioxidant and anti-inflammatory properties of DMSO and inhibitory effects of DMSO on inflammatory cytokine secretion in autoimmune arthritis have also been shown.[5-7] As the monocytes and T-cells play a key role in inflammation,[26] the anti-inflammatory effects of DMSO may be partly due to its cytotoxic effects on these cells as was shown in our study. Additionally, there are numerous reports of antitumor effects of DMSO and induction of antitumor immunotherapy by DMSO.[8-10,12] The antitumor effects of DMSO might be in part owing to its direct cytotoxic effects on neoplastic cells as we showed in present study.

On the other hand, pretreatment by DMSO potentiated the toxic effect of cisplatin on sensory hair cells.[13] Furthermore, antitumor effects of two ruthenium (II)-DMSO-chalcone complexes has been described.[10,14] The augmentation of antitumor effects of some anticancer drugs by DMSO may probably result form its cytotoxic effects which acts synergistically in cancer therapy.

Consistent to our results, treatment of mouse hepatocellular carcinoma cell line with 2% DMSO, depressed cell proliferation.[12] However, in contrast to our study, there was no reduced cell viability.[12] The difference between our results and the mentioned study may be due to the type and number of the cells and the methods used for the assessment of cell viability. Jiang and colleagues used 1×10^5, 1×10^4 and 1×10^3 mouse hepatocellular carcinoma cell/well and used CCK-8 reagent to screen cell viability, while we used 2×10^4 cell/well of human leukemic cells and used trypan blue dye exclusion test for assessment of cell viability.

In our study, all of the used leukemic cell lines showed time-dependent sensitivity to DMSO at ≥2% concentrations. Taken together our findings suggest that DMSO might be a cytotoxic agent for leukemic cells. According to the results of the present study, DMSO might be a useful candidate in design of chemotherapeutic protocols for leukemia as well as other cancers.

However, additional studies such as evaluation of DMSO toxicity on normal and other tumor cells are required for further conclusions. As DMSO is commonly used as a solvent, it is noteworthy to investigate its toxicity on different cells at lower concentrations especially between 1% and 2% in various time intervals in-vitro to prevent inaccuracy in evaluating the characteristics (such as cytotoxicity) of other drugs/ agents which have been dissolved in DMSO.

Conclusion

DMSO shows cytotoxic effects on leukemic cells and might be a useful candidate in design of chemotherapeutic approaches for leukemia as well as other cancers.

Conflict of Interest: None declared.

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ORIGINAL ARTICLE

Association between Red Cell Distribution Width and Mortality in Pediatric Patients Admitted to Intensive Care Units

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ABSTRACT

Background: Red cell distribution width (RDW) is a routine laboratory measure that could be used as a predictor of mortality in critically ill patients. Identification of patients at risk for mortality early in the course of PICU admission is an important step in improving the outcome. We aimed to assess the use of RDW as an early biomarker for outcome in pediatric critical illnesses.
Methods: A retrospective study by extracting administrative and laboratory data from patients admitted to PICU of an academic pediatric teaching hospital was accomplished. After exclusion of 64 patients according to our exclusion criteria, 304 pediatric patients with PICU admissions over the 6 months of study period were included in the study.
Results: The mean RDW for all patients was 14.9%±2.5%. PICU mortality was 13.3%. The rate of mortality in the quartile of RDW>15.7% was 20.1%. Elevated RDW was associated with longer duration of PICU admission (P<0.001). Tracheal intubation and ventilator support was needed in 34.2% of the patients. This was also correlated with elevated RDW (P=0.043).
Conclusion: We observed that higher RDW was strongly linked to higher mortality risk in pediatric patients admitted in PICU. Higher RDW was associated with longer duration of PICU admission.

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Introduction

Pediatric intensive care units (PICU) with growing life-sustaining technologies have resulted in advanced care for children and adolescents. Moreover, characterizing the disease severity at admission and assessing risk factors correlating with mortality can help improve the quality of patient care. By means of simple laboratory values this goal seems to be attainable.

Red cell distribution width (RDW) is a laboratory parameter which expresses the variability in red blood cell size and is calculated as the standard deviation in red blood cell (RBC) size divided by the mean corpuscular volume (MCV). Clinically, it is a widely available and low-cost test. Its normal range is between 11.5–14.5%. Reference ranges may vary depending on the individual laboratory and patient's age. Elevated RDW on complete blood count reflects marked anisocytosis on peripheral blood smear review, which can be caused by any disease involving red blood cell (RBC) destruction or production.[1]

Studies have revealed that RDW could be used as a predictor of mortality in critically ill patients.[2,3] Although the mechanism of this relationship is not fully apparent, it seems that in critical illnesses, the acute systemic inflammatory response can alter both erythropoiesis and erythrocyte maturation.[4-9] In different contexts including sepsis, cardiovascular disease, cancer, and

chronic lower respiratory tract disease, RDW has shown to have association with increased risk of mortality.[4,10-15] In patients admitted to PICU, RDW is associated with risk of death and is suggested as an independent prognostic marker.[14,16-18] The prognostic value of RDW in adult patients with medical conditions such as heart failure admitted to ICU has been studied previously;[19,20] however, information about the value of RDW as a predictor of clinical outcomes in pediatric patients is more limited. We aimed to study the association between RDW parameter in pediatric patients admitted to PICU with mortality.

Materials and Methods

A retrospective study by extracting administrative and laboratory data from patients admitted to PICU of an academic pediatric teaching hospital between September 2015 and February, 2016 was accomplished. Approval for the study was obtained from the Institutional Review Board of Mofid Children's Hospital.

