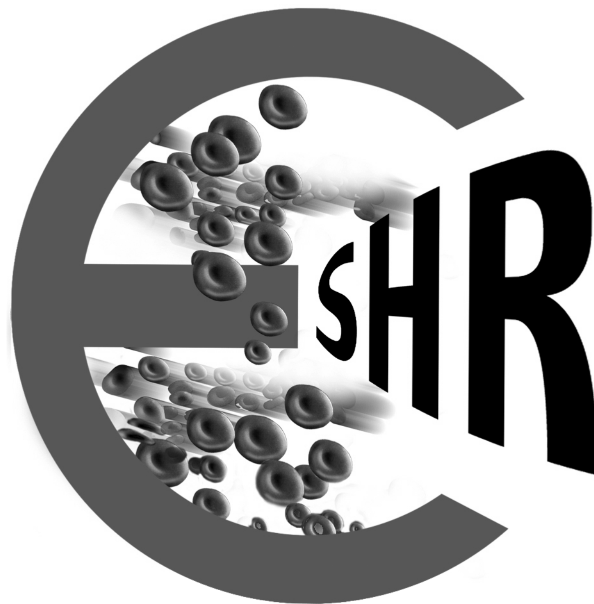
The Journal of the Egyptian Society of Haematology & Research



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New Concept of the Role of Angiogenesis in the Pathogenesis of Chronic Lymphoproliferative Diseases

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ABSTRACT

Background: The importance of angiogenesis for the progressive growth and viability of solid tumours is well established, while little is known about angiogenesis in leukemia. Recent studies suggested that angiogenesis may be involved in hematological malignancies.

Aim of the Work: The aim of this study was to evaluate the role of angiogenesis and its significance in the pathogenesis of lymphoproliferative neoplasms.

Patients and Methods: The study included 26 patients with chronic lymphoproliferative disorders, 20 with non-Hodgkin lymphoma, and 6 patients with CLL. The study also included 10 lymphoma patients with normal bone marrow biopsy as control group. Immunohistochemical staining of bone marrow blood vessels using anti-vWF, anti-thrombomodulin and VEGF expression in bone marrow trephine biopsy sections were done to all patients and controls.

Results: The number of bone marrow blood vessels per high power field when using anti vWF was significantly higher in cases of follicular lymphoma (FL) ($p<0.001$), diffuse large cell lymphoma (DLCL) ($p<0.001$) and chronic lymphocytic leukemia (CLL) ($p<0.001$) with median and inter-quartile range (IQR) of 11.5 (2.75), 12.0 (1.75) and 10.0 (2.0) respectively as compared with the control group 2.5 (1.5).

On using anti-thrombomodulin as endothelial cell marker, bone marrow blood vessels per high power filed was significantly higher in FL ($p<0.001$), DLCL ($p<0.001$) and CLL ($p<0.001$) with median and IQR of 12.0 (2.5), 11.0 (2.75) and 9.5 (3.5) respectively as compared with the control group with a median of 3.0 (2.0).

There was a positive correlation between the number of bone marrow blood vessels counted using immunohistochemical staining with anti-vWF and anti thrombomodulin antibodies as endothelial cell markers.

Vascular endothelial growth factor (VEGF) expression (intensity of the reaction and percentage of positive cells) was statistically higher in cases of FL ($p<0.001$), DLCL ($p<0.001$) and CLL ($p<0.001$) with a median IQR of 40 (17.5), 40.0 (10.0) and 35.0 (20.0) respectively when compared with the control group 4.0 (4.0). Immunoreactive score (IRS) was also increased in FL, DLCL and CLL as compared to the control group. There was also a positive correlation between VEGF expression and the number of bone marrow blood vessels counted by immunohistochemical staining with anti-vWF and antithrombomodulin antibodies.

Conclusion: The increase of bone marrow blood vessels, which is measured by immunohistochemical staining with anti-vWF and antithrombomodulin, and the increased expression of VEGF suggests that increased angiogenesis may play a role in the pathogenesis of the disease.

Key Words: *CLPD – NHL – Anti-vWF – Thrombomodulin – VEGF.*

INTRODUCTION

Lymphoproliferative neoplasms are a group of clonal diseases that arise as a result of somatic mutation in lymphocyte progenitor. The progeny of the affected cell carry the phenotype of a B, T or natural killer cell as judged by immunophenotyping [1]. According to the National Cancer Institute of Cairo, lymphoma and leukemia constitute 12% of all cancers [2]. In 2008 WHO classified lympho-proliferative malignancies as B and T cell disorders [3].

General clinical features of chronic lymphoproliferative disorders (CLPD) include fever, malaise, weight loss, splenomegaly and lymphadenopathy. Lymph node biopsy and/or splenic and bone marrow biopsies are essential for pathological diagnosis and staging of the disease. For proper categorization of different subtypes of CLPD immunophenotyping, immunohistochemistry and genetic studies are mandatory [1]. Different staging systems are established for clinical staging of lymphoproliferative disorders including Ann-Arbor, Binnet, Rai, and Modified Rai [1].

Angiogenesis is the formation of new blood vessels from the pre-existing ones, it is a critical process that occurs in the body both in health and disease. Physiologically it is important for wound healing and formation of placenta. The healthy body controls the formation of new blood vessels through the balance between positive regulators as fibroblast growth factors and vascular endothelial growth factor and negative regulators as transforming growth factor B and platelet factor [4].

Endthothelial cell markers such as thrombomodulin, von Willebrand factor and VEGF play an important role in malignancy. Thrombomodulin is an integral membrane protein expressed on the surface of endothelial cells. It functions as a cofactor in thrombin induced activation of protein C in the anticoagulant pathway. As it is expressed in high density by a restricted number of cells including endothelial and mesothelial cells it is used as endothelial cell marker [5].

Von Willebrand factor plays an important role in primary hemostasis by promoting platelet adhesion to the subendothelium at site of vascular injury. It is stored in alpha granules of megakaryocytes and endothelial cells (Weibel-Palade bodies). It is used as an endothelial cell marker to highlight the endothelial cells [6].

Vascular endothelial growth (VEGF) factor plays an essential role in vasculogenesis during embryogenesis, physiologic angiogenesis and the neovascularization of malignancy. It is a tumour derived angiogenic factor that promotes the formation of endothelial lining of tumour vessels by recruitment of highly proliferative circulating endothelial precursors (CEPs, angioblast) from the bone marrow, haematopoetic stem cells, progenitor cells, monocytes and macrophages [7].

Aim of the work:

The aim of this work is to study the role of angiogenesis in the pathogenesis of CLPD.

MATERIAL AND METHODS

This study was carried out on 26 patients with chronic lymphoproliferative neoplasms (14 male and 12 female) 12 patients had FL, 8 with DLCL, and 6 CLL. Cases were selected from the Hematology Department of the Medical Research Institute, Alexandria University over a period of 18 months. All selected cases have infiltrated bone marrow biopsies. Ten bone marrow biopsy sections for lymphoma patients showing no infiltration were also included in the study as control group.

Exclusion criteria:

- Antiangiogenic drugs.
- Other causes of angiogenesis as hepatitis C.

All patients and controls were subjected to the following:

1- Medical examination:

Detailed history taking and thorough clinical examination to assess the presence of lymph node enlargement, the presence of hepatomegaly and splenomegaly.

2-Routine investigations:

A- Imaging investigations:

Plain X-ray chest, abdominal ultrasound and CT abdomen.

B- General laboratory investigations including:

Complete blood picture [8], ESR [8], serum LDH [9], liver functions tests [10] and kidney functions tests [11].

C- Specific laboratory investigations:

Bone marrow aspiration and biopsy [12].

Immunohistochemical staining of infiltrated bone marrow using the following monoclonal antibodies:

1- Mouse anti-human CDC141, Thrombomodulin clone (1009) [13]:

Applied at 1:25 dilutation for 60min at room temperature.

Positive control: Mesothelioma.

Staining pattern: Cell membrane.

2- Factor VIII related antigen/von Willbrand factor rabbit antibody [14].

Applied at 1:100 dilution for 10min at room temperature.

Positive control: Tonsil.

Staining pattern: Cytoplasmic.

3- Vascular endothelial growth factor epitope specific rabbit antibody [15].

Ready to use for 10min at room temperature.

Positive control: Tumor cells in hemangiosarcoma.

Staining pattern: Cytoplasmic, cell surface and extra-cellular matrix.

Principle of immunohistochemical staining:

The method consists of a labeled streptavidin biotin immunoenzymatic antigen detection system. This technique involves the squentional incubation of the specimen with an unconjugated primary antibody specific to the target antigen, a biotinylated secondary antibody which reacts with the primary antibody, enzyme-labeled streptavidin and substrate-chromogen.

Intensity of immunocytochemical staining was evaluated by taking both intensity of the color reaction and percentage of cells which exhibited the positive reaction. Final results represented the product of the two parameters to calculate the IRS.

% positive cells	Grading of positivity	Intensity of the reaction
No positive cells	0	No positive reaction 0
<10 positive cells	1	Faint color reaction 1
10-50% positive cells	2	Moderate color reaction 2
51-80% positive cells	3	Intense color reaction 3
>81% positive cells	4	

Statistical analysis:

After data entry into a specially designed sheet using Microsoft Excel, a print out of the data was thoroughly revised and data entry mistakes were corrected. Then the file was transferred into Statistical Package for Social Science (SPSS) version 17 format and data explore was carried out. Testing normality using Kolmogorov-Smirnov test proved that data is abnormally distributed, so median and inter-quartile range (IQR) were used for descriptive statistics and non-parametric (Kruskal-Wallis and Mann-Whitney) tests were used for comparison. When Kruskal-Wallis test is significant multiple inter-group comparison (pair-wise comparison) were carried out and correction of p value for multiple comparison (Bonferroni correction) was done. As only comparison with the control group was significant other inter-group significant which proved to be non-significant were not mentioned in the tables. Kendall's-Tau bivariate correlation was also performed. The study adopted a 0.01 level of significance (alpha error) and beta error was set to be 20%.

RESULTS

The present study was conducted on 26 patients presented to the Medical Research Institute with chronic lymphoproliferative neoplasms. Their ages ranged from 26 to 67 years with a mean of 51.5±10.3 years, they were 14 male and 12 female with a ratio of 1.16 to 1.0.

The studied patients were classified into: Twelve patients with FL (46.15%), eight patients with DLCL (30.75%) and six patients with CLL (23.10%).

The clinical presentation of the studied patients were lymphadenopathy in 25 patients (96.15%), splenomegaly in 24 (92.3%), hepatomegaly in 21 (80.67%) and B symptoms in 19 (73.10%).

Bone marrow trephine biopsy:

The patterns of bone marrow infiltration of the studied group showed the following: Focal pattern of bone marrow infiltration in 7 (26.9%), interstitial pattern in 7 (26.9%), diffuse pattern in 6 (23.10%) and mixed pattern in 6 (23.10%). As regards the type of infiltrating cells, 12 (46.15) were infiltrated by mixed small and large cells, 9 (34.6%) were infiltrated by small cells, and 5 (19.23%) were infiltrated by large cells.

Immunohistochemical staining of the bone marrow blood vessels using the anti von Willebrand factor:

When comparing the number of blood vessel in the bone marrow sections on using anti-vWF as endothelial marker between the studied cases and the control group, Table (1), a statistically significant difference was detected (p=0.000). Cases of FL, DLCL and CLL showed statistically significant difference when each group was compared with the control group. (ZMW= 3.979, p=0.000), (ZMW=3.591, p=0.000) and (ZMW=3.286, p=0.001) respectively. (Table 1).

Immunohistochemical staining of the bone marrow blood vessels using antithrombomodulin:

On using anti thrombomodulin as endothelial cell marker there was also statistically significant difference between patients and the control group (p=0.000). On comparing the different histopathological groups with the control group, cases of FL, DLCL and CLL each group showed a statistically significant difference when compared with the control group (ZMW=3.987, p=0.000), (ZMW=3.591, p=0.000) and (ZMW= 3.293, p=0.001) respectively (Table 1).

Vascular endothelial growth factor expression in the studied cases:

VEGF expression (% of positive cells) showed statistically significant difference between cases and control group. On comparing the different histopathological groups with the control group, cases of FL, DLCL and CLL showed statistically significant difference when comparing each with the control group (ZMW= 3.983, p=0.000), (ZMW=3.580, p=0.000), (ZMW=3.273, p=0.000) respectively. Regarding the IRS, it was significantly higher in cases as compared with the control group (p<0.001), also cases with FL, DLCL and CLL each group was statistically significant when compared with the control group. (Table 1).

On comparing the different histopathological groups with each other (FL, DLCL and CLL) using anti-vWF, antithrombomodulin and vascular endothelial growth factor. There was no significant statistical difference between the three studied groups.

Immunoreactive score (IRS):

In FL cases, it ranged from 2-9 with a median (IQR) of 4.0 (2), in cases of DLCL it ranged from 2-6 with a median (IQR) of 4.0 (2), while in the six cases of CLL it ranged from 2-4 with a median (IQR) of 4.0 (2) and in the control group the median was 1 (1). (Table 1).

Correlation between various immunohisotchemical stains:

A positive correlation was encountered between the number of blood vessels detected by anti-vWF, antithrombomodulin and VEGF (Figs. 1-3).

Examples of positive immunohistochemical staining with anti-vWF, antithrombomodulin and VEGF expression are presented in (Figs. 4-9).

DISCUSSION

Development of tumours is a highly complex process in which several molecular events are required for tumour cells to achieve independent growth. One of such events is the enhancement of angiogenesis [3].

Angiogenesis and proangiogenic growth factors have a known role in solid neoplasia, and there is increasing evidence that they also play a role in hematolymphoid neoplasia. Increased microvessel density has been noted in a range of hematolymphoid disorders, including multiple myeloma, non Hodgkin lymphoma, acute and chronic leukemias of lymphoid and myeloid lineages and myelodysplastic disorders [4].

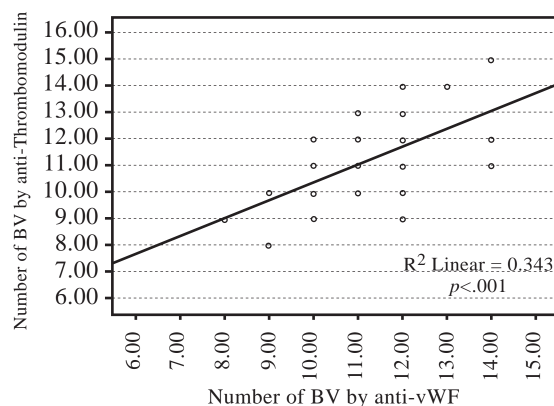

Fig. (1): Positive correlation between immunohistochemical staining of bone marrow blood vessels by anti-vWF and anti-thrombomodulin.

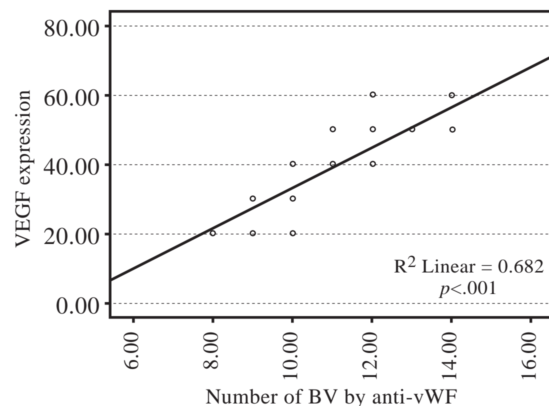

Fig. (2): Positive correlation between immunohistochemical staining of bone marrow blood vessels by anti-vWF and VEGF expression (% of positive cells).