The medical records of all patients were reviewed for the following data: Demographic data, vital signs including body temperature, blood pressure, respiratory rate and pulse rate, a CBC, including RDW, measured within 24 hours of PICU admission, blood gas results, blood bank reports, microbiology reports, mortality, and duration of PICU admission.

RDW is reported as a coefficient of variation (percentage) of red blood cell volume. The normal reference range for RDW in this hospital laboratory was 11.5-14.5%. Patients were categorized into four RDW quartiles based on previously published studies as a priori cut-points (RDW<13.4%, 13.4–14.3%, 14.4–15.7%, and >15.7%).[4,13,14] Anemia was defined in accordance with World Health Organization (WHO) recommendations.[21]

Exclusion criteria were: Age more than 16 years, chronic renal failure, chronic metabolic disease, cancer, chronic hematologic diseases with the potential to change RDW, history of RBC transfusion within previous 72 hours.

SPSS software, version 16.0, was adopted for statistical analysis. The obtained measurement data in line with the normal distribution were expressed as mean±standard deviation. Univariate analysis was performed using Mann-Whitney U and Chi-square tests when appropriate. P<0.05 was considered statistically significant.

Results

After exclusion of 64 patients according to our exclusion criteria, 304 pediatric patients with PICU admissions over the 6 months of study period were included in the study. Demographic, clinical, and laboratory characteristics of the patients are summarized in Table 1. The mean age of the patients was 2.9±3.6 years and 42.9% were female. Nonsurgical, surgical and neurosurgical diseases were recorded in 50.5%, 29.3% and 20.1% of the cases, respectively. The mean RDW for all patients was 14.9%±2.5%. The RDW range was between 11.6%-25%.

Overall PICU mortality was 13.3%. However, the rate of mortality in the quartile of RDW>15.7% was 20.1%. Elevated RDW was significantly more encountered in nonsurgical patients (P=0.046).

The median length of PICU stay was 7.2 days. Elevated RDW was associated with longer duration of PICU admission (P<0.001). Tracheal intubation and ventilator support was needed in 34.2% of patients. This was also correlated with elevated RDW (P=0.043).

Anemia was detected in 52.7% of the patients; it was more frequent in patients with elevated RDW (P=0.048). Thrombocytopenia and thrombocytosis was observed in 13.1% and 19.6% of the patients, respectively. Abnormal platelet counts significantly correlated with elevated RDW (P=0.001).

Leukocyte counts lower than 5×10^3 /µL was reported in 9.3% of patients and leukocyte counts more than 15×10^3 /µLin 24%. There was no correlation between RDW and leukocyte counts.

The patients in the quartile of RDW>15.7% had significantly more hypotension according to their age, but RDW did not correlate with body temperature and pulse rate at the time of PICU admission.

Discussion

Elevated RDW reflects anisocytosis and higher variability in size of circulating RBCs. The results of our study was compatible with the literature that elevated RDW in pediatric patients admitted in PICU is associated with a higher risk for mortality in critically ill pediatric patients.[2,3]

Many studies have evaluated diverse prognostic markers for early recognition of ICU patients who have high morbidity and mortality risk. A variety of approaches including clinical scoring systems such as the Pediatric Risk of Mortality (PRISM) score, and Pediatric Index of Mortality score and also specific routine laboratory tests have been evaluated in former studies for identifying their potential role in prediction of outcome in critically ill pediatric patients.[22,23]

RDW has been proposed to be a prognostic factor influencing mortality in a spectrum of diseases including cardiovascular, pulmonary, renal, infectious and oncologic diseases and also in critically ill patients.[24-27] Studies have shown that RDW is an independent predictor of mortality and its addition to the "Acute Physiologic and Chronic Health Evaluation (APACHE)" score; which is one of the most commonly used ICU scoring systems, has improved its power for mortality prediction.[28]

Although the precise pathophysiological mechanism of the correlation between higher RDW and mortality is vague, it seems that chronic subclinical inflammation affects iron metabolism as well as bone marrow function and its response to erythropoietin. On the other hand, erythrocyte maturation is suppressed by the inflammatory cytokines and high oxidative stress leading to the entry of newer, larger reticulocytes into the circulation and elevation of RDW.[10] Additionally, RBC membrane glycoproteins and ion channels are altered by inflammation contributing to the change of RBC morphology.[29,30]

Previous studies have established that RDW values increased with age.[17] This relationship; although not fully defined, could depend on several factors