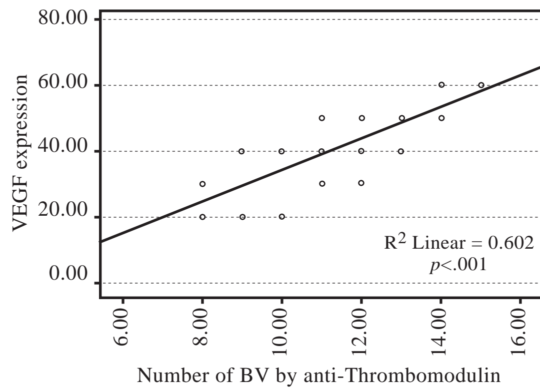

Fig. (3): Positive correlation between immunohistochemical staining of bone marrow blood vessels by anti-thrombomodulin and VEGF expression (% of positive cells).

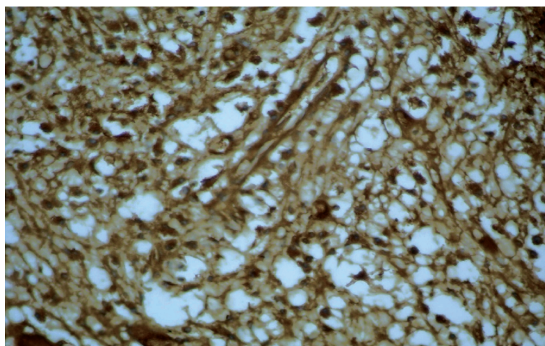

Fig. (4): Bone marrow trephine biopsy in follicular lymphoma showing positive immunohistochemical staining of bone marrow blood vessels with anti-vWF (400X).

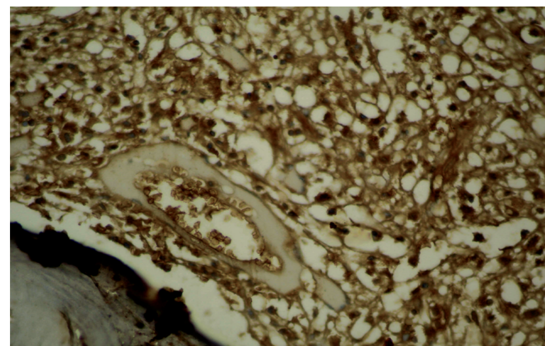

Fig. (5): Bone marrow trephine biopsy in follicular lymphoma showing positive immunohistochemical staining of bone marrow blood vessels with anti-vWF (400X).

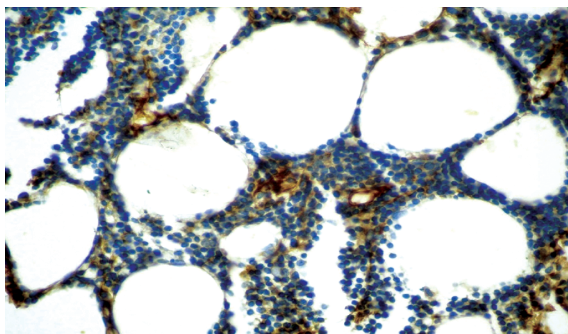

Fig. (6): Bone marrow trephine biopsy in follicular lymphoma showing positive immunohistochemical staining of bone marrow blood vessels with anti TM (400X).

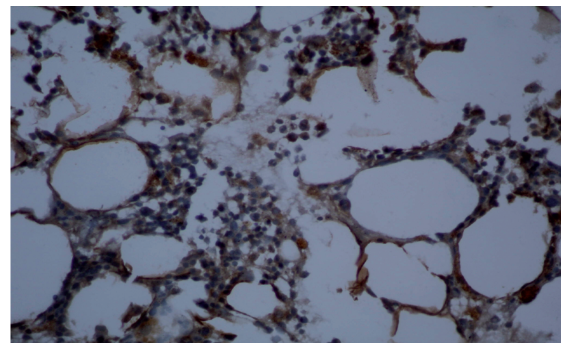

Fig. (7): Bone marrow trephine biopsy in diffuse large cell lymphoma showing positive immunohistochemical staining of bone marrow blood vessels with anti TM (400 X).

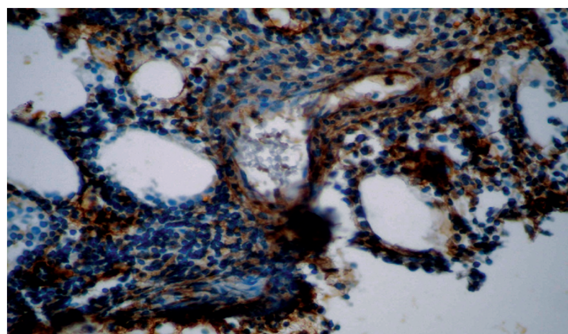

Fig. (8): Bone marrow trephine biopsy in diffuse large cell lymphoma showing positive immune histochemical staining with vascular endothelial growth factor (the positivity was 60%).

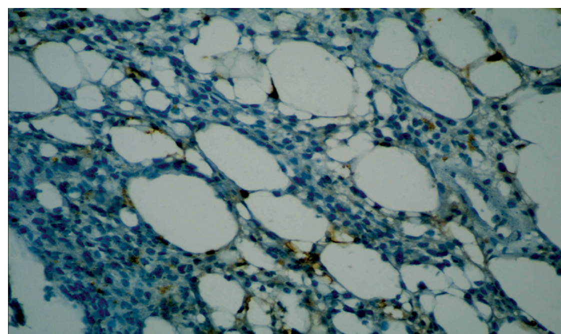

Fig. (9): Bone marrow trephine biopsy in chronic lymphocytic leukemia showing positive immunohistochemical staining with vascular endothelial growth factor (the positivity was 20%).

Table (1): Comparison of immunohistochemical staining of bone marrow BV with anti-vWF and anti-thrombomodulin and VEF expression among the studied patients with different histopathology.

	Patients with FL (n = 12)	Patients with DLCL (n = 8)	Patients with CLL (n = 6)	Control group (n = 6)	Significance
Anti-vWF:					
Median	11.5	12.0	10.0	2.50	X^2(KW)= 27.703
IQR	2.75	1.75	2.0	1.50	*p*= 0.000*
Z_{MW}	3.979	3.591	3.286		
p value	0.000*	0.000*	0.001*		
Anti-Thrombomodulin:					
Median	12.0	11.0	9.50	3.0	X^2(KW)= 26.861
IQR	2.50	2.75	3.50	2.0	*p*= 0.000*
Z_{MW}	3.987	3.591	3.293		
p value	0.000*	0.000	0.001*		
VEGF (%):					
Median	40.0	40.0	35.0	4.0	X^2(KW)= 25.826
IQR	17.5	10.0	20.0	4.0	*p*= 0.000*
Z_{MW}	3.983	3.580	3.273		
p value	0.000*	0.000*	0.001*		
IRS:					
Median	4.0	4.0	4.0	1.0	X^2(KW)= 26.216
IQR	2.0	2.0	2.0	1.0	*p*= 0.000*
Z_{MW}	4.061	3.661	3.293		
p value	0.000*	0.000*	0.001*		

IRS : Immunoreactive score.
IQR : Interquartile range.
$X^2_{(KW)}$: Chi square of Kruskal-Wallis test.
$Z_{(MW)}$: Z of Mann Whitney test (comparing each group with the control group). There is no other intergroup significance.
p : Probability of error (level of significance).
* : Significant difference (after correction for multiple comparisons).

The present study was conducted on twenty six patients with chronic lymphoproliferative neoplasms: Twelve patients with FL, eight patients with DLCL, and six patients with CLL.

Immunohistochemical staining of bone marrow blood vessels using anti von Willibrand factor and antithrombomodulin as well as histochemical studies of VEGF expression in bone marrow trephine biopsy sections were investigated in our patients as markers of angiogenesis.

In the present study we detected a significant increase in the number of bone marrow blood vessels when compared to the normal controls ($p<0.001$).

Our results are in agreement with El-Sorady et al., **[16]** who studied angiogenesis in 20 patients with NHL and 20 patients with Hodgkin disease, using immune-histochemical staining of bone marrow blood vessels by anti-vWF as endothelial cell marker in bone marrow biopsy sections. They observed that patients with hematological malignancies generally had significantly higher bone marrow microvessel counts when compared with the control group.

Foss et al., **[17]** stated that malignant lymphomas are heterogenous with respect to their microvasculature. They observed that vascularity was prominent in HD but not in most cases of low grade B cell lymphoma.

In the present study immunohistochemical staining using anti TM as endothelial cell marker in cases with FL as well as cases with DLCL showed a high statistically significant increase in number of BM blood vessels when compared with the control group.

Ribatti et al., **[18]** in their study concerning angiogenesis in NHL demonstrated that more intense vascularization has been described in B-cell NHL compared with the benign lymphadenopathies. Our results are also in line with other reports which demonstrated increased bone marrow blood vessels in bone marrow biopsy sections in different hematological malignancies, when compared to the control group. **[19,20]**.

These results are also concomitant with Gratzinger et al. (2007), **[21]** who assessed angiogenesis in cases of diffuse large B cell lymphoma, however they assessed the vascu-

larity immunohistochemically in lymph node biopsy sections. They stated that diffuse large B cell lymphoma specimens showed higher microvessel density when compared with the controls, implying that lymphoma cells induce local tumour angiogenesis. Moreover, they also stated that this microvessel densities showed a broad distribution which may offer differential access to the vascularly distributed nutrients, growth factors and chemotherapeutics.

In this study we could not observe a statistically significant difference in the number of BM blood vessels counted by using anti-vWF and those counted by using anti TM antibodies, and this could be attributed to the small sample size.

List et al., [22] in their study on lymph node biopsies stated that cellular expression of VEGF is common in cases with NHL. El-Sorady et al., [16] measured VEGF levels in the serum of their NHL patients, and they demonstrated that they significantly exceeded those of the control group. They stated that VEGF appears to be one of the most relevant angiogenic factors, since it's expression is closely related with the vessel density in most hematological malignancies. Foss et al., [17] found that VEGF expression in lymphomas proved to be almost entirely restricted to reactive cells, which are probably fibroblasts. They suggested that the process of angiogenesis differs in epithelial and lymphoid tumours and that the induction of angiogenesis through VEGF in malignant lymphomas is an indirect process involving reactive cells.

In this study we detected a strong positive correlation between the number of blood vessels counted by anti-vWF and thrombomodulin and the degree of VEGF expression. Cases which showed the highest number of BM blood vessels also had the highest VEGF expression.

These results are in accordance with Gartzinger et al., [21] although they assessed the relationship among the mirovessel density and expression of vascular endothelial growth factor in lymph node biopsy sections. They stated that diffuse large B cell lymphoma specimen showing higher local vascular endothelial growth factor expression showed correspondingly higher microvessel density. In addition, they also found that local vascular endothelial growth factor expression was higher in those specimens showing higher expression of the receptors of the growth factor, suggesting an autocrine growth promoting feedback loop.

In the present study VEGF expression did not show any statistical difference between cases of FL and cases DLCL.

Ruan et al. (2008), [23] reported that VEGF expression by neoplastic cells had been demonstrated in aggressive subtypes of lymphoma including peripheral T cell lymphoma, diffuse large B cell lymphoma, mantle cell lymphoma, primary effusion lymphoma and indolent histologies such as CLL/SLL, they also stated that only a minority of indolent follicular lymphoma cases showed variable expression of VEGF, and that increased VEGF expression had been associated with areas of transformation from indolent B cell lymphoma to aggressive DLBC and poor prognostic subgroups within DLBCL [23].

In contrast Gratzinger et al. in another study (2008), [24] investigated the prognostic significance of VEGF expression, VEGF receptors and microvessel density in diffuse large B cell lymphoma treated with anthracycline-based chemotherapy, and they stated that no correlation between increased MVD and VEGF expression has been found. The investigations also concluded that increased tumour vascularity was associated with poor overall survival and was independent of the international prognostic index (IPI) score. Other studies found no correlation between baseline MVDs and VEGF expressions, IPI score or clinical outcome. These studies provided a glimpse into the heterogeneity and complexity of the angiogenic processes in DLBCL [24].

Koster et al., [25] stated that in follicular lymphoma, it is well accepted that MVDs are significantly higher in interfollicular as opposed to intrafollicular regions. They also reported that increased vascularity pretreatment predicted favorable outcome.

Another possible explanation for Koster et al., [25] findings is that patients with follicular lymphoma with increased angiogenesis and which are characterized by a high MVD are more susceptible to the antiangiogenic effects of IFN-α2b therapy than those with low vascularity.

In the present study patients with CLL showed significant increase in the number of blood vessels in the bone marrow sections using anti-vWF and anti TM, compared with normal controls ($p<0.001$). Molica et al., [26] found significant increase of BM angiogenesis in cases of B-CLL, using anti-vWF as endothelial marker. They also stated that level of BM angiogenesis in hematologic malignancies is a complex process related to an interaction of an array of angiogenic and antiangionic factors released into the microenviroment. They also reported that the extension of microvessel area predict the risk of progression of the Binet stage, suggesting that the microvessel area should be preferred for prognostic assessment.

Kini et al., [27] found a significant positive correlation between the Rai clinical stage at the time of the biopsy, and microvessel density so they correlated the extent of BM involvement by B-CLL and the microvessel numbers, and reported that there was positive correlation between both. Regarding our cases, in the present study they were all in Binnet stage C, so similar correlation between the stage of the disease and microvessel count could not be done. Moreover Frater et al., [28] found in their study that CLL patients had higher MVD compared to controls.

The present study detected a strong positive correlation between microvessel counts obtained by anti-vWF and anti TM antibodies, two highly specific endothelail cell markers, no significant difference was found between them. Other studies have also reported that vWF, although highly specific for the vasculature, was partially absent in the capillary endothelium of tumour tissue, this explained the lower microvessel count they found in the bone marrow sections stained by this marker. They also stated that megakaryocytes are stained with anti-vWF but they were easily distinguishable by their morphology so they suggested that TM staining was a reliable tool for quantification of angiogenesis.

In contrast to these results Aguayo et al., [29] studied angiogenesis in cases of chronic leukemias and they used anti-vWF to highlight endothelial cells, they stated that they found no increase in BM neovascularisation in cases of CLL despite increase in cellularity. When they compared BM cellularity with vascularity, there was no correlation and vascularity appeared independent of cellularity. They suggested that other factors in bone marrow stroma or leukemic process may be important in determining the level of vascularity in these diseases.

As regards VEGF expression in CLL patients, there was statistically significant increase in the percentage of positive cells, intensity of the reaction and immunoreactive score.

Chen et al., [30] studied angiogenesis in CLL cases, they stated that VEGF expression is increased up to 7 folds. VEGF produced by B-CLL cells stimulates endothelial cell proliferation and angiogenesis.

In another report by Kay et al., [31] they demonstrated increase in VEGF and basic fibroblast growth factor (bFGF) in the culture supernatant of CLL cells grown in vitro and upregulation of mRNA encoding VEGF and its receptors as well as bFGF, suggesting that angiogenic factors are important in the biology of the malignant B-cell clone [30].