Table 1: Patient characteristics

Characteristic	All patients	RDW Quartile <13.4	RDW Quartile 13.4-14.3	RDW Quartile 14.4-15.7	RDW Quartile >15.7	P value
Number (%)	304	85(28)	75(24)	68(22)	76(25)	
Age(years)	2.9(0.1-16)	3.4(0.15-16)	2.13(0.1-14)	2.1(0.1-13)	3.1(0.1-16)	0.046
Gender (%)						0.9
Male	169(55.)	50(58.8)	41(54.7)	36(52.9)	42(55.3)	
Female	135(44.)	35(41.2)	34(45.3)	32(47.1)	34(44.7)	
Admit category (%)						0.004
Nonsurgical	140(46.)	37(43.5)	26(34.7)	31(45.6)	46(60.5)	
Surgical	94(30.9)	20(23.5)	28(37.3)	23(33.8)	23(30.3)	
Neurosurgical	70(23)	28(32.9)	21(28)	14(20.6)	7(9.2)	
CRP						0.01
<10	68(58.6)	22(73.3)	12(66.7)	23(63.9)	11(34.4)	
>10	48(41.4)	8(26.7)	6(33.3)	13(36.1)	21(65.6)	
Anemia (%)	159(52.)	39(45.9)	35(46.7)	35(51.5)	50(65.8)	0.048
WBC						0.128
<5000/mm3	25(8.2)	8(9.4)	5(6.4)	3(4.4)	9(11.8)	
5000-15000/mm3	205(67.4)	61(71.8)	54(72)	49(72.1)	41(53.9)	
>15000/mm3	74(24.3)	16(18.8)	16(21.3)	16(23.5)	26(34.2)	
Platelet						0.001
<150000/mm3	35(11.6)	3(3.6)	4(5.3)	13(19.1)	15(19.7)	
150000-450000/mm3	207(68.5)	67(79.8)	61(80.6)	38(55.9)	41(53.9)	
>450000/mm3	61(20.1)	14(16.7)	10(13.3)	17(25)	20(26.3)	
PICU Length of stay mean (days)	7.2(0.1-90)	5.39(0.5-60)	6.4(0.5-56)	9.1(1-51)	8.45(0.1-90)	<0.001
PICU Length of stay >48 hrs	164(53.9)	45(52.9)	30(40)	41(60.3)	48(63.2)	0.022
Mortality(%)	34(11.1)	8(9.4)	5(6.6)	5(7.3)	16(21.1)	0.016
Respiration						0.043
Normal	153(50.3)	46(54.1)	45(60)	34(50)	28(36.8)	
Tachypnea	51(16.8)	17(20)	12(16)	8(11.8)	14(18.4)	
Intubation	100(32.9)	22(25.9)	18(24)	26(38.2)	34(44.7)	
Tachycardia(%)	185(60.9)	53(62.4)	45(60)	42(61.8)	45(59.2)	0.975
Tempreture	37.36(35-40.5)	37.36(36.39.4)	37.22(35-40)	37.43(35-40)	37.44(35.4-40.5)	0.7
Blood pressure						0.017
Normal	267(87.8)	79(92.9)	67(89.3)	59(86.8)	62(81.6)	
Hypotention	11(3.6)	0(0)	1(1.3)	2(2.9)	8(10.5)	
Hypertention	28(8.6)	6(7.1)	7(9.3)	7(10.3)	6(7.9)	
PTT						0.017
<35 sec	134(76.6)	42(89.4)	33(78.6)	30(76.9)	29(61.7)	
>35 sec	41(23.4)	5(10.6)	9(21.4)	9(23.1)	18(38.3)	
INR						0.017
<1.5	167(91.3)	49(98)	45(95.7)	34(89.5)	39(81.3)	
>1.5	16(8.7)	1(2)	2(4.3)	4(10.5)	9(10.8)	
Albumin						0.003
<3.5 gr	47(44.3)	7(28)	13(56.5)	5(21.7)	22(62.9)	
>3.5 gr	59(55.7)	18(72)	10(43.5)	18(78.3)	13(37.1)	

including inflammation, anemia, nutritional status and age associated diseases.[31,32] In a study by Buyukkocak an colleagues, the correlation between RDW and age in patients admitted in ICU was significant especially among the patients who had expired.[16] In our study, age correlated positively with RDW and the mean age of patients in the quartile of RDW>15.7% was significantly higher.

An association between increasing levels of acute phase reactants such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and interleukin-6 with elevated RDW has been confirmed in adults.[4-6,33,34] In our study, elevated RDW was correlated positively with raised CRP but not with ESR.

In the study by Ramby et al. Al in Italy the Overall PICU mortality was 6.5% which was much less than what we had in our hospital. They also found that there was a significant increase in mortality rate across all RDW quartiles and RDW measured within 24 hours of PICU admission was independently associated with PICU duration of admission >48 hours and higher mortality

in a general PICU population.[35] In our patients, elevated RDW was also associated with longer duration of PICU admission more than 48 hours.

Increase in RDW may be a sign of cytomembrane instablity which may cause multiple organ dysfunction, consequently leading to a poorer prognosis and increase in mortality.[36] Instability of cell membrane could be due to lack of some materials such as blood albumin and cholesterol.[19,37] In our study hypoalbuminemia was associated with elevated RDW (P=0.003).

Different cohorts reveal that poor medical conditions requiring mechanical ventilation is one of the most important risk factors of mortality in PICU patients.[38] In our study, tracheal intubation and mechanical ventilation was associated with elevated RDW (P=0.043).

In summary, we observed that higher RDW was associated with higher mortality rate in pediatric patients admitted to PICU. This study should prompt further prospective evaluation of the association between high RDW and prediction of mortality in pediatric patients in order to improve risk-stratification of the ill patients.

Conflict of Interest: None declared.

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ORIGINAL ARTICLE

Association between Percentage of TCD4 and TCD8 Lymphocytes with Iron Status in Female Adolescents

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ABSTRACT

Background: Iron deficiency impairs the proliferation and function of T lymphocytes. This study was conducted to assess the relationship between serum iron with percentage of TCD4 and TCD8 lymphocytes in peripheral blood of female high school students in Hamadan.
Methods: In this cross-sectional study, 355 female high school students with an age range of 15-18 years were enrolled from January 2016 to March 2017. After approval by the ethics committee of Hamadan University of Medical Sciences, taking written consent of parents, and completion of a questionnaire involving demographic information, serum iron profile, the percentage of TCD4 and TCD8 cells, and TCD4/TCD8 ratio were measured using standard methods. The results were analyzed by SPSS software, version 13.
Results: The prevalence of iron deficiency anemia was 16.1% in 355 female high school students of Hamadan. There was no correlation between transferrin saturation with percentage of TCD4 lymphocytes and TCD4/TCD8 ratio in the two groups of students with and without iron deficiency ($P>0.05$). However, a significant correlation was found between Tfs with percentage of TCD8 lymphocytes in the group of patients with iron deficiency anemia ($P<0.05$).
Conclusion: This study indicated an increased percentage of TCD8 lymphocytes with reduced Tfs in patients with iron deficiency anemia. In addition to reduced Tfs, other factors may be associated with the alterations in percentage of TCD4 and TCD8 lymphocytes and TCD4/TCD8 ratio.

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Introduction

Iron is involved in multiple cellular processes including erythropoiesis, thrombopoiesis, leukopoiesis, oxidative metabolism, CNS development, and immune responses.[1-7] Negative consequences of iron deficiency on hematopoietic tissues and the nervous system are well proven, while there is controversy regarding the effects of iron deficiency on the immune system.[8] Children, female adolescents, and women of childbearing age are more predisposed to iron deficiency anemia (IDA) because of the increased requirement and loss of Iron. According to a WHO survey between 1993 and 2005, nearly 30.2% of non-pregnant women aged 15-60 years were anemic.[9]

Low concentrations of serum iron reduce neutrophil bactericidal capacity and proliferation of lymphocytes,[10-12] leading to increased susceptibility to infectious diseases and even development of malignancies.[8] However, the exact role of iron in the regulation of immune system responses, especially in the groups predisposed to iron deficiency anemia has not been fully recognized.

Given the importance of iron deficiency and its health consequences, we aimed to determine the values of serum iron profile, percentage of CD4 positive T cells (TCD4) and CD8 positive T cells (TCD8), TCD4/TCD8 ratio in

peripheral blood, the prevalence of IDA and eventually evaluation of the relationship between serum iron with percentage of TCD4 and TCD8 lymphocytes in female high school students in Hamadan (northwestern Iran) during 2016-2017.

Materials and Methods

This cross-sectional study was conducted during 2016-2017 after obtaining the approval of the Ethics Committee of Hamadan University of Medical Sciences. 355 female students of Hamadan high schools were enrolled in this study using two-step cluster method. First, 16 high schools for girls were randomly selected from the city of Hamadan and the students of each high school participated in the study by stratified random sampling. We included students who had not received iron supplements, hematinics and multivitamin in the past six months, those who did not have a history of acute or chronic bleeding and chronic diseases and infections and were not in the menstruation period, who did not have any symptoms of malnutrition, had no history of receiving immunosuppressive drugs, radiation, and chemotherapy, and were not married.

Written informed consent was taken from the parents and the questionnaire including demographic data was completed. Hematological indices and serum iron profile were measured by standard methods.[13] 5 mL whole blood was drawn using standard methods in tubes without anticoagulant to isolate the serum, as well as in EDTA-containing tubes for complete blood count (CBC). The percentage of TCD4 and TCD8 lymphocytes, as well as TCD4/TCD8 ratio were determined by flow cytometry. CBC was done by Sysmex coulter counter (KX-21N Japan). The serum iron profile, including serum iron, total iron-binding capacity (TIBC), Transferrin saturation (Tfs) was determined by an Autoanalyzer (Hitachi 912, Japan).

EDTA anticoagulated blood samples were used to determine the percentage of TCD4 and TCD8 lymphocytes and TCD4/TCD8 ratio. For this purpose, specific monoclonal antibodies (CD4-FITC & CD8-PE DAKO, Denmark) were used in Attune NxT flow cytometry device (USA). Briefly, 20 λ of relevant antibody was added per 50 λ of blood sample. The level of TCD4 and TCD8 lymphocytes was evaluated by the device after 45 minutes of incubation, adding 100 λ of lyser, and 15 minutes of incubation in the dark. Mouse IgG1 FITC/PE (DAKO, Denmark) was used as the isotype control. IDA was diagnosed upon decreased hemoglobin, MCV, MCH, RBC, serum iron, transferrin saturation with iron and ferritin along with increased TIBC.

Examination of more than 120 samples has been preferentially proposed to obtain the reference values in a society. In the present study, 121 normal samples were used to obtain reference values for serum Tfs, percentage of TCD4 and TCD8 lymphocytes and TCD4/TCD8 ratio. The relationship between Tfs with the percentage of T lymphocyte subsets was analyzed using Pearson's correlation test. P-value less than 0.05 was considered significant. Finally, the results were analyzed by SPSS software, version 13.