In conclusion, the increase in bone marrow blood vessels, which is measured by immunohistochemical staining with anti-vWF and anti-thrombomodulin and the increased expression of VEGF suggests that increased angiogenesis may play a role in the pathogenesis of the disease.

Serial immunohistochemical staining of bone marrow microvessel densities in the follow-up of cases is recommended that might be of prognostic value. Evaluation of angiogenesis after treatment is also recommended to judge patient's response to therapy and to help the decision of adding anti-angiogenic drugs to protocols of treatment.

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Endothelial and Platelet-Derived Microparticles in Preeclamptic and Normal Pregnant Women

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ABSTRACT

Background: Preeclampsia (PE) is a vascular disorder with uncertain etiology. Increased levels of circulating microparticles (MPs) have been suggested as a possible cause of vascular damage.

Aim: To explore the MPs involved in the control of hemostatic equilibrium: platelets and endothelial MPs as well as total MPs, defined as annexin V positive MPs, in pregnant women.

Patients and Methods: The study was conducted on 25 women with PE and 25 normal pregnant women (NP); another 25 normal non-pregnant women served as a normal control. Endothelial microparticles (EMPs) and platelets microparticles (PMPs) were measured by flowcytometry using monoclonal antibodies (anti-PECAM-1, FITC-CD31 for EMPs; anti-GPIIbIIIa, PE-CD41 for PMPs; and FITC-labeled annexin V for total MPs).

Results: The mean level of EMPs ($CD31^+/CD41^-$) was significantly elevated in PE group compared to NP group ($p<0.001$). EMPs level in PE positively correlated with systolic blood pressure ($p=0.006$; $r=0.36$) and diastolic blood pressure ($p=0.001$; $r=0.42$). The mean level of PMPs ($CD31^+/CD41^+$) as well as mean level of the total MPs were not different among the three groups. No significant correlation was found between PMPs or total MPs and mean arterial pressure in cases or control subjects.

Conclusions: The significant elevation in EMPs supports the theory of endothelial injury in the pathogenesis of PE. Further studies are needed to evaluate diagnostic and prognostic value of EMPs in PE at earlier gestational ages.

Key Words: *Preeclampsia – Circulating microparticles – Platelets – Endothelial microparticles.*

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INTRODUCTION

Normal pregnancy is a hypercoagulable state, which is associated with increased levels of coagulant factors and decreased levels of naturally occurring anticoagulants and fibrinolysis **[1]**. Preeclampsia (PE) is a multiorgan vascular disorder that complicates 5 to 7% of pregnancies and presents with hypertension, proteinuria, and fetal growth delay **[2]**. Approximately 10% of pregnancies are associated with hypertension, 75% of them are related to PE **[3]**.

Despite extensive research, the mechanisms involved in the vascular dysfunction in PE are not well understood. An endothelial dysfunction because of placental ischemia was proposed **[4]**. More recently, elevated plasma concentration of shed membrane microparticles (MPs) during PE has been reported **[5]**.

MPs are sub-cellular fragments (0.1-1.0 mm in diameter) that are released from the plasma membrane of stimulated or apoptotic cells into body fluids **[6-9]**. Elevated levels of MPs may reflect either increased cell activation or impairment of clearance by the reticuloendothelial system **[10]**. In addition, MPs exert prothrombotic activity by exposing negatively charged phospholipids and tissue factor. Thus, MPs are increased in conditions involving hypercoagulation, or systemic inflammation such as idiopathic thrombocytopenia, sepsis, and metabolic syndrome **[11-14]**. Normal pregnancy as well as PE, enhances MPs release from intravascular cells **[15,16]**.

Endothelial MPs (EMPs) provide markers of activated endothelium and increased levels

were seen in diseases associated with endothelial injury such as atherosclerosis, acute coronary syndromes, hypertension, and PE [12,17-19]. In addition, a proportion of women with pregnancy loss had elevated EMPs suggesting that endothelial damage or activation might be involved in the pathogenesis of pregnancy loss [20].

Circulating platelet MPs (PMPs) could be markers of platelet activation [17]. In addition, increased levels of PMPs were demonstrated in patients with thrombotic disorders such as transient ischaemic attacks and myocardial infarction [11]. However, the physiological significance of PMPs is still unclear.

PE is a serious pregnancy complication in less developed countries due to the poor antenatal care and low socioeconomic state. In Egypt, PE was reported as an important cause of maternal and fetal mortality as well as premature deliveries [21]. Additionally, more than 83% of Egyptian women with toxemia of pregnancy reported severe form of PE [22]. The present study aimed to explore the MPs involved in the control of hemostatic equilibrium, i.e. platelets-derived, endothelial-derived, as well as total MPs (annexin V positive) micropaticles, in both PE and normal pregnant women.

PATIENTS AND METHODS

This is a case control study that was carried out on women attending the outpatient clinic as well as the internal department of Obstetrics and Gynecology at Suez Canal University Hospital in the period from October 2007 to April 2008.

The institutional review board (of Suez Canal University) had approved the study. All patients have given an informed consent. The study included 3 groups as follows: (Group 1) pregnant women diagnosed with PE (PE, n=25), (Group 2) normal [normotensive] pregnant women (NP, n=25) and, (Group 3) non-pregnant women as a control group (C, n=25). The following matches were considered: a) Age (±5 years) and parity (for women in the three groups), b) gestational age (±2 weeks) for PE and NP groups.

Definition of PE:

A- de novo appearance of hypertension (diastolic blood pressure ≥110mmHg on any occasion, or ≥90mmHg on two separate occasions (at least four hours apart).

B- New onset of proteinuria (at least 0.3g protein/24 hours or ≥2+ on dipstick/24 hours) detected for the first time after 20 weeks of gestation.

C- Symptoms of PE developed after 20 weeks gestational age in a previously normotensive woman (according to standard criteria, Brown et al., 2001).

Patients with preexistent hypertension, gestational diabetes mellitus, coagulation disorders, previous renal or hepatic disease, intra-uterine growth retardation, patients in labor, and patients on regular drug treatment other than antihypertensive medications (i.e. oral contraceptive pills and aspirin) were excluded from the study. The control group consisted of healthy women not using any medications including oral contraceptives.

Methods:

Venous blood was drawn with minimal stasis into citrated vacutainer tubes (BD Biosciences; Oxford, UK) containing 0.5mL of 3.8% 0.129 mol/L trisodium citrate, 9:1 v/v.

Platelet-poor plasma (PPP) was obtained by centrifugation at 1500g for 15 min within 15min. of venipuncture. Plasma samples were divided in 250µl aliquots, snap frozen in liquid nitrogen to preserve MPs structure, and stored at –80°C until further analysis.

Flowcytometer analysis of MPs:

A- *Isolation of MPs:* MPs were isolated from plasma samples as described previously [23]. A sample of 250µl frozen plasma was centrifuged for 30 minutes at 19000g at 20°C to pellet the MPs. After centrifugation, 225µl of the supernatant was removed. The MPs pellet and remaining supernatant were re-suspended in 225µl phosphate-buffered saline with citrate (154mmol/L NaCl, 1.4mmol/L phosphates, 10.9mmol/L trisodium citrate, pH 7.4). After centrifugation for 15 minutes at 13000g at 20°C, 225µl of the supernatant was removed again. The MPS pellet was then re-suspended in 75µl

citrated PBS, of which five µl was used per incubation.

B- *Labeling of MPs:* Five µl of MPs suspension was diluted in 35µl of PBS containing calcium chloride (2.5mol/L), and separated equally into two tubes (A and B): In tube (A), 5µl FITC-labelled annexin V (BD Biosciences pharmingen, USA) was added to measure total MPs. In tube (B), 5µl of the monoclonal antibody against endothelial and platelet antigens were used (anti-PECAM-1, FITC-CD31, and anti-GPIIbIIIa, PE-CD41, Diaclone, France) to measure EMPs and PMPs respectively. Samples were incubated in the dark for 15 minutes at room temperature. After incubation, 200µl citrate-containing PBS was added to tube (A) while, 200µl of calcium-containing PBS was added to tube (B).

C- *Analysis:* Samples were analysed in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software (Becton Dickinson, San Jose, CA). Both forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. To identify marker positive events, thresholds were set based on microparticle samples incubated with same concentrations of isotype matched control antibodies (fluorescein isothiocyanate (FITC)-labelled IgG1 and phycoerythrin (PE)-labelled IgG1, Becton Dickinson, San Jose, CA) and an internal standard was added immediately prior to flow cytometry (Enumeration beads 1.01mm in diameter), (Sigma, USA). Calculation of the number of microparticles per ul plasma is based upon the particle count per unit time, the flow rate of the flowcytometer, and the net dilution of the microparticle suspension. MPs were identified on basis of their size (< mean diameter of the latex beads), density and capacity to bind to a cell type-specific antibody. The inter-assay and intra-assay coefficients of variation were <8% and <5%, respectively.

D- *Interpretation of the results:* Events with 0.1- to 1-µm size on a FS-SS graph were gated as MPs. MPs were estimated as the difference in labeling between specific antibody and their isotype. Mps expressing only CD31 ($CD31^+/CD41^-$) were defined as endothelial-derived MPs, Particles co-expressing both antigens ($CD31^+/CD41^+$) were defined as platelet-derived MPs while, (annexin V^+) MP were defined as prothrombotic MPs [23].

Statistical analysis:

Statistical analyses were performed with the use of the software SSPS for windows 11.5. Values were expressed as median, range, and percentages as appropriate. Mann-Whitney U Test was used to compare MPs between two groups. While, Kruskall Wallis test was used to compare MPs among the three groups. Correlation between EMPs and blood pressure was performed by linear regression analysis. *p*-values of 0.05 or less were regarded as significant.

RESULTS

Baseline characteristics of study participants are shown in (Table 1). Blood pressure was significantly higher in PE group compared to NP group ($p<0.001$).

Hematologic parameters were compared between PE group and NP group. Platelet count was significantly lower in PE group compared to NP group ($p=0.02$), however, it was within the normal range for NP and C groups. Comparing NP group to C group revealed a significant decrease in all hematologic parameters in NP group compared to C group ($p=0.002$, 0.002, 0.004 and 0.03 for hemoglobin levels, red blood cells, white blood cells and platelet counts respectively) (Table 2). The total number of prothrombotic circulating MPs were analyzed based on whether they were annexin V positive or not. The mean level of MPs showed no significant difference among the three groups (Table 3, Fig. 1). The mean level of Platelets microparticles (PMPs) ($CD31^+/CD41^+$ PMPs, counts $x10^3$/ml) were not significantly different among the three groups ($p=0.20$) (Table 3, Fig. 2). In addition, no correlation was found between PMPs levels and either systolic or diastolic blood pressure ($p=0.47$, $p=0.67$ respectively). The mean level of endothelial cell microparticles (EMPs) ($CD31^+/CD41^-$ EMPs, count $x10^3$/ml) revealed a statistically significant difference among the three groups ($p=0.04$). EMPs was significantly higher in PE group compared to NP group ($p=0.001$). While, no difference between NP group and C group was noted ($p=0.62$). EMPs levels in PE group positively correlated with both systolic and diastolic blood pressure ($r=0.36$; $p=0.006$) and ($r=0.42$; $p=0.001$) respectively (Table 3, Figs. 2-4).

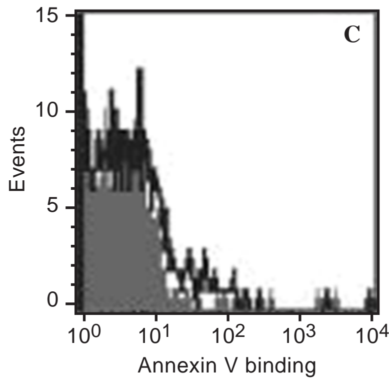


Fig. (1): Representative histogram of positive FITC annexin-V microparticles in PE group (preeclampsia).

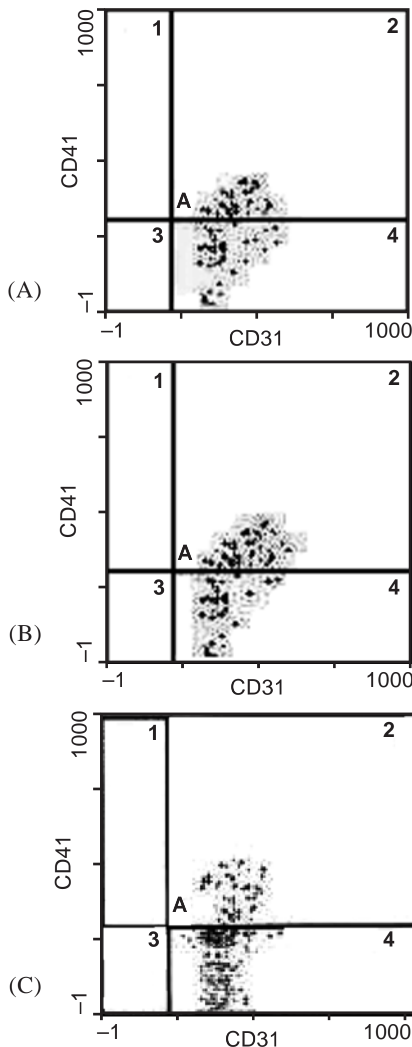


Fig. (2): Flow cytometric determination of EMP ($CD31^+/CD41^-$) and PMP ($CD31^+/CD41^+$): (A) C group (non-pregnant); (B) NP group (normal pregnant); (C) PE group (preeclampsia). Region 4 (lower right) is $CD31^+/CD41^-$, which represents EMP. Region 2 (top right) is $CD31^+/CD41^+$, which represents PMP.

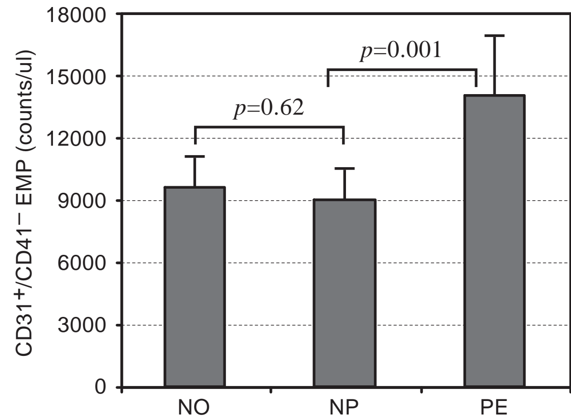
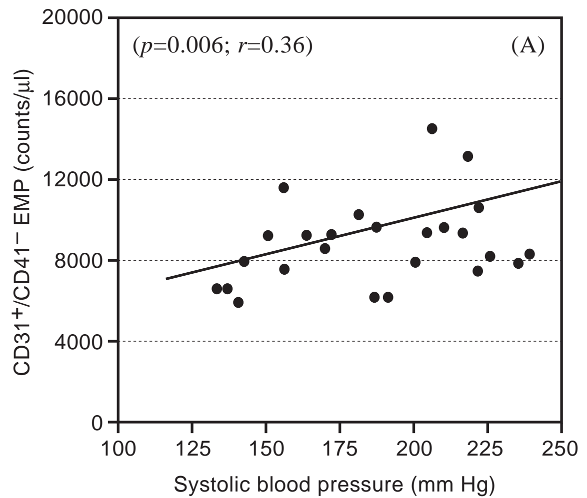


Fig. (3): Endothelial microparticles in: Non pregnant group (C), normal pregnant group (NP) and preeclampsia group (PE).