Results

In this study, from a total of 382 samples, 360 female

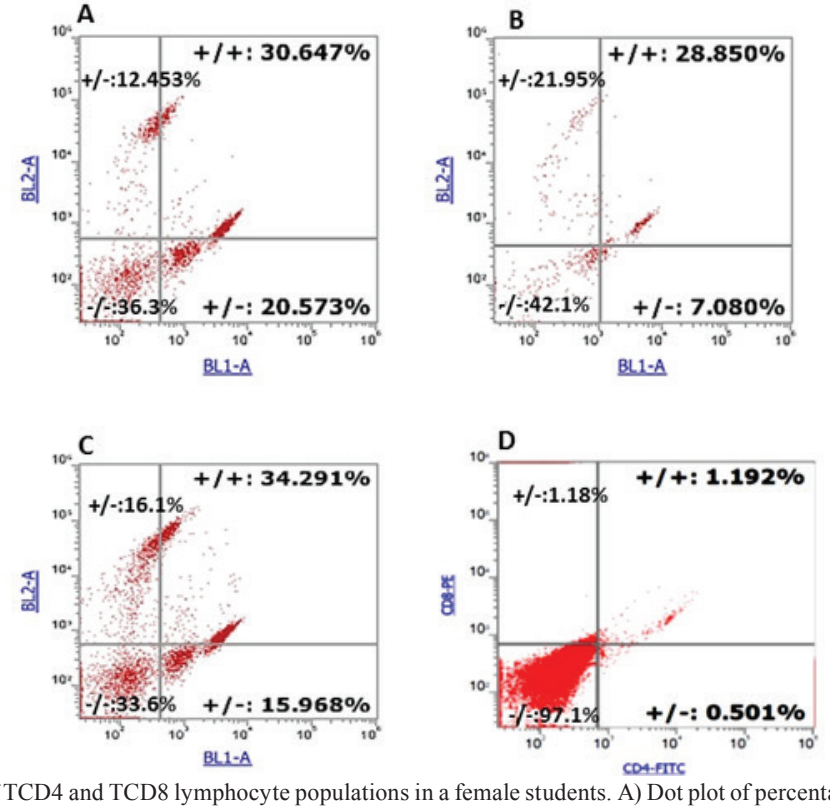

Figure 1: A dot plot of TCD4 and TCD8 lymphocyte populations in a female students. A) Dot plot of percentage of TCD4 and TCD8 in the NID group, B) Dot plot of percentage of TCD4 and TCD8 in IDA group, C) Dot plot of percentage of TCD4 and TCD8 in IDA group, D) Dot plot of isotype control of TCD4 and TCD8 antibodies.

students aged 15-18 were recruited from January 2016 to January 2017. Five out of 360 students were excluded from the study since they were diagnosed to have an anemia other than IDA. Among 355 students, 57 had IDA and 298 were normal. In this study, the students were divided to two groups of iron deficient (ID) and non-iron deficient (NID), including all students with IDA and 121 NID students for descriptive analysis of variables, as well as Pearson's correlation between Tfs and percentage of lymphocyte subtypes, respectively. Among 355 students of this study, the prevalence of IDA was 16.1%. Flow cytometric analysis and iron measurement were conducted for 121 NID students and 57 students with IDA, and the data were analyzed.

In figure 1, a percentage histogram of TCD4 and TCD8 lymphocytes of a female patient is presented. In accordance with international and WHO standards,[13] the group of normal students included those having hematological parameters in a normal range. ID students and those with IDA had Tfs lower than 12%.

The difference in Tfs and percentage of TCD8 lymphocytes was significant between NID and ID groups. As shown in table 1, mean values of TCD8 and Tfs were significantly different between the two groups of NID and those with IDA (in other words, mean TCD8 and Tfs values in students having IDA were significantly lower than normal students). However, mean TCD4 and TCD4/TCD8 ratio were not significantly different between NID and IDA students (P=0.230).

In NID students, no correlation was found between the percentage of TCD4 and TCD8 lymphocytes, as well as TCD4/TCD8 ratio with Tfs. In group of patients with IDA, there was also no significant correlation between TCD4 percentage and TCD4/TCD8 ratio with Tfs; however, a significant negative correlation was observed between TCD8 percentage with Tfs (P<0.001, table 2).

We found a significant difference in correlation coefficients of Tfs and TCD8 cells between NID and ID students (table 3). In figure 1, a percentage histogram of TCD4 and TCD8 lymphocyte populations of a female student is presented.

Discussion

During their rapid growth period, early adolescents need more iron and other primary materials to generate red blood cells. Ignorance of serum iron profile and not preventing or treating iron deficiency anemia can have unfavorable outcomes on hematopoietic tissue, nervous system, and immune competence, especially the decreased number and function of T lymphocytes, increased risk of infections and even malignancy.[8] In our study the prevalence of IDA was 16.1%. But in other studies in Iran, the prevalence of IDA

Table 1: Descriptive statistics of hematological, immunological and iron storage parameters in ID and NID groups

	Groups	No	Mean	SD	Max	Min	P value
Hb	ID	57	13.1	1.2	15.8	9.7	>0.05
	NID	121	13.8	0.8	17.1	12.0	
Hct	ID	57	40.4	3.0	45.60	30.0	>0.05
	NID	121	41.1	2.0	46.7	36.0	
T CD4+ (%)	ID	57	37.6	22.2	90.0	12	0.122
	NID	121	45.3	19.2	97.0	6	
T CD8+(%)	ID	57	28.5	3.3	32.0	23	0.001
	NID	121	33.8	13.5	66.0	8	
CD4+/CD8+ ratio	ID	57	1.3	0.7	3.0	0.5	0.230
	NID	121	1.5	0.9	5.0	0.37	
Tfs (12%)	ID	57	4.1	2.0	7.5	1.5	<0.001
	NID	121	26.0	9.8	67.1	12.3	
Ferritin	ID	57	6.0	3.2	11.0	2.0	0.001
	NID	121	51	3.3	92.0	15.0	

Table 2: Pearson correlation between serum transferrin saturation and percentage of lymphocyte subsets in NID and IDA patients

Groups		Non-iron deficient Students			Students with Iron deficiency Anemia		
		T CD4+	T CD8+	CD4/CD8	T CD4+	T CD8+	CD4/CD8
Transferrin saturation	Pearson correlation	0.161	0.027	0.015	-0.165	-0.723	-0.092
	Number	121	121	121	57	57	57
	P value	0.109	0.786	0.882	0.501	<0.001	0.707

Table 3: Comparison of Pearson correlation between serum transferrin saturation and percentage of lymphocyte subsets in normal and ID students

	Non-iron deficient Students	Students with Iron deficiency Anemia	P value
Transferrin saturation/TCD4	0.161	-0.165	0.222
Transferrin saturation/TCD8	0.027	-0.723	<0.001
Transferrin saturation/CD4 to CD8 ratio	-0.092	0.015	0.691

was 9.3% and 4.5% in Yazd and Semnan, respectively.[13] The main objective of this study was to determine normal values of serum iron profile, the percentage of TCD4 and TCD8 lymphocytes, TCD4/TCD8 ratio, as well as to detect the correlation between serum iron with percentage of TCD4 and TCD8 lymphocytes.