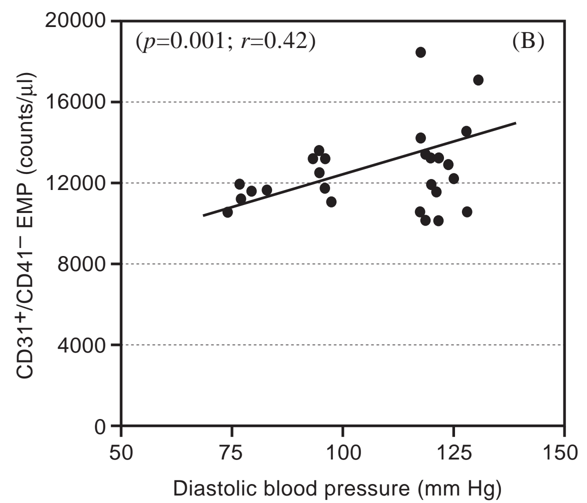


Fig. (4): Correlation between endothelial microparticles and systolic blood pressure (A), diastolic blood pressure (B) in Preeclamisa.

Table (1): Study population characteristics.

Characteristic	Preeclamptic (PE) (n = 25)	Normal pregnant (NP) (n = 25)	Control (C) (n = 25)
Demographic data:			
Patient age (yrs)	29 (27.7-30.4)	29 (28.0-30.6)	30 (23.5-34)
Gestational age (weeks)	35 (33.9-36.3)	36 (33.7-36.3)	–
Blood pressure (mmHg):			
Systolic	160 (148-200)	110 (109-118)	108 (95-133)
Diastolic	100 (97-103)	70 (68-74)	69 (65-85)
Proteinuria (g/L)	3.2 (0.4-5.2)	–	–
Parity:			
Primiparous	15 (60%)	12 (48%)	116 (64%)
Multiparous	10 (40%)	13 (52%)	99 (36%)

Values are given as medians, ranges and percentages.

Table (2): Hematological data of the study groups.

Hematologic data	Preeclamptic (PE) (n=25)	Normal pregnant (NP) (n=25)	P^a	Control (C) (n=25)	P^b
Hemoglobin (g/dl)	11.9 (10.9-12.6)	11.9 (11-12.3)	0.80	13.1 (12.6-13.8)	0.002
Red cell count ($x10^6$/L)	3.8 (3.6-3.9)	3.9 (3.7-4.2)	0.36	4.6 (4.2-4.8)	0.002
White cell count ($x10^3$/L)	8.7 (7.6-10.4)	8.4 (7.7-10.0)	0.65	6.5 (4.9-8.2)	0.004
Platelet count ($x10^3$/L)	154 (93-229)	203 (186-340)	0.02	288 (223-390)	0.03

Values are given as medians and ranges.
Statistical significant difference at $p<0.05$.
P^a = Preeclamptic group (PE) compared to normal pregnancy group (NP): Mann-Whitney U test.
P^b = normal control group (C) compared to normal pregnancy group (NP): Mann-Whitney U test.

Table (3): Microparticles in the study groups.

	Preeclamptic (PE) (n = 25)	Normal pregnant (NP) (n = 25)	Non pregnant (C) (n = 25)	P	P^b	P^c
Total MPs [a] (counts $x10^3$/µl)	23.4±9.1	19.8±7.6	22.7±8.8	0.62	–	–
PMPs [a] (counts $x10^3$/µl)	10.6±6.5	8.8±4.4	9.9±5.4	0.20	–	–
EMPs [a] (counts $x10^3$/µl)	13.9±7.9	8.5±3.0	9.8±2.6	0.04	0.001	0.62

MPs = Microparticles; PMPs = Platelet-derived microparticles; EMPs = Endothelial microparticles.
a =Values are given as mean ± SDÆ
P : Statistical difference between all groups (Kruskal-Wallis test).
P^b : Statistical difference between PE and NP groups (Mann-Whitney test).
P^c :Statistical difference between NP and C groups (Mann-Whitney test).

DISCUSSION

Preeclampsia (PE) is a major obstetrical health problem that threatens the survival of both mother and baby and, it becomes even more serious in less developed countries **[5]**. According to a WHO study on 7993 pregnancies in developing countries, hypertensive disorders of pregnancy was one of the most common obstetric events leading to perinatal deaths **[24]**.

PE is relatively a disorder of unknown etiology; however, many markers of endothelial dysfunction have been reported in women who develop PE, suggesting that PE is an endothelial cell disorder **[25,26]**. In theory, circulating MPs as pro-coagulant factors could cause the exaggerated hemostatic response 'hypercoagulability' seen in PE **[15]**.

Data about MPs in normal pregnancy and preeclampsia are controversial. Up to our knowledge, this is the first report about the pattern of MPs in Egyptian pregnant women with or without PE. In the present study, the total numbers of prothrombotic circulating MPs showed no

difference among the three studied groups, with no correlation with arterial blood pressure. This result came in conformity with the results of VanWijk et al. [15,27] who investigated the cellular origin and numbers of circulating MPs in normal pregnancy and reported no correlation between the total number of circulating MPs and blood pressure. In contrast, Bretelle et al., [28] was the first to demonstrate that normal pregnancy was associated with increased numbers and procoagulant activity of cell-derived MP. Unexpectedly according to the same study, pathological pregnancies did not show higher levels of MPs, instead they were associated with lower number of total (annexin V+) MPs. However, Desprez et al., [29] reported a progressive increase in procoagulant MPs level during normal pregnancy but these high levels did not exceed MPs level obtained in non-pregnant women. Recently, Redman and Sergent, [30] had found that MPs were increased during normal pregnancy, and they increased further with PE. A recent study reported a decrease in MPs at 12 weeks in normal pregnancy, and then returned to normal values postpartum. While a significant decrease in MPs in PE was observed at 28 and 36 weeks [31]. In our study, the mean gestational age was 35 weeks for normal pregnancy and 36 weeks for PE, which means that we might have missed the period of possible changes in MPs according to the previous study. The differences between the results of the previous studies may be related to the differences in number of study population, methods used to detect MPs, and gestational age during the study.

Previous studies showed that the levels of PMPs were reduced [28], increased [5], or unchanged [18] between PE and normal pregnancy. In this study, numbers of platelets-derived MPs were neither different among the three study groups nor related to arterial blood pressure. Same results were reported previously [18,32]. However, others reported significant fewer absolute numbers of PMPs in PE women compared to normal pregnant women [28,31,33]. Surprisingly, PMPs were not significantly lower only in PE, but also in non-pregnant women compared to normal pregnancy [27].

Recently, Lok et al. [16] suggested that the decrease in PMPs in PE women is possibly due to the concurrent decrease in platelet numbers. They reported a ratio of 0.02 between PMPs count and platelets number that was consistent among all the studied groups suggesting a direct association between number of circulating platelets and number of PMPs. Another possible explanation was that PMPs from PE patients might attach to leucocytes via p-selectin glycoprotein ligand-1 (PSGL-1) and being removed from circulation. In our study, platelet counts were significantly lower in PE compared to normal pregnancy and control group, yet it was within the normal range of platelets count in NP and C groups. Thus, the absence of a difference in PMPs in our patients could be explained partially by the absence of a true decrease in platelet count. Also, there is a possibility that PE may be associated with increased procoagulant potential of MPs without a change in their total number, as reported previously [15,27,28]. In favor of the latter explanation is the lack of a correlation between numbers of MPs and severity of PE suggesting that MPs numbers alone do not explain the reported vascular effects of MP [31].

The measurement of plasma EMPs is emerging as a useful marker of endothelial injury. Normally, EMPs represent 10-15% of the total microparticle population, and exist within a concentration range of 1-70 x10^3 (EMPs/ml) [28,34,35]. In the current study, EMPs showed a significant increase in PE group compared to normal pregnant group with significant correlation to both systolic and diastolic blood pressure. The same results were recorded by González-Quintero et al. [18]. These results are in favor of the theory that suggested EMPs as an important pathogenetic factor in the development of endothelial cell disorder (ECD). Previously, circulating EMPs were reported to directly affect the endothelium via impairment of ACh-induced vasorelaxation and nitric oxide production and thus not only act as a marker for ECD but also aggravate preexisting ECD [36].

Accordingly, a prospective study on patients with PE, and those with gestational hypertension reported a significant elevation in $CD31^+$/$CD42^-$ EMPs in PE compared to both gestational hypertension and normal pregnancy. In addition, PE plasma elicited a significantly greater level of $CD31^+$ EMP release from the cultured renal microvascular endothelial cells compared to gestational hypertension or control plasma

[37]. However, Bretelle et al. [28] enumerated EMPs using specific monoclonal antibody directed against αvβ3 (CD51). They reported a significant increase in plasma EMPs in normal pregnant women compared to non-pregnant controls; in the mean time, no difference was observed in PE group compared to normal pregnant women.

In this work systolic and diastolic blood pressure significantly correlated with the levels of $CD31^{+}/CD41^{-}$ EMP, indicating that the severity of hypertension is likely indicative of progressive endothelial damage. This supports previous findings in which non-pregnant patients with severe hypertension exhibited elevated EMP values when compared with mild hypertension and control groups [19]. This increase in blood pressure goes hand in hand with the elevation of EMPs since both reflect the possible endothelial damage in PE patients.

Some limitations to our study included the cross sectional type (tests were done in a single time point), the relatively small population number, and the late gestational age (35-36 weeks). Further prospective studies using larger population of pregnant women at different maternal and gestational ages are needed to enhance our results and, evaluate MPs diagnostic and prognostic value in PE. Still, this is the first study investigating MPs levels in Egyptian pregnant and preeclamptic women. In conclusion, the significant elevation in EMPs in this study supports the theory of endothelial injury in the pathogenesis of PE.

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Chromosomal Aberrations in Operating Room Nurses Exposed to Waste Anesthetic Gases

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ABSTRACT

Background: Although eliminated rapidly from the body due to low solubility in blood and tissues, anesthetic gases have been reported to be neurotoxic, genotoxic, teratogenic and carcinogenic.

Aim of the Work: To evaluate genotoxic risk of occupational exposure to anesthetic gases in a group of operating room nurses.

Subjects and Methods: A group of 27 operating room nurses exposed to waste anesthetic gases and 18 control nurses were examined for chromosome aberrations and sister chromatid exchanges (SCE) in peripheral blood lymphocytes.

Results: A significant increase in chromosomal damage in exposed nurses as detected by total chromosomal aberrations, gaps, deletion and endomitosis was detected while the increase in centromere separation and chromatid breaks was not significant. There was an increase in sister chromatid exchange frequency in exposed nurses compared to control even though it was not significant. Most of these parameters of genetic damage in exposed nurses were positively correlated with age and duration of exposure to inhaled anesthetics.

Conclusion: The results of our study suggest that exposure to waste anesthetic gases has the potential to cause changes in human genome including chromosomal aberrations and SCE.

Key Words: *Genotoxicity – Operating room personnel – Waste anesthetic gases – Chromosomal aberrations – Sister chromatid exchange (SCE).*

INTRODUCTION

Waste anesthetic gases are small amounts of volatile anesthetic gases that leak from the patient's anesthetic breathing circuit into the air of operating rooms during delivery of anesthesia. These gases may also be exhaled by patients recovering from anesthesia. Waste anesthetic gases include both nitrous oxide and halogenated anesthetics such as halothane, enflurane, isoflurane, desflurane, sevoflurane, and methoxyflurane [1].

There is a great concern that patients, physicians, and the operating room personnel might be exposed to health risks due to exposure to anesthetic gases. However, whether chronic exposure to waste anesthetic gases is hazardous to the health of anesthetic room personnel is still controversial [2].

Exposure to high concentrations of waste anesthetic gases, even for a short time, may cause the headache, irritability, fatigue, nausea, drowsiness, and impairment in judgment and coordination [1].

Although some studies report no adverse health effects from long-term exposure to low concentrations of waste anesthetic gases, several studies have linked such exposure to miscarriages, genetic damage and cancer among operating-room workers. Studies have also reported miscarriages in the spouses of exposed workers and birth defects in their offsprings. This reproductive and carcinogenic action in exposed operating room personnel may be related to genetic toxicity of inhalation anesthetics [3].

A meta-analysis suggested that chronic exposure to trace concentrations of anesthetic gases might cause mutations in DNA [4]. Some studies reported an association between occupational exposure to waste anesthetic gases and an increase in sister chromatid exchanges (SC-

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Es) in lymphocytes for staff working in unscavenged operating room (OR) [5]. While others did not support such association [6].

Hence, the undesirable health effects caused by anesthetic gases in human are of special concern. Among these are the genotoxic effects, including cancer and several other genetic diseases. Genetic biomonitoring of population exposed to potential carcinogens is an early warning system for genetic diseases or cancer. It also allows identification of risk factor at a time when control measures could still be implemented. Human biomonitoring can be performed using different genetic markers. Biomarkers such as chromosomal aberrations, micronucleus test, comet assay and sister chromatid exchange are among the most extensively used markers of genotoxic effects in molecular epidemiologic studies [7,8].

SCE analysis in peripheral blood lymphocytes is a well established technique aimed at evaluating human exposure to toxic agents. Its sensitivity and reliability have made SCE analysis one of the most popular methods in toxicology and human biomonitoring [6].

SCEs are interchanges between DNA replication products at apparently homologous loci. Although the precise molecular mechanisms underlying SCE formation are not fully understood, it has been suggested that they reflect either DNA damage, DNA repair or both [2].

Aim of the work:

The present study was carried out to estimate the genotoxic risk of occupational exposure to anesthetic gases in a group of operating room nurses and to investigate the possible relation of these findings with age and duration of exposure.

SUBJECTS AND METHODS

Over a 3 months period starting from April 2009 till June 2009, a cross sectional study was conducted at Kasr El-Aini hospital.

The study involved 45 subjects classified into 2 groups. The first group consisted of 27 nurses exposed to waste anesthetic gases in the operating room. The exposed nurses were exclusively females, with an age ranged from 20-50 years and a median of 32 years. They work 8 hours/day for 6 days/week. The median duration of their employment in the operating theatre was 14 years (range 2-31 years).

All the operating rooms had no active waste anesthetic gas scavenging system. The most commonly used anesthetics were nitrous oxide, isoflurane, sevoflurane and desflurane.

The control group consisted of 18 females nurses who were selected randomly from the same hospital with no history of occupational exposure to anesthetic agents, with an age ranged from 28-53 years and a median of 38 years. The median duration of their employment in the operating theatre was 25 years (range 6-35 years). The operating room personnel and the controls did not statistically differ from each other except for occupational exposure.

All examined nurses were non-smokers.

The studied groups were subjected to the following:

- Full history taking, including standard demographic data (age, marital status, etc...) as well as history of medical exposure to X-ray, vaccination or medications, occupational history (working hours/day, years of exposure, use of personnel protective measures, ventilation status of the workplace).
- The study was approved by the local Ethics Committee on human research. Informed consent was obtained from each nurse before the beginning of the study.
- Structural and numerical chromosomal aberrations in peripheral blood lymphocytes using the G-banding technique and determination of Sister chromatid exchange.

Chromosomal aberrations (CA) and SCE assay in peripheral blood lymphocytes:

Venous blood sample (3ml) was collected once from all the exposed and control group subjects using heparinized syringes. Blood samples were coded to avoid possible bias. The samples were transported to the laboratory and were processed within 2h after collection.

The CA analysis was conducted following a standard protocol with slight modifications. Half ml heparinized whole blood was cultured in RPMI with L-glutamine medium supplemented with 20% fetal bovine serum (FBS) (Eurolone, Europe), 200ul phytohaemaglutinin, 100ul penicillin and streptomycin, 100ul antimycotic

and 25ul preserved heparin. Each culture was incubated in 5% CO_2 incubator at 37°C for 72 hours. Metaphases were obtained by adding colcemide to the cultures at a final concentration 0.4ug/ml 2 hours before harvesting. The cells were collected by centrifugation, re-suspended in a pre-warmed hypotonic solution (0.075 M KCI) for 30min at 37°C and fixed in acetic acid-methanol (1:3 v/v). Chromosome preparations were stained using 4% Giemsa stain. The slides were analyzed using the high power of the light microscope and 25 metaphases were screened per each individual. Cells with 46 chromosomes were scored for CA. The analysis of CA included chromatid and chromosome breaks, chromatid gap, chromatid deletions, chromatid rings, dicentrics, centomere separation and endomitosis [9].

SCE assay was analyzed as follow: Bromodeoxyuridine (Sigma) was added to a final concentration of 10µg/ml at the start of the cultures for SCE analysis. The cultures were harvested after 72 hours. Harvesting was done as CA but with avoiding excessive light. Slides were stained using 50ug/ml hoechest dye and 4% Giemsa stain then analyzed with high power of light microscope. Twenty five metaphases were screened per each individual. Cells with 46 chromosomes were scored for SCE [10]. Examples of the SCE and chromosomal abnormalities are presented Figs. (1-3).

Statistical analysis:

Data were checked, coded, entered and analyzed using computer based statistical package for social sciences (SPSS) for windows 7.5 program.

Comparison between quantitative data of the study groups was done using student's *t*-test, while comparison between qualitative data was done by chi-square test. Pearson correlation coefficient was used for testing the association between two continuous variables. The "*p*" value of 0.05 was considered the limit below which the difference of the values would be statistically significant [11].

RESULTS

The results of occupational exposure to waste anesthetic gases on the levels of genetic damage were assessed by CA and SCE analysis.

In the current study, there were three female nurses out of 27 exposed nurses who were found to have offspring with congenital anomalies. One of the exposed subjects had a pituitary tumor and she was on treatment. Four of them had a past history of abortion.

Table (1) shows that the prevalence of headache, drowsiness, irritability, fatigue and syncopal attack were more frequently experienced by the exposed group as compared to the control group. The differences were found to be statistically significant ($p<0.05$).

Table (2) shows the frequencies of CA (gap, break, deletion, centromere separation, endomitosis and total chromosomal aberrations). In the operating room nurses, a significant increase in total CA, chromosomal deletion and endomitosis were observed in comparison with controls ($p<0.05$). As regards centromere separation and chromatid breaks, there was an increase in their frequencies in exposed nurses in comparison with the control group but not reaching the significant level ($p>0.05$).

As regards the frequencies of SCE, they were slightly higher in the exposed nurses as compared to the control group but the difference was not statistically significant ($p>0.05$).

No correlation was encountered between age or duration of exposure on one side and chromatid breaks, gaps, deletion, centromere separation or total chromosomal aberrations on the other side. On the other hand, a fair positive correlation was encountered between age and duration of exposure on one side and SCE on the other side (r=0.48 & 0.39 respectively and $p<0.05$).

Table (1): Frequency of clinical manifestations among nurses exposed to waste anesthetic gases.

Parameters	Exposed group		Control group		*p*
	No.	%	No.	%	
Headache	18	66.6	2	11.1	<0.05*
Drowsiness	17	62.9	3	16.6	<0.05*
Fatigue	7	25.9	2	11.1	<0.05*
Syncopal attack	6	22.2	1	5.5	<0.05*
Irritability	13	48.1	2	11.1	<0.05*

*S = Significant.

Table (2): Structural chromosomal aberrations among nurses exposed to waste anesthetic gases.

Parameters	Exposed group		Control group		*p*
	Range	(Mean ± SD)	Range	(Mean ± SD)	
Total chromosomal Aberrations	1-9	4.3±3.3	1-9	2.3±1.4	<0.05*
Breaks	0-2	2.1±2.2	0-1	1.8±1.1	>0.05
Gaps	0-9	3±2.7	2-4	1.5±0.9	<0.05*
Centromere separation	0-5	0.81±1.46	0-2	0.55±0.85	>0.05
Deletion	0-2	0.51±0.80	0	0.01±0.02	<0.05*
Endomitosis	No.	%	No.	%	<0.05
	5	18.5	0	0	

*S = Significant.

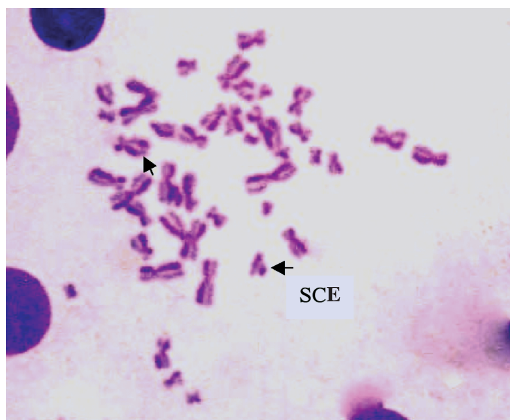

Fig. (1): Hoechst-Giemsa preparation from peripheral blood lymphocytes of a nurse exposed to waste anesthetic gas. The arrow points to sites of Sister Chromatid exchange.

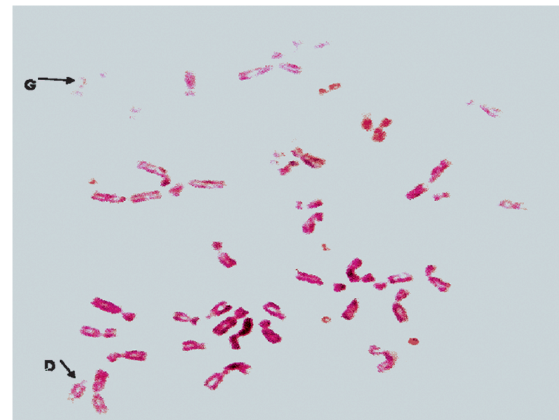

Fig. (2): Giemsa stained chromosomal preparation of a nurse exposed to waste anesthetic gas. Arrow G refers to gap while arrow D refers to deletion.

Fig. (3): Giemsa stained chromosomal preparation of a nurse exposed to waste anesthetic gas. The arrow points to chromosomal break.

DISCUSSION

The possibility of a potential mutagenic or carcinogenic action of chronic exposure to low concentrations of inhalational anesthetics has been previously studied, with conflicting results **[6]**.

Workers exposed to excessive amounts of anesthetic gases complain about feeling as if they themselves are anesthetized. They experience drowsiness, irritability, depression, headache, nausea, fatigue and impaired judgment **[12]**.

These behavioral modifications are of great concern, particularly in the operating room, where they can compromise surgical success and the health of the operating-room personnel. Assessing the long-term effects of exposure to anesthetic agents is more difficult. The chronic effects of anesthetic gas exposures are usually identified through retrospective epidemiological studies, followed by confirmational animal studies. The conclusions that could be drawn in some studies of chronic low-level exposures have been limited due to lack of quantitative exposure data and heavy reliance on information from questionnaires **[13]**.

However, chronic exposure to waste anesthetic gases has been associated with increased risk of spontaneous abortion in exposed women

workers and the wives of exposed men. Other adverse reproductive effects among exposed females include involuntary infertility and infants with low birth weights and with congenital abnormalities [3].

In the present study, only operating room nurses have been chosen as exposed subjects for this work, because they have the most exposure to waste anesthetic gases emanating from the apparatus as they spend more time than other persons working in the operating room (e.g. anesthetists, surgeons, etc…). If ventilators are not used, the level of anesthetic gases in the operating room is 76% higher near the apparatus than elsewhere [14].

Female nurses were only selected in the present study as many researchers concluded that there is a higher sensitivity to hazards of anesthetic gases in women. Rozgaj et al. (2001) reported that there was a significantly increased relative risk values for chromosomal aberrations and micronucleus for women [8]. Also, Bonassi et al. (1995) confirmed that there was a genetic damage due to exposure to inhaled anesthetics which was significant in women and not in men [15].

We excluded smoker subjects from our study as several researches demonstrated that smoking had a significant effect on DNA damage as both smoking persons exposed to anesthesia and smoking control persons presented increased rates of DNA damage [7,16]. The smoking index correlated significantly with the frequency of chromosomal aberrations [5]. This supports the importance of minimizing the risk of unwanted habitual variability (smoking habit), as in our study.

Our results showed that the exposed group reported high frequency of headache, drowsiness and other neurological manifestations (irritability & syncopal attack); the difference was statistically significant ($p<0.05$).

This is in agreement with Zacny et al. (1996) who reported that long term exposure to inhalation anesthetic agents may cause headache, depression, anxiety, loss of appetite, loss of memory and also changes in intellectual function [17].

In vitro experiments corroborated those results as Ozer et al. (2006) observed that chronic exposure to sub-anesthetic concentrations of sevoflurane and desflurane is associated with behavioral changes in rats [18].

Nitrous oxide exposure was shown to be associated with impaired neurobehavioral performance [19]. Even lower levels of exposure to anesthetic gases can cause an impairment of neurobehavioral performance [20].

In the current study, there were three female nurses out of 27 exposed nurses who were found to have offspring with congenital anomalies. One of the exposed subjects had a pituitary tumor and she was on treatment. Four of them had a past history of abortion.

In accordance with our findings, several researchers reported reduced fertility, increased risk of spontaneous abortion and the development of congenital abnormalities in the offsprings of operating room personnel exposed to waste anesthetic gases [3,21].

The results of studies of possible genotoxic effects of anesthetics on occupationally exposed subjects are controversial. Rozgaj et al. (2001) reported that the increase in sister chromatid exchange frequency was not significant while chromosome aberrations and micronucleus frequency increased significantly in personnel exposed to anesthetic gases [8].

Also, Chandrasekhar et al. (2006) reported a statistically significant increase in DNA damage as shown by chromosome aberrations, micronucleus frequency and the comet assay in operating room personnel exposed to anesthetic gases [7].

In this study, we found that most of the chromosomal aberrations were significantly more frequent in nurses exposed to waste anesthetic gases than in the unexposed nurses of the same hospital.

Our findings show that the frequency of SCE was only slightly higher in nurses exposed to waste anesthetic gases than in controls, this increase was insignificant. Similar results were obtained by many workers [5,22-24,28]. While others reported a significant increase in SCE frequency in medical workers exposed to volatile anesthetics [6,25-27].

Wroska-Nofer et al. (2009) reported that occupational exposure to nitrous oxide is asso-

ciated with increased DNA damage in female nurses exposed to anesthetics [29].

Contrary to what was expected, Pasquini et al. (2001) found in their study a lower frequency of SCE in male anesthesiologists than in controls but micronucleus frequency was significantly higher in female, but not male, anesthesiologists than in controls [30].

The mechanism by which the anesthetics induce DNA damage is still unclear. When isoflurane reacts directly with DNA, the most feasible alkali-labile modifications may be alkylation at the N-7 position of purines. Another explanation could be that, anesthetic gases undergo a residual metabolic oxidation or reduction giving rise to reactive products. Radical mediated reactions may also be involved in DNA damage induction [31].

Nitrous oxide may interfere with DNA synthesis by irreversibly oxidizing the cobalt atom of vitamin B_{12} and reducing methionine and thymidylate synthetase activity [32].

In the operating room nurses of the present study, age and duration of exposure positively correlated with genetic damage as presented by frequency of SCE. Similarly, investigation of operating room personnel found a positive correlation between chromosomal aberrations and years of employment [5].

On the contrary, a study on operating room personnel using micronucleus test showed that age and duration of employment did not correlate with micronucleus frequency [33] or DNA damage [7].

In conclusion our study showed that exposure to waste anesthetic gases may result in an increased risk of genetic damage which may lead to increased morbidity.

A limitation of our study, as of all other studies on this topic, is that it is not clear whether the observed genotoxic effect is attributable to the exposure to nitrous oxide, volatile anesthetics, or a mixture of both. Further studies should be performed in personnel solely exposed to nitrous oxide or (single) volatile anesthetics.

The outcome of our study indicates the danger of exposure to waste anesthetic agents in the hospital, this outcome is associated with our poorly equipped operating rooms (not having a central high-flow scavenging system and low leakage anesthesia machines, and not having facilities to use low-flow and closed-circuit anesthesia).

Our study suggest that anesthesia practices should be designed to further minimize environmental concentrations of anesthetic gases. The waste anesthetic gas scavenger and air conditioning equipment should be included in the operating theater and sufficient ventilation should be provided. Further, preventive health examination of all exposed personnel should be carried out periodically, including genetic biomonitoring.

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Study of Multidrug Resistance Protein, Lung Resistance Protein, and Cyclin A2 in Adult Acute Lymphoblastic Leukemia

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ABSTRACT

Background: Multidrug resistance agents: Multidrug resistance 1 (MDR1) gene and lung associated-resistance protein (LRP) are associated with unsuccessful treatment of acute lymphoblastic leukemia (ALL), however, their prognostic role is still largely unknown. Cyclin A2 is a member of the G2 cyclins that are involved in the cell cycle control and has been postulated to be associated with the chemosensitivity of leukemic blast cells. Its prognostic significance in adult ALL remains to be clarified.

Objective: Our aim in this study is to evaluate the frequencies of occurrence of multidrug resistance agents MDR1, and LRP, and cell proliferation marker cyclin A2 in Egyptian adult ALL patients, and to correlate them with disease prognosis.

Material and Methods: In this study, we measured the expression of MDR1 protein (P-gp), LRP, and cyclin A2 in 40 de novo adult ALL patients using flow cytometry.

Results: MDR1 protein was expressed in 20% of all cases and constituted 15% of complete remission (CR) cases, and 28.6% of non remission (NR) cases. LRP was positive in 32.5% of all cases, 23.1% of CR cases, and 50% of NR cases. Cyclin A2 was positive in 62.5% of all cases, 65.4% of CR cases, and 57.1% of NR cases. LRP showed significant correlation with cyclin A2 in all cases. There was also highly significant correlation between each of the 3 parameters with each other in NR cases. The 3 parameters showed no correlation with CR rate. None of the 3 parameters had any correlation with either of age, WBC count, Hemoglobin, BM blasts, or BM cellularity.

Conclusion: Our study revealed significant correlation between LRP and cyclin A2 in all adult ALL patients as well as highly significant correlation between the 3 parameters with each other in cases with no response to treatment. The exploration of such correlations could be further expanded with studies including more parameters.

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INTRODUCTION

Drug resistance is a major obstacle in the successful treatment and an important cause of death in acute leukemia. Such resistance may be present before beginning treatment or may develop during chemotherapy. Drug resistance that extends to structurally and functionally unrelated drugs is termed multidrug resistance [1].

Several molecular biological mechanisms have been identified as being associated with multidrug resistance [2]. P-glycoprotein (P-gp) is a product of the multidrug resistance1 gene (MDR1) and is an ATP-dependent pump capable of expelling drugs out of cancer cells [3,4]. P-gp is a transmembrane glycoprotein conferring cross-resistance to a variety of mechanistically and structurally unrelated cytotoxic drugs, such as anthracyclines, taxanes, vinca alkaloids and epipodophyllotoxins [5]. Another protein, the multidrug resistance related protein (MRP) is structurally similar to P-gp and belongs to the same transmembrane transporter superfamily [6]. In addition to these two proteins, a 110 kDa protein has been identified in a P-gp -negative multidrug resistant lung cancer cell line. This protein was termed the lung resistance protein (LRP) and acts as a major vault protein in humans [7]. The function of these vaults has been associated with nuclear-cytoplasmic transport [8]. More recently, the number of vaults was shown to be elevated in drug-resistant cell lines [9].