Recent studies have given conflicting results on the impact of iron deficiency and IDA on cellular and humoral immunity in humans and animals.[14] The impact of iron deficiency on immune system is investigated intensively by different groups. Some researchers have shown that iron deficiency mainly affects the function of the lymphocytes and others showed that iron deficiency primarily affects the number of lymphocytes rather than their functions.[8] One study showed that the percentage of TCD4 lymphocytes and TCD4/TCD8 ratio in children with IDA was significantly decreased compared to the control group.[15] Another study on non-pregnant premenopausal women with IDA showed that absolute count of T-cells, TCD4, and TCD8 cells were significantly decreased in comparison to the control group; however, TCD4/TCD8 ratio was not significantly different between the two groups.[16] The study of Ekiz and colleagues was conducted in Turkey on 2005 to investigate the effect of iron deficiency on immune system function and showed no significant difference in the percentage of CD3/CD4, CD3/CD8, and CD3/CD19 lymphocyte subsets between patients with IDA and the control group.[8] Van Heerden and colleagues in 1981, in line with the findings of Ekiz et al. indicated no abnormality in percentage of B-cells and T-cells neither in children with IDA nor in those only with ID.[17]

We found a significant association between TCD8 with Tfs in students with IDA. In other words, reduced Tfs led to increased TCD8 percentage. No significant difference was found between Tfs with percentage of TCD4 and TCD4/TCD8 ratio between normal students with those with iron deficiency. In our study, there was a significant association between Tfs and percentage of TCD8 in the IDA group in comparison with NID, which was consistent with some studies,[8,17] but inconsistent with Karamati and colleagues' study.[16] Regarding the TCD4/TCD8 ratio, the results of our study were consistent with the findings of Karamati and colleagues' study.[16]

Differences in sample size, location where the study was performed and other factors that affect the immune system function such as zinc and vitamin A deficiency or infections may account for different results of this study which requires further investigation. In this study, the association between serum iron levels with percentage of TCD4 and TCD8 lymphocytes and TCD4/TCD8 ratio was assessed, which was merely a quantitative assessment of cellular immunity. Therefore, it is suggested to evaluate the relationship between iron saturation with T-cell function, B-cell count and function or humoral immunity in another study with a larger sample size.

Conclusion

The findings of our study showed that TCD8 percentage is increased in students with IDA along with reduced Tfs. Therefore, decreas ed Tfs may be associated with increased cellular immune response or other factors may be involved in the alterations in percentage of TCD4 and TCD8 cells, as well as TCD4/TCD8 ratio, which requires further investigations.

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CASE REPORT

Multiple Myeloma Presenting as Respiratory Stridor

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ABSTRACT

Extramedullary plasmacytoma occurs in 18% of patients with multiple myeloma. Laryngeal involvement in multiple myeloma is rare, and only a few cases have been reported. We present a case of a 44-year-old women with multiple myeloma who presented with stridor due to a mass involving the larynx which was initially proven to be plasmacytoma on biopsy. She had evidence of multiple myeloma of IgA lambda subtype. She was treated with bortezomib containing chemotherapy followed by lenalidomide as maintenance therapy. She attained complete remission and is alive in remission at 3 years of treatment.

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Introduction

Plasmacytoma is a discrete solitary mass of monoclonal neoplastic plasma cells occurring either in bone (solitary plasmacytoma of bone) or in soft tissues (extramedullary plasmacytoma/EMP). EMP occurs in 18% of patients with multiple myeloma.[1] The most common sites of EMP are head and neck region, upper respiratory tract, gastrointestinal tract, and central nervous systems. EMP accounts for less than 1% of all malignant head and neck tumours. Multiple myeloma (MM) usually presents with anemia, bone pain, and renal failure. Laryngeal involvement in multiple myeloma is rare, and only a few cases have been reported.[2-7] We present a woman with multiple myeloma who presented with stridor due to plasmacytoma involving the larynx.

Case Report

A 44-year-old woman presented with hoarseness of voice for 3 months and a period of two weeks of stridor. There was no dysphagia. Computed tomogram scan of neck showed a soft tissue mass (3 x 3.3 x 3.4 cm) in the left pyriform sinus and aryepiglottic fold encasing hyoid bone, thyroid cartilage, and vocal cord on left side along with bilateral regional lymphadenopathy (figure 1). She underwent emergency tracheostomy. Indirect laryngoscopy revealed a mucosa-lined growth involving the left arytenoids, aryepiglottic fold and pyriform sinus with fixed left hemilarynx (figure 2). A biopsy from the mass showed plasmacytoid cells loosely arranged in a fibrocollagenous stroma. On immunohistochemistry, the tumor cells were strongly positive for CD138 and negative for cytokeratin, thus a preliminary diagnosis of plasmacytoma was made for the patient (figure 3A & 3B). Skeletal survey revealed multiple lytic bone lesions and bone marrow aspiration showed a population of about 45% immature plasma cells which resulted in final diagnosis