Despite the identification of these proteins, the pathways that result in drug resistance in leukemic cells remain largely uncharacterized.

While drug resistance genes expression has been studied in acute leukemia [10-13], the value of MDR1, MRP and LRP gene expression as independent predictors of treatment success is still controversial.

The regulation of the cell cycle is of particular importance for hemopoietic system. Critical components of the basic cell cycle regulations have been identified including cyclins, cyclin dependent kinases (CDKs), and cyclin dependent kinase inhibitors (CDKIs) [14]. Cyclin A2 is a member of the G2 cyclins that are involved in the control of the G2/M cell cycle transition and mitosis, as well as S-phase progression [14,15]. A previous study indicated that expression of cyclin A2 mRNA is a marker of cell proliferation in several hematological malignancies, and shows a highly significant correlation between expression of either cyclin A2 mRNA or protein and the cumulative percentage of cells in the S phase [17]. More recent studies show the correlation of lower levels of cyclin A2 with acute leukemia resistant to treatment [18], and recurrent ALL [19]. High levels correlated positively with complete remission and high levels of topoisomerase II [19].

Our aim in this study is to evaluate the frequencies of occurrence of multidrug resistance agents P-gp, and LRP, and cell proliferation marker cyclin A2 in Egyptian adult acute lymphoblastic leukemia patients, and to correlate them with disease prognosis and clinical and laboratory variables.

PATIENTS AND METHODS

Patients:

Forty patients with de novo acute lymphoblastic leukemia, who presented to the National Cancer Institute, Cairo University, in the period between September 2006 and March 2008, were included in this study, after an informed consent. They were 31 male and 9 female. Their ages ranged from 18 to 63 years, with a median of 29 years.

All patients were subjected to thorough history taking and full clinical examination. In addition radiological examination in the form of chest X-ray, abdominal ultrasound and CT scan whenever needed were performed.

Complete blood picture, bone marrow aspiration and morphological examination, liver and kidney function tests were also done. Acute lymphoblastic leukemia was diagnosed according to the criteria revised by the French-American-British (FAB) classification and immunological classification.

Immunophenotyping was done by flow cytometry (Partec III from DAKO cytomation), on marrow blast cells with a panel of monoclonal antibodies, purchased from DAKO (Denmark), including FITC and PE conjugated CD19 and CD20 for B ALL, and CD3 and CD7 for T ALL. Specific isotype control for FITC, PE conjugated monoclonal antibodies was used. Results were expressed as a percentage of cells showing positive expression. The monoclonal antibodies for P-gp, and cyclin A2 were also purchased from DAKO (Denmark), and that for LRP were purchased from Santa-Cruse biotechnology.

All patients were followed-up and classified according to treatment response, into complete remission (CR) group and no or incomplete remission (NR) group.

Complete remission in ALL was defined using the following criteria developed by an International Working Group [20-22] as follows:

- Normal values for absolute neutrophil count (>1000/µl) and platelet count (>100,000/µl), and independence from red cell transfusion.
- A bone marrow cellularity reveals normal maturation of all cellular components (i.e., erythrocytic, granulocytic, and megakaryocytic series).
- Less than 5 percent blast cells are present in the bone marrow.
- The absence of a previously detected clonal cytogenetic abnormality (i.e., complete cytogenetic remission, CRc) confirms the morphologic diagnosis of CR but not currently a required criterion.

Some patients may fulfill all of the above criteria for CR but may not recover peripheral blood counts to the required level. These are denoted as CRi, or CR with insufficient hematological recovery (platelets or neutrophils). CRp describes a subset of patients with CRi, where patients fulfill all criteria for CR except that platelet counts are <100,000/µl).

Patients who fail to achieve CR or CRi may experience a partial remission (PR), defined as a ≥50 percent decrease in bone marrow blasts with normalization of peripheral blood counts, or some other measure of hematologic improvement. A PR in ALL is generally expected to be of short duration, and in most circumstances, is unlikely to serve as a surrogate reasonably likely to predict for clinical benefit.

Methods:

Sampling: 5ml of peripheral blood were withdrawn under aseptic precautions, and were delivered into EDTA vacutainer tubes for complete hemogram, and flow cytometric analysis and for P-gp, LRP, and cyclin A2 measurement.

Surface study of MDR1 and LRP protein expression by flow cytometry:

A hundred µl of the heparinized blood were mixed with 10µl anti-MDR1 (monoclonal mouse antihuman MDR1 [SC-1313]) or anti-LRP (monoclonal mouse antihuman LRP [SC-18701]). An irrelevant monoclonal antibody of the same iso-type and protein concentration was used as a negative control. The tube was incubated at room temperature in the dark for 30min, washed twice with PBS; the supernatant was aspirated, leaving approximately 100µl fluid. Sheath liquid was added and analyzed by flow cytometry.

Cytoplasmic study for Cyclin A2 protein expression by flow cytometry:

Fifty µl of the diluted anticoagulated blood were added to 100µl intra-stain reagent A (fixation), vortexed gently, incubated at room temperature for 15 minutes, washed in PBS and then the supernatant aspirated, leaving approximately 50µl of fluid. A hundred µl DAKO intra-stain reagent B (permeabilization) (Fixative A and permeabilization B, DAKO Cytomation) were added, then 10µl PE conjugated monoclonal mouse antihuman cyclin A2 (SC-239) were also added. An irrelevant monoclonal antibody of the same iso-type and protein concentration was used as a negative control. The tubes were incubated in the dark at room temperature for 15 minutes, and washed twice by PBS. The pellets were resuspended in a sheath fluid for flow cytometric analysis.

As a measure for the intensity of staining, the mean fluorescence index (MFI) was used, which represents the ratio between the mean fluorescence intensity of cells stained with the specific antibody and that of cells stained with the isotype-matched control antibody, the case was considered over expressing or positive for P-gp at a ratio of ≥1.1 [23], and was considered positive for LRP when the ratio exceeds 0.3 [24] and for cyclin A2 when the ratio exceeds 0.2 [25].

Statistical analysis:

The data were coded entered and processed on an IBM-PC compatible computer using SPSS (version 15).

Student's *t*-test was used to assess the statistical significance of the difference between two population means in a study involving independent samples.

Correlation analysis: Assessing the strength of association between two variables. The correlation coefficient denoted symbolically *r*, defines the strength and direction of the linear relationship between two variables. The level $p<0.05$ was considered the cut-off value for significance.

RESULTS

Forty patients with de novo acute lymphoblastic leukemia were included in this study. They were 31 male and 9 female. There ages ranged from 18 to 63 years, with a median of 26 years, and a mean of 31.95±12.5 years.

Their WBC count was 82.6±85.7 x10^9/L, and ranged between 11.2 and 315.4 x10^9/L. Their hemoglobin level was 8.5±1.84g/L and ranged between 5.3-11.4g/L. Their BM blasts was 73.2%±14.32 with a range of 35-91%. Thirty three (82.5%) of them had hypercellular marrow and 7 (17.5%) had normocellular marrow.

After induction therapy 26 (65%) patients achieved complete remission (CR group), and 14 (35%) did not achieve it (NR group).

The mean fluorescent intensity (MFI) of P-gp in all ALL cases was 16.28±38.46, that of LRP was 8.62±22.95, and that of cyclin A2 was 3.20±6.28. The case was considered over expressing or positive for P-gp at a ratio of ≥1.1, and was considered positive for LRP when the ratio exceeds 0.3, and for cyclin A2 when the ratio exceeds 0.2.

The cellular expression of P-gp, LRP, and cyclin A2 proteins in all patients group, CR group, and NR group is shown in Table (1).

As regards the correlation between P-gp, LRP, and cyclin A2 in all cases, LRP showed significant correlation with Cyclin A, (*r*=0.67, *p*=0.001), and non significant correlation with MDR1, (*r*=0.37, *p*=0.10). MDR showed no significant correlation with cyclin A2 (*r*=0.36, *p*=0.1).

The correlations between cyclin A2, P-gp, and LRP in cases with complete remission, were not statistically significant. However, the correlations between each of P-gp and LRP and cyclin A2, were highly significant in cases with no response to treatment (all *r*=0.99, *p*<0.0001).

We found no significant difference in MFI of P-gp, LRP, and cyclin A2 in patients with CR, and those with NR (*p*=0.94, 0.36, 0.72 respectively) (Table 2).

Tables (3) describes correlation studies between each of cyclin A2, P-gp, and LRP, respectively, and age, TLC, Hb, and BM blasts. They revealed no correlation.

As well, when comparing the levels of cyclin A2, P-gp, and LRP in relation to BM cellularity, no statistically significant difference was found between the levels in normocellular, and hypercellular marrows, (*p*>0.05) (Mann-Whitney test) Table (4).

Table (1): Expression of Pgp, LRP, cyclin A2 in all, CR, and NR cases in adult ALL.

Group	All cases	CR cases	NR cases
P-gp	8/40 (20%)	4/26 (15.4%)	4/14 (28.6%)
LRP	13/40 (32.5%)	6/26 (23.1%)	7/14 (50%)
Cyclin A2	25/40 (62.5%)	17/26 (65.1%)	8/14 (57.1%)
P-gp & LRP	5/40 (12.5%)	3/26 (11.5%)	2/14 (14.3%)

Table (2): Correlation between levels of P-gp, LRP, Cyclin A2 in NR and CR groups in adult ALL.

	Response to treatment		*p*
	NR (14)	CR (26)	
	Median	Median	
Cyclin A2 MFI	1.20	1.15	0.72
MDR MFI	1.29	1.25	0.94
LRP MFI	1.00	1.10	0.36

Table (3): Correlation of P-gp, LRP and Cyclin A2 with clinical and laboratory data in 40 adult ALL patients.

	MFI					
	MDR		LRP		Cyclin A2	
	r	*p*	*r*	*p*	*r*	*p*
Age	–0.33	0.15	–0.30	0.18	0.10	0.67
TLC	0.49	0.49	0.19	0.41	0.31	0.17
Hb	–0.11	0.63	–0.23	0.31	0.00	1.00
BM blasts	0.30	0.21	0.23	0.35	0.22	0.37

Table (4): Correlation between levels of P-gp, LRP, Cyclin A2 and BM cellularity in 40 adult ALL patients.

	BM Cellularity		*p*
	Normocellular	Hypercellular	
	(3)	(19)	
Cyclin A2 MFI	1.46	1.15	1.4
MDR MFI	1.33	1.25	1.38
LRP MFI	1.40	1.10	1.05

DISCUSSION

Studies on the treatment of adult ALL have shown only modest improvements over the last 2 decades, with the actual cure rate still ranging between 15% and 40%. The resistance of tumor cells to chemotherapeutic drugs is a major limitation in cancer treatment. Multidrug resistance phenotype is the most frequently studied mechanism for intrinsic drug resistance, yet the prognostic role of P-gp and other multidrug resistance-associated proteins in adult ALL is still largely unknown **[26]**.

On the other hand deregulation of the cell cycle is a prerequisite for the formation of most if not all malignant tumors. When tumor cells proliferate abnormally, cyclins, including cyclin A2, may be expressed abnormally **[23]**. Several reports show direct correlation between the expression of cyclins and better prognosis **[18,19, 22,23]**. Some authors **[29]** remarked that these results are counterintuitive considering that these cyclins may enhance cellular proliferation by accelerating entry into S phase. However, they postulated that the detailed implications of overexpression of cyclin A2 in leukemic cells are still unknown. They proposed that CDK2-cyclin A2 complexes may exhibit negative regulation for S phase progression, or, alternatively,

cyclin A2 overexpression may contribute to the increased chemosensitivity of leukemic cells by stimulating these cells into S phase of the cell cycle. It has been also postulated that cyclin A2 may be associated with the chemosensitivity of leukemic blast cells for the following reasons: (1) Expression levels of cyclin A2 mRNA have a tendency to decrease after relapse compared with the primary leukemia; (2) Expression levels of cyclin A2 have a positive correlation with topoisomerase II mRNA; (3) Expression levels of cyclin A2 have an inverse correlation with multidrug resistance 1 (mdr1) RNA expression, and elevated levels of the latter are characteristic of refractory acute leukemia cells **[31]**.

Our aim in this study was to evaluate the multidrug resistance effect of MDR agents versus the postulated chemosensivity effect of cyclin A2, through studying the frequencies of occurrence of MDR1 protein, LRP, and cyclin A2 in Egyptian adult acute lymphoblastic leukemia patients, and their correlation with disease prognosis and clinical and laboratory variables. Our patients were grouped into 2 groups according to response to induction therapy: Those who achieved complete remission (CR group), and those who did not achieve it (NR group).

P-gp expression was found positive in 8/40 patients (20% of all ALL cases), of them, 4/26 achieved complete remission (CR) which makes 15% of CR cases, and 4/14 cases with no response to treatment (NR) which makes 28% of NR cases.

A multicenter study, on adult ALL cases at diagnosis, found 21.7% of patients positive for P-gp, with lower CR rate among positive cases (53.5%) than among negative cases (79.6%) **[25]**.

Some authors, in another study on adult ALL cases at diagnosis, found 47% of patients positive for P-gp, with similar CR rates in positive and negative cases **[27]**.

While other authors studied MDR1 mRNA expression by RT-PCR in adult and childhood acute leukemia. They found that MDR1 mRNA was expressed in 25% of cases at diagnosis. Of the positive cases, 71% achieved CR, compared to 78% of the negative cases. They stated that MDR1 expression appeared to have no statistically significant effect on patient outcome following induction chemotherapy **[28]**.

A different group, studied P-gp expression by flow cytometry on childhood ALL cases and found 26% of cases positive at diagnosis, with no difference between positive and negative cases in CR **[23]**.

The percentage of expression in all of the above studies is widely variable. This conclusion was also described by two groups of authors who attributed its causes to the use of different techniques and methods, different cut-off values and pooling of heterogenous groups of patients such as AML and ALL, initial and relapse samples, and adult and pediatric cases **[32,33]**.

Our study showed that LRP was expressed in 13 (32.5%) patients, of them, 6/26 had CR (46.8% of all CR cases), and 7/14 had NR (53.2% of all NR cases). A multicenter study, found that 60.5% of cases were positive for LRP, and that LRP expression had no influence on CR **[26]**. Some authors reported the frequency of LRP expression 18% of adult ALL cases. They stated that the positive cases were too few to make statistical comparison between CR and NR cases **[27]**. A different group, in a study on acute leukemia patients, found 15% of cases expressing LRP, making 33% of CR cases, and the LRP negative cases made 84% of NR cases **[29]**.

Dual expression of P-gp and LRP was detected in our study in 5 cases (12.5% of all cases), two of them were in CR (2/26 of all CR cases, 7.7%), and three cases were in NR (3/14 of all NR cases, 21.4%). Although the result is suggestive of significant difference, the sample size does not support statistical confirmation.

Some authors reported 21.1% coexpression of MDR1 and LRP with no significant influence on CR rate **[26]**. However, two different groups concluded from their studies that coexpression of LRP and MDR1 might result in worse prognosis **[29,37]**.

We had 25/40 cases expressing cyclin A2 most of them had CR (17/26, 66% of CR group), and 15/40 negative for cyclin A2 (8/14, 57.1% of NR group).

Another group found 50/75 of AL cases expressing cyclin A2. They also found CR rate

87.9% in cases with over expression of cyclin A2, and 38% of the negative cases had CR **[19]**.

Our study revealed significant correlation between LRP and cyclin A2 (p=0.001) in all ALL cases, while P-gp correlation with cyclin A2 was not significant. Both P-gp and LRP did not have any significant correlation with cyclin A2 in the CR group. However both had a highly significant correlation with cyclin A2 in NR group.

Some authors discovered a negative correlation between the gene expression levels of MDR1 and cyclin A2 (r=–0.37, p=0.029) in NR group. Their study was conducted on AL patients using RT-PCR **[19]**.

Another group in a study on ALL patients revealed no significant correlation between MDR1 and cyclin A2 **[35]**.

Our results showed no significant difference between CR group and NR group in the expression of P-gp, LRP, and cyclin A2.

In agreement with our results, a study group stated that CR rates were similar in MDR1 positive and negative patients **[27]**. Also another group found the difference in CR rates between LRP positive and negative patients was not significant, (p>0.05) **[36]**. Cyclin A2 expression rates showed no difference between newly diagnosed AL patients and patients in CR, in the study conducted by a different group **[30]**.

However, two different groups disagree with the previous results stating in their studies that patients with negative MDR1 expression had a significantly higher CR rate than patients with positive ones **[26,29]**. Same conclusion was deduced by a third group regarding LRP who reported that CR rate in LRP positive was lower than LRP negative patients **[37]**. Finally, some authors found that LRP expression was associated with lower CR rate, while MDR1 appeared to have statistically no significant effect on CR **[28]**.

No correlation was detected between P-gp, LRP, and cyclin A2 and known prognostic markers such as age and WBCs count, which represent tumor cell mass, as well as hemoglobin and BM blasts.

Our results are in accordance with two different groups who revealed no relation between each of P-gp and LRP with either age or WBCs count **[32,33]**.

Conclusion:

The present study demonstrates a significant correlation between LRP and cyclin A2 in adult ALL patients, as well as highly significant correlation between the 3 parameters with each other in cases with no response to treatment. Significance of correlation of dual expression of MDR1, and LRP on leukemic cells was not supported statistically due to small sample size.

For better understanding of the impact of the factors involved in multidrug resistance we recommend, in a future study, involving MRP with MDR1 and LRP.

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Gestational Antioxidants Reduce Pre-Eclampsia Associated Coagulopathy and Improve Neonatal Outcome

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ABSTRACT

Background: Oxidative stress is blamed in the pathogenesis of pre-eclampsia. However, it is less clear what effect gestational antioxidants would have on pre-eclampsia associated coagulopathy, and on neonatal outcome.

Aim of the Study: To investigate the effect of antioxidants supplementation on coagulopathy during pre-eclampsia and to evaluate maternal and neonatal outcome.

Patients and Methods: The study was performed as a randomized, controlled, blinded trial; 251 high risk pregnant women were randomized to receive either antioxidants (1000mg vitamin C, 400IU vitamin E, 100µg Selenium and 1500IU vitamin A) or placebo. Primary maternal outcome was pre-eclampsia or one of its complications. Newborns for both groups were followed through the neonatal period. In each trimester, detailed blood chemistry lipogram, and coagulation profile were done. Antioxidants blood levels (vitamins A, C, and E) were measured immediately before delivery.

Results: Incidence of pre-eclampsia did not differ between the two groups. However, a significant reduction in disease severity was noticed. Antioxidants reduced the levels of D-dimer, von Willibrand factor, and fibrinogen significantly. Platelets activity showed a significant reduction in the supplemented group. Antioxidants were significantly higher in blood of the supplemented group. Concerning neonatal outcome, low birth weight, need for neonatal intensive care and neonatal hyperbilirubinaemia were significantly reduced in newborns of the supplemented group.

Conclusion: Supplementing high risk women with antioxidants during pregnancy may help to counteract the oxidative stress and control coagulopathy. However, it does not prevent the disease. This study suggests potential benefits for gestational antioxidants as regards neonatal outcome in pre-eclampsia.

Key Words: *Coagulopathy – Antioxidants – Pre-eclampsia – Neonatal jaundice – Vitamins.*

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INTRODUCTION

Pre-eclampsia is a complex multisystem disorder that affects 2-8% of all pregnancies and causes about 30% of all maternal deaths **[1]**. Despite all effort, the only successful therapy, once the diagnosis of pre-eclampsia has been established, is termination of pregnancy. Accordingly, pre-eclampsia remains a major cause of several neonatal problems, such as, preterm birth, intrauterine growth restriction (IUGR), intrauterine hypoxia and perinatal death **[2]**.

The cause of pre-eclampsia is not fully understood; however, several environmental, nutritional and genetic factors have been suggested to trigger pre-eclampsia through initiating placental ischemia and endothelial cells dysfunction. Pathologically, pre-eclampsia is characterized by vasoconstriction, cell damage and coagulation disorders **[3]**.

During normal pregnancy, hemostasis shifts in the direction of hypercoagulability, thus decreasing bleeding complications during delivery, and in this aiding the uterine muscle contraction, the primary factor responsible for interrupting blood flow **[4,5]**. In this regard several noticeable changes in the hemostatic balance have been noticed. Thrombocytopenia, increased endogenous thrombin generation, acquired activated protein C resistance, decreased activated partial thromboplastin time (aPTT) and increased prothrombin complex level with international normalized ratio of less than 0.9 have been reported **[6]**. With the exception of factor XI, most coagulation factors including fibrinogen are increased

during normal pregnancy [4,7]. Although platelet count remains within the normal range during the first and second trimesters, benign gestational thrombocytopenia (80-150 X 10^9/L) can be observed in the third one. This is associated with activation of platelets, release of beta-thromboglobulin and platelet factor-4, and an unchanged bleeding time [8]. The level of both plasminogen activator inhibitor-1 from endothelial cells and plasminogen activator inhibitor-2 from placenta are increased. Prothrombin fragment 1+2, TAT complex, soluble fibrin and D-dimer increase as well. All these reflect activation of blood coagulation and simultaneous increase in fibrinolysis, which normalize 4-6 weeks after delivery without signs of organ dysfunction [7,8].

Any factor that causes a change in the delicate hemostasis balance during pregnancy can exaggerate hypercoagulability state and increase risk of disseminated intravascular coagulopathy (DIC) and multiorgan dysfunction. This is observed in situations like pre-eclampsia, eclampsia, and intrauterine fetal death [9,10]. It has been noticed that an increase in oxygen free radicals is associated with exaggeration of hypercoagulability state [11], suggesting a compromise in antioxidant capacity in such situations. Indeed, lipid peroxidation resulting from endothelial damage during pre-eclampsia leads to an increased very low density lipoprotein and low antioxidants level [12].

On the other hand, several neonatal problems that largely influence neonatal mortality and morbidity, such as chronic lung disease, necrotizing enterocolitis, retinopathy of prematurity and intracranial hemorrhage, are thought to be related to the action of reactive oxygen species, especially in preterm infants [13]. It is thought that neonates, especially prematurely born, have an overstressed underdeveloped antioxidant system. Neonates depend on maternally transferred antioxidants that do not cross the placenta in sufficient amount until the third trimester of gestation [14]. In pre-eclampsia maternal antioxidant system is exhausted and there is a higher risk of preterm delivery. Another neonatal condition that is thought to be related to oxidative stress in neonates is neonatal idiopathic hyperbilirubinaemia [15]. It was proven that the decrease in plasma bilirubin was contemporary with an increase in plasma antioxidant capacity and decrease in oxidative stress in preterm infants [16].

Despite the fact that several hematological and biochemical studies suggested that oxidative stress may be involved in the pathogenesis of pre-eclampsia and its effects [17-19], it is less clear whether intake of antioxidant vitamins can protect against pre-eclampsia and improve neonatal outcome. On one hand, an observational study had shown an increased risk of pre-eclampsia among women with an intake of vitamin C below the recommended dietary allowance [20], and another randomized controlled trial had shown a lower occurrence of pre-eclampsia in high-risk women who were supplemented with high doses of the antioxidative vitamins C and E [21]. On the other hand, other studies failed to show any difference in the incidence of pre-eclampsia between treated and untreated women [22,23]. The effect regarding neonatal outcome was most confusing as one trial showed an increase in the rates of low birth weight in the antioxidant supplemented group [22].

Our aim was to study the potential effects of antioxidants supplementation on coagulopathy-associated pre-eclampsia and its effect on both maternal and neonatal outcomes.

PATIENTS AND METHODS

Study population:

Study population was recruited form Sohag University Hospital obstetric outpatient clinic in the period from July 2006 to June 2008. Only pregnant women with high risk for pre-eclampsia were considered. The inclusion criteria were gestational age between 6-10 weeks with one or more of the following risk factors: pre-eclampsia in the pregnancy preceding the current one, eclampsia in any previous pregnancy, essential hypertension requiring medication, diagnosis of HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count), and chronic renal disease pre-pregnancy or during pregnancy. Exclusion criteria included maternal liver disease, diabetes mellitus and possible materno-fetal fetus Rh incompatibility.

The assigned ladies were counseled about their participation in the study. Written informed consent was obtained prior to recruitment. Women had the right to refuse to participate and/or

withdraw from the study at any time without being denied or their babies for regular full clinical care. Personal information and medical data collected were confidential and were not made available to a third party. After delivery, newborns were followed-up and included in the study.

Study design and protocol:

This study was a prospective randomized controlled blinded trial. Participating women were randomly assigned to receive either antioxidants cocktail (group I) containing 1000mg vitamin C, 400IU vitamin E, 100μg Selenium and 1500IU vitamin A; or identical placebos (group II). This supplementation was given daily from enrolment to delivery and was continued even after pre-eclampsia or hypertension was diagnosed. Participating women were seen once a month for clinical evaluation according to the standardized antenatal care protocol. The definition of pre-eclampsia was in accordance with the American College of Obstetricians **[2]**. In the last visit in the first and second trimester, and immediately before delivery blood samples were drawn for laboratory investigations.

A total of 251 women consented to the study of which 126 women were recruited to the antioxidant arm (group I) and 125 to the placebo (group II). Of group I, 105 women were followed up until delivery while 21 women were lost during follow-up (7 abortions and 14 did not come for antenatal care). Of group II, 102 women were followed-up until delivery while 23 women were lost to follow-up (10 abortions and 13 did not come for antenatal care). Blood sample for hematological and biochemical testing were drawn from 126 women in group I in the first trimester (group Ia), 110 women in the second trimester (group Ib), and 105 women in the third trimester (group Ic). For group II, blood samples were drawn from 125 women in the first trimester (group IIa), 114 women in the second trimester (group IIb), and 102 women in the third trimester (group IIc). Vitamins levels were done only in the last sample drawn in the third trimester.

Routine investigations included complete blood count (CBC) done on H-Max Coulter system, coagulation profile including prothrombin time (PT) and activated partial thromboplastin time (aPTT) on Sysmex Dade Behring fully automated system; random blood glucose, liver function tests, renal function tests, lipogram [cholesterol, triglycerides (TG), high density lipoprotein (HDL-c) and low density lipoprotein (LDL-c)] on Beckman-Synchrone CX-9 fully automated chemical autoanalyzer; and urine for protein. Research investigations including plasma fibrinogen, thrombin time (TT), and D-dimer done on Sysmex Dade Behring fully automated system; von Willibrand Factor (vWF) by latex agglutination method supplied by Dade Behring; platelet activation by ADP/Collagen on PFA-100 Dade Behring; and vitamins assay including vitamins A and C by high performance liquid chromatography (HPLC) and vitamin E assay by gas chromatography **[24]**.

All newborns were evaluated by detailed full clinical examination. Neonates were followed-up every week for the first four weeks of life or until discharge from neonatal care if the period of admission to neonatal care extended beyond the first four weeks of life. Neonatal outcomes were recorded. Neonates were grouped into two groups, group I, whose mothers received antioxidants during pregnancy; and group II, whose mothers did not receive antioxidants during pregnancy. Of the newborns included in the study, 98 were followed-up in group I for the whole neonatal period while only 90 newborns completed the neonatal follow-up in group II. Blood sample was drawn whenever clinical jaundice was apparent. A total bilirubin level above 13mg/dl was used to diagnose neonatal hyperbilirubinaemia.

Details of blood sampling and laboratory technique:

The pregnant females were sampled before breakfast on light dinner. Blood was withdrawn in appropriate vacutainers for different tests. For the coagulation profile, the citrated samples were centrifuged in cooling centrifuge system at 4000 rpm for 10min at 20°C to prepare platelet poor plasma, then the plasma was delivered to SYSMEX DADE BEHRING Fully Automated system for PT, aPTT, TT, fibrinogen by clotting method and D-dimer by turbidimetric method, Kits were supplied by DADE BEHRING. Normal values were as follows: PT 10.5-13sec., aPTT 26-38sec., TT 14-17sec., fibrinogen 180-360mg/L, D-dimer <20μg/dL. vWF was assayed by latex agglutination method supplied by DADE BEHRING with a normal value of 70-150%. For platelets activation, the second citrated tube was delivered to PFA-100 DADE

BEHRING as whole blood platelets activation, using cartilage for ADP/Collagen, normal closure time is up to 175sec. For blood picture the K-EDTA blood tube was delivered to H-Max Coulter system fully automated system.

For vitamins assays the heparinized tube was rapidly centrifuged at 4°C and immediately separated in the dark and kept at –70°C for HPLC. Vitamins assays were done according to previously published method [24]. Vitamin A assay was carried out using a stainless steel column 0.125m long and 4mm in internal diameter packed with octa-decylsinlyl silica gel for chromatography R (5µm). Flow rate used was 1mL/min, with detector of spectrophotometer set at 325nm and retention time of 3min. The reference range was 0.35-0.75µg/dL. Vitamin C assay was carried out using 0.8mL/min flow rate at an ambient temperature with a detection of 254nm and retention time of 6min. The reference range was 280-1100mmol/L. Vitamin E assay was carried out using a fused silica column 30mm long and 0.25mm in internal diameter using helium as a carrier gas. Flow rate used was 1mL/min at split ratio of 1:100. Temperature used was 280°C for the column and 290°C for the injection port and detector. Run time was adjusted at twice the retention time (15min). The reference range was 5-20µg/mL.

Main outcome measures:

Our maternal primary outcome was the occurrence of one or more of the following: Pre-eclampsia, severe pre-eclampsia (defined as severe gestational hypertension plus proteinurea), delivery for pre-eclampsia at or before 34 weeks' gestation, eclampsia, HELLP syndrome and severe proteinuria defined as excretion of 5000mg or more of protein over 24h. The neonatal outcomes were preterm delivery before 37 weeks' gestation, low birth weight (<2500g), small for gestational age (<10th centile of the WHO recommended standard [25]), need for neonatal intensive care admission and neonatal death before hospital discharge.

Statistical analysis:

Statistical analysis was performed using SPSS software, version 10 (SPSS, Chicago). Only ladies and newborns, who completed the follow-up were included in the analysis of outcome. Summary data were presented by group as number (%) or mean (SD) and range when appropriate. We presented outcomes analyses (maternal and neonatal) as simple risk ratios with 95% CIs. The independent sample *t*-test was used to assess the significance of the difference between continuous variables in the two groups. The χ^2 test or the Fisher exact test was used to assess the statistical significance of categorical variables. $p<0.05$ was considered statistically significant.