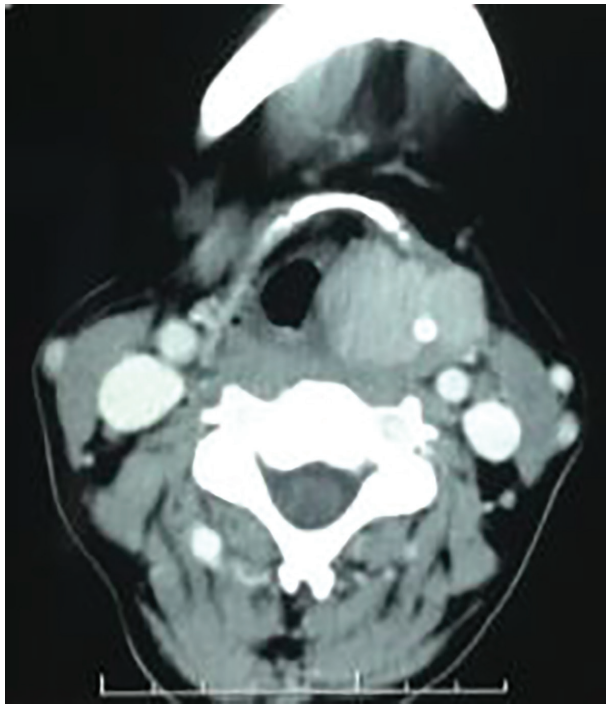

Figure 1: CT-scan of the neck showing a soft tissue mass in left pyriform sinus and aryepiglottic fold encasing hyoid bone, thyroid cartilage and vocal cord on the left.

of MM, since bone and bone marrow involvement were detected. The 24-hr urine protein was 2.49 gm/day, and β2-microglobulin was 14.5 mg/dl. Serum electrophoresis showed M-band with M protein level of 7.8 gm/dl. Serum Immunoglobulin (Ig) A was 11955 mg/dl, IgG was 333 mg/dl, IgM was <25mg/dl, free kappa chain was 15.5 mg/L. and free lambda chain was 114.3 mg/L. Immunofixation electrophoresis demonstrated the presence of IgA lambda monoclonal gammopathy. She received bortezomib and dexamethasone for 6 cycles. A repeated CT-scan after 6 cycles of chemotherapy showed complete resolution of the laryngeal lesion. She also achieved complete remission of MM at this point. She was planned for consolidation with high-dose melphalan and autologous peripheral stem cell transplantation, however she refused. She was scheduled to receive maintenance with lenalidomide for

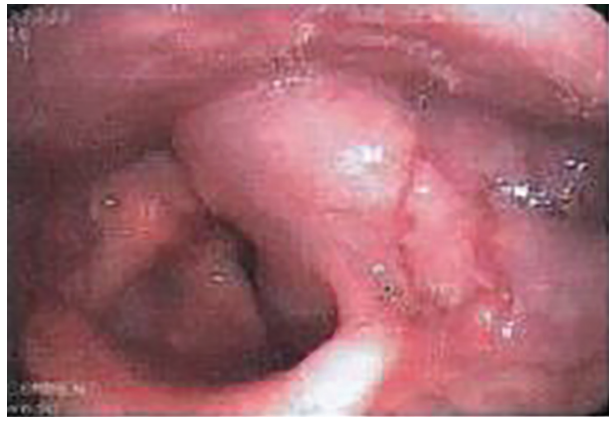

Figure 2: Indirect laryngoscopy showing a mucosa covered growth involving the left arytenoids, aryepiglottic fold and pyriform sinus.

1 year. Currently she is alive in complete remission at 3 years. Patient has given written informed consent for publishing her case details.

Discussion

Neoplasms originating from plasma cells are rare in the head and neck region. Approximately 80% of these EMPs involve the paranasal sinuses, pharynx, nasal cavity or gum and oral mucosa and only 10% of EMP occur in larynx.[8] EMP of larynx represent only 0.04-0.45% of malignant laryngeal tumors. Laryngeal involvement is rare in plasma cell neoplasms and the sites involved are the epiglottis, vestibular fold, arytenoids, aryepiglottic folds, and the subglottis.[2] The peak age of incidence is the 6th decade of life with a male preponderance of 3:1.[8] The clinical presentation varies according to the location of the mass and includes hoarseness, cough, dyspnoea, and stridor.

There are only 2 cases of laryngeal EMP among 22 cases of EMP of the head and neck observed over 20 years.[3] A 65-year-old man with IgG myeloma presenting with stridor has been described.[4] Meanwhile, a 58-year-old man with IgA smoldering myeloma presenting with dysphonia; which was found to be due to laryngeal involvement of MM, has also been reported.[5] A 62-year-

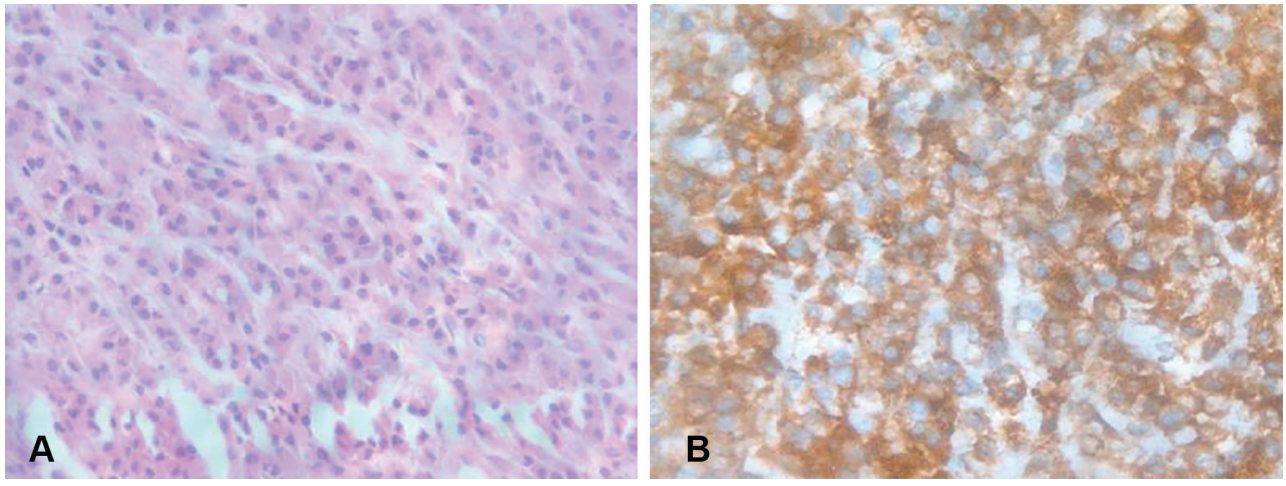

Figure 3: **A)** H&E x40: Section from the laryngeal mass showing plasmacytoid cells. **B)** Immunohostochemistry showing tumor cells positive for CD138.

old woman with dysphonia was also detected to have a plasmacytoma arising from her left true vocal fold through fiberoptic laryngoscopy.[6] There is also a case of plasmacytoma of the larynx being treated with radiotherapy.[7] Our patient presented with stridor whose initial diagnosis of plasmacytoma was positive for CD138; however, she was further diagnosed with MM due to bone marrow involvement with about 45% plasma cells.

Treatment options for isolated EMP of larynx include radiotherapy, laser surgery, endoscopic or conservative surgery and chemotherapy. Plasmacytoma is highly sensitive to radiation and enables voice preservation. The prognosis is related to the location of the tumor and cartilage or bone destruction. Survival is higher in patients with localized disease than in those with MM.[4] Our patient was treated with bortezomib containing chemotherapy in view of the systemic disease. She achieved complete remission and maintenance therapy was continued after achieving remission in place of autologous stem cell transplantation.

Conclusion

Multiple myeloma rarely presents with symptoms related to the primary site of an EMP, as in this case who presented with stridor due to EMP involving the larynx. This entity should be kept in mind in patients with hoarseness and dysphonia.

Conflict of Interest: None declared.

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PHOTO CLINIC

Rhabdomyosarcoma of the Lower Eye Fornix and Conjunctiva in a Child

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A 6-year-old boy was presented with a small polypoid growth in inner conjunctival fornix of the left eye. The parents noted first the lesion as conjunctival congestion associated with dilated episcleral vessels three weeks ago. He received a trial of topical corticosteroid which was not helpful. Examination of the eye described a papillomatous fleshy tissue arising from the caruncle, extending onto bulbar conjunctivae, occupying about three clock hours (figure 1). A conjunctival punch biopsy was approached under general anesthesia which was compatible with embryonal rhabdomyosarcoma (RMS). Immunohistochemical analysis was positive for desmin and myoglobin. Orbital MRI scan showed an enhanced thickening of the lower part of the conjunctiva without orbital infiltration. Extensive staging work-up was negative. According to Intergroup RMS study classification, he was considered as stage III and was treated with chemotherapy alone. One month after chemotherapy eye examination was almost normal (figure 2). The child is in complete remission after 4 years.

RMS is the most common soft-tissue sarcoma of the head and neck in childhood and contains 4% of pediatric malignancies, with 10% of all cases arising in the orbit. Most ocular RMS arise in the soft tissues of the orbit but seldom can occur in other ocular adnexal structures and even within the eye.[1] Orbital RMS is usually extraconal (37–87%) or both intra- and extraconal (13–47%) and more commonly superonasal in location especially for embryonal RMS (inferior location is more common for

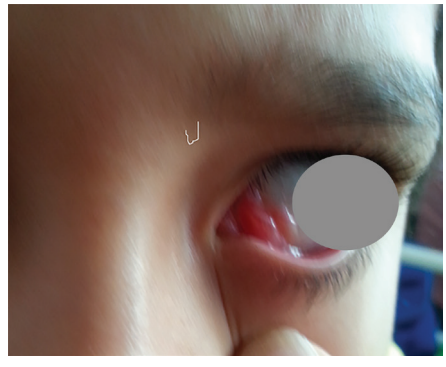

Figure 1: Tumoral mass in the lower eyelid at forniceal conjunctive at diagnosis.

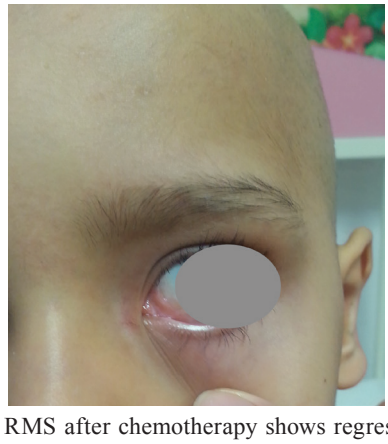

Figure 2: RMS after chemotherapy shows regression of the tumoral mass.

alveolar).[2] It may present with misleading signs such as alterations of the eyelid, conjunctiva or even caruncle,[3] the same as our case.

Orbital RMS has a favorable prognosis because of its anatomic site, (symptoms become apparent at an early stage), favorable histology, the histologic subtype (80% embryonal) and also the age of the patient.[1] Overall survival is excellent for groups I, II and III (92% at 5 years and 87% at 10 years).[4]

Conflict of Interest: None declared.

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