RESULTS

Baseline characteristics and distribution of risk factors showed no significant differences at study entry between ladies recruited in the two groups (Table 1). The most common risk factors were similar in both groups, being chronic hypertension (39.9% and 34.4) followed by a history of pre-eclampsia (31.8% and 33.6). Multiple risk factors were seen in 20.6% and 18.4% of cases followed-up until delivery in group I and group II, respectively.

Table (2) showed the maternal outcomes for the pregnant women who were followed-up until termination. Pre-eclampsia was seen in 19 (18.09%) versus 22 (20.95%) women. Treatment with antioxidant did not reduce this risk. The risk of severe pre-eclampsia and HELLP syndrome was significantly lower in the supplemented group (p=0.02 and 0.04, respectively). Early onset pre-eclampsia (delivery for pre-eclampsia <34 weeks' gestation) was similar between both groups. Three (2.85%) women taking antioxidant developed severe proteinurea compared with five (4.90%) on placebo, without any significant difference among both groups. Other serious morbidities were similar in antioxidant versus placebo groups including eclampsia and maternal admission to intensive care unit.

Among the neonatal outcome (Table 3) all preterm deliveries, small for gestational age tend to be similar in both groups, while maternal supplementation with antioxidant significantly reduced the occurrence of low-birth weight, admission to NICU and neonatal hyperbilirubinaemia (p=0.02, 0.004 and 0.003; respectively). Gestational age, birth weight and age at time of blood sampling of newborn infants, who developed hyperbilirubinaemia, were similar in both groups. A significant ($p<0.05$) decrease in the serum level of creatinine, cholesterol and LDL-C in the supplemented group was observed only in the third trimester (Table 4).

Hematological and coagulation variables in the study groups were shown in Table (5) and Fig. (1). The hematocrite showed a significant (p<0.05) decrease in the supplemented group in 3rd trimester when compared to the placebo group of the same trimester. Platelet count showed a highly significant (p<0.005) decrease in the 3rd trimester of both supplemented and non supplemented placebo groups when compared to their corresponding groups of 1st trimester. Platelet activity reflected by closure time on PFA-100 showed a highly significant (p<0.005) reduction (as shown by the significant increase in time) in the supplemented group in 2nd and 3rd trimesters when compared to the control (placebo) group for the same trimester. Within the non-supplemented group there was highly significant (p<0.005) increase in platelet activity reflected by the decrease in closure time when the 1st trimester group was compared to both 2nd and 3rd trimester groups. Fibrinogen, vWF and D-dimer levels showed a steady increase in both supplemented and placebo groups as we proceed through pregnancy from 1st, 2nd to the 3rd trimester. However, the supplemented group showed lower levels of the three parameters when compared to placebo group in the same trimester. This reached significance for Fibrinogen and D-dimer (p<0.005 for 1st and 3rd trimesters and p<0.05 for 2nd trimester).

Table (6) showed the antioxidant levels in the third trimester in both groups. There is a significant increase in the level of all of vitamins supplemented (p<0.05 for vitamin A, p<0.003 for vitamin C and p<0.05 for vitamin E). The correlations between antioxidants levels with hematological variables and lipogram in supplemented group are given in Table (7). Significant correlations were found between vitamin A level and both leukocytic count (p=0.002) and D-dimer (p=0.003). Also vitamin C correlated significantly with fibrinogen level (p= 0.004), D-dimer (p=0.002), ADP/collagen closure time (p=0.002) and LDL-C (p=0.004), while vitamin E level correlated significantly (p=0.005) to both fibrinogen and D-dimer and to ADP/collagen closure time (p=0.003).

Table (1): Base line characteristic and distribution of risk factors in the studied groups.

	Group I (n = 126)	Group II (n = 125)
Age (years)	29.3±6.2	30.6±7.4
Parity:		
Primipara	88 (69.9%)	81 (64.8%)
Multipara	38 (30.1%)	44 (35.2%)
Body mass index (BMI)	30.6±5.8	30.2±4.5
Chronic hypertension	49 (39.9%)	43 (34.4%)
Previous pre-eclampsia	40 (31.8%)	42 (33.6%)
Previous eclampsia	5 (4.0%)	7 (5.6%)
Previous HELLP	10 (7.9%)	8 (6.4%)
Previous chronic renal disease	3 (2.4%)	2 (1.6%)
Multiple pregnancy	11 (8.7%)	8 (6.4%)
BMI >30	24 (19.0%)	21 (16.8%)
Multiple risk factors	26 (20.6%)	23 (18.4%)

Data are presented as mean ± SD or number (%) as appropriate, No significance differences between any variable could be detected.

Table (2): Maternal outcome for the cohort who completed the follow-up.

	Group I (n = 105)	Group II (n = 102)	Risk ratio (95% CI)	p-value
Pre-eclampsia	19 (18.09%)	22 (20.95%)	0.839 (0.498-1.454)	0.53
Severe pre-eclampsia	3 (5.71%)	12 (7.84%)	0.242 (0.070-0.835)	0.02
Gestational hypertension	8 (7.61%)	10 (9.80%)	0.777 (0.319-1.890)	0.57
Eclampsia	1 (0.95%)	3 (2.94%)	0.328 (0.034-3.062)	0.32
HELLP	1 (0.95%)	8 (7.84%)	0.121 (0.015-0.953)	0.044
Delivery related to pre-eclampsia (<34 wks)	5 (4.67%)	6 (5.88%)	0.809 (0.255-2.569)	0.72
Severe proteinurea	3 (2.85%)	5 (4.90%)	0.582 (0.143-2.375)	0.45
Maternal admission to intensive care unit	2 (1.9%)	5 (4.90%)	0.400 (0.079-2.014)	0.27
Maternal mortality	0	1 (0.98%)	0.333 (0.013-7.860)	0.50

Data are presented as number (%).

Table (3): Neonatal outcomes of therapy the cohort who completed neonatal follow-up.

	Group I (n = 98)	Group II (n = 90)	Risk ratio (95% CI)	p-value
Preterm delivery (<37wks)	3 (3.06%)	8 (8.88%)	0.344 (0.094-1.258)	0.16
Low birth weight <2500gm	17 (18.88%)	29 (32.22%)	0.538 (0.318-0.910)	0.02
Admission to neonatal intensive care unit	4 (4.08%)	16 (17.77%)	0.229 (0.079 0.661)	0.004
Hyperbilirubinaemia	11 (11.2%)	32 (35.6%)	0.315 (0.169-0.588)	0.003

Data are presented as number (%).

Table (4): Blood chemistry in the studied groups.

	First trimester		Second trimester		Third trimester	
	Group Ia (n = 126)	Group IIa (n = 125)	Group Ib (n = 110)	Group IIb (n = 114)	Group Ic (n = 105)	Group IIc (n = 102)
Serum albumin (g/dL)	4.2±0.98 (3.8-5.5)	4.0±1.1 (3.2-5.3)	3.9±1.23 (3.3-5.4)	3.9±1.1 (3.2-5.1)	3.1±0.77 (3.2-4.9)	3.1±0.87 (2.8-4.6)
Serum creatinine (μm/L)	87.39±39 (73-120)	101.8±22.4 (74-150)	99.5±25.93 (61-156)	109.26±29.61 (55-157)	88.9±19.14* (58-131)	125.15±23.55* (69-163)
Uric acid (mg/dL)	3.4±0.59 (3-5.2)	3.92±0.72 (2.1-5)	4.13±0.58 (3-5.1)	4.4±0.63 (2.7-5.1)	4.3±0.69 (2.3-5.3)	4.6±0.66 (2.8-5.5)
Cholesterol (mg/dL)	175.48±35.9 (131-242)	184.1±23.4 (126-231)	185.8±35.76 (146-260)	193.83±27.88 (140-260)	196.3±24.61* (150-247)	226.45±37.08* (160-310)
Triglycerides (mg/dL)	142.69±40.6 (79-228)	139±38.85 (60-218)	142.8±40.6 (79-228)	140.73±47.92 (72-280)	146.9±42.36 (70-243)	152.87±40.62 (80-228)
LDL-C (mg/dL)	102.69±40.6 (54-128)	89.22±9.09 (70-110)	87.6±15.39 (65-128)	97.76±13.28 (78-123)	91.2±16.12* (65-130)	122.27±91.81* (79-165)
ALT (IU/L)	26.76±8.43 (23-44)	27.55±9.87 (20-46)	34.56±6.87 (22-44)	35.54±7.56 (25-46)	45.47±21.48 (31-100)	48.5±28.63 (33-160)
AST (IU/L)	22.76±5.44 (18-33)	30.43±5.87 (25-45)	32.4±7.43 (18-45)	35.87±7.55 (26-48)	42.66±18.65 (28-80)	44.32±24.52 (30-140)
Serum Bilirubin (mg/dL)	0.44±0.035 (0.4-0.8)	0.43±0.098 (0.5-0.55)	0.52±0.090 (0.5-0.63)	0.62±0.07 (0.6-08)	0.73±0.078 (0.7-2.8)	0.77±0.085 (0.6-3.2)

Data are presented as mean ± SD (range), *$p<0.05$.

Table (5): Hematological and coagulation variables in the studied groups.

	First trimester		Second trimester		Third trimester	
	Group Ia (n = 126)	Group IIa (n = 125)	Group Ib (n = 110)	Group IIb (n = 114)	Group Ic (n = 105)	Group IIc (n = 102)
Leukocytes ($X10^9$/L)	7.6±2.16 (4.6-13)	7.89±1.49 (4.7-11)	7.65±2.16 (4.6-13)	7.48±1.68 (4.6-119)	8.61±2.11 (5.4-149)	8.58±2.1 (5.4-139)
Hemoglobin (g/dL)	12.02±1.24 (9-15)	11.88±0.91 (9-14)	11.43±1.15 (9-14.3)	12.18±1.0 (10.4-14.2)	11.9±1.55 (9-15)	14.79±1.33 (9-15)
Hematocrite (%)	36.43±4.8 (26-47)	35.36±3.83 (30-43)	36.45±4.55 (27-47)	37.4±4.41 (27-47)	37.46±4.07* (32-47)	47.27±17.08* (32-54)
Reticulocytes (%)	1.07±0.4 (0.5-2.8)	1.03±0.34 (0.5-2)	1.44±0.59 (0.5-3)	1.34±0.62 (0.5-3)	1.21±0.56 (0.5-3)	1.28±0.68 (0.5-3.3)
Platelets ($X 10^9$/l)	270±84.9** (143-432)	267.4±74.26** (155-432)	263±83.4 (140-432)	235.7±47.5 (155-334)	219.7±65.8** (100-378)	220.5±65.8** (90-376)
ADP/Collagen Closure Time (sec.)	101.4±4.5 (82-159)	98.7±35.9** (69-179)	104.4±28.7** (94-170)	75.5±12.8** (65-130)	108.4±27.4** (90-175)	75.5±19.6** (58-126)
PT (sec.)	12.5±1.27 (10.4-15)	11.73±0.81 (10.4-13)	12.26±0.12 (10.4-14.6)	11.75±0.68 (10-12.8)	12.12±.15 (10.3-19)	11.99±3.14 (10.3-22)
aPTT (sec.)	35.2±3.8 (27-41)	33.92±3.81 (27-41)	34.22±3.95 (27-41)	33±3.33 (27-38)	34.37±6.68 (27-50)	34.37±7.73 (27-55)
TT (sec.)	14.89±1.53 (13-19)	13.86±0.93 (13-16)	14.32±1.43 (12-17)	13.99±0.82 (12-15.7)	14.84±1.82 (11-19)	14.7±1.71 (11-19)
Fibrinogen (mg/dL)	337.56±62.99** (200-450)	361.86±32.38** (270-420)	375.4±49.49* (250-450)	430.3±40.9** (330-540)	390.6±75.23** (180-460)	480.25±88.7** (150-520)
vWF (%)	87.7±10.37 (70-120)	90.83±10.32* (80-120)	94.47±15.21 (70-130)	101.03±13.74* (75-125)	90.24±14.52 (70-130)	105.7±14.79* (70-130)
D-dimer (μg/dL)	28.33±12.19** (10-59)	34.15±18.88** (18-70)	35.17±12.96* (10-70)	46.43±16.87* (26-109)	44.48 ±4.52** (10-76)	55.88±0.66** (40-100)

Data are presented as mean ± SD (range), *$p<0.05$, **$p<0.005$, (for platelets count Ia versus Ic and, IIa versus IIc; for ADP/collagen closure time Ib versus IIb, Ic versus IIc, and IIa versus IIb and IIc; for Fibrinogen Ia versus Ib, Ia versus Ic, IIa versus IIb, IIa versus IIc; for vWF IIa versus IIb, and IIa versus IIc; for D-dimer Ia versus Ib, Ia versus Ic, IIa versus IIb, and IIa versus IIc).

Table (6): Antioxidants level in the 3rd trimester.

	Group Ic n(105)	Group IIc n(102)	*p*-values
Vitamin A (µg/dL)	0.43±0.06 (0.26-0.90)	0.33±0.09 (0.17-0.63)	<0.05
Vitamin C (mmol/L)	650.38±144.19 (430.65-1080)	209.9±76.64 (237.7-548.5)	<0.003
Vitamin E (µg/mL)	12.5±5.3 (6.3-20.2)	8.5±3.6 (6.8-13.4)	<0.05

Data are presented as mean ± SD (range).

Table (7): Correlation between the antioxidant levels (vitamin A, C and E) and various hematological and lipogram values in antioxidants supplemented group ladies.

	Hemoglobin (g/d)	Platelet (n)	Leukocytes (n)	Fibrinogen mg/dL	D-dimer µg/dl	vWF (%)	ADP/collagen Closure time (s)	Cholesterol (mg/dL)	TG (mg/dL)	LDL-c (mg/dL)	HDL-c (mg/dL)
Vitamin A (µg/dl)	*r*=0.090 *p*=0.19	*r*=0.326 *p*=0.08	*r*=0.655 *p*=0.002	*r*=0.138 *p*=0.10	*r*=0.745 *p*=0.003	*r*=0.089 *p*=0.23	*r*=0.089 *p*=0.12	*r*=0.328 *p*=0.09	*r*=0.090 *p*=0.07	*r*=0.260 *p*=0.08	*r*=0.140 *p*=0.09
Vitamin C (mmol/L)	*r*=0.212 *p*=0.06	*r*=0.143 *p*=0.09	*r*=0.232 *p*=0.06	*r*=0.750 *p*=0.004	*r*=0.649 *p*=0.002	*r*=0.213 *p*=0.08	*r*=0.723 *p*=0.002	*r*=0.098 *p*=0.08	*r*=0.087 *p*=0.10	*r*=0.750 *p*=0.004	*r*=0.250 *p*=0.35
Vitamin E (µg/mL)	*r*=0.354 *p*=0.06	*r*=0.275 *p*=0.08	*r*=0.135 *p*=0.27	*r*=0.627 *p*=0.005	*r*=0.680 *p*=0.005	*r*=0.245 *p*=0.230	*r*=0.834 *p*=0.003	*r*=0.095 *p*=0.21	*r*=0.210 *p*=0.09	*r*=0.200 *p*=0.56	*r*=0.095 *p*=0.06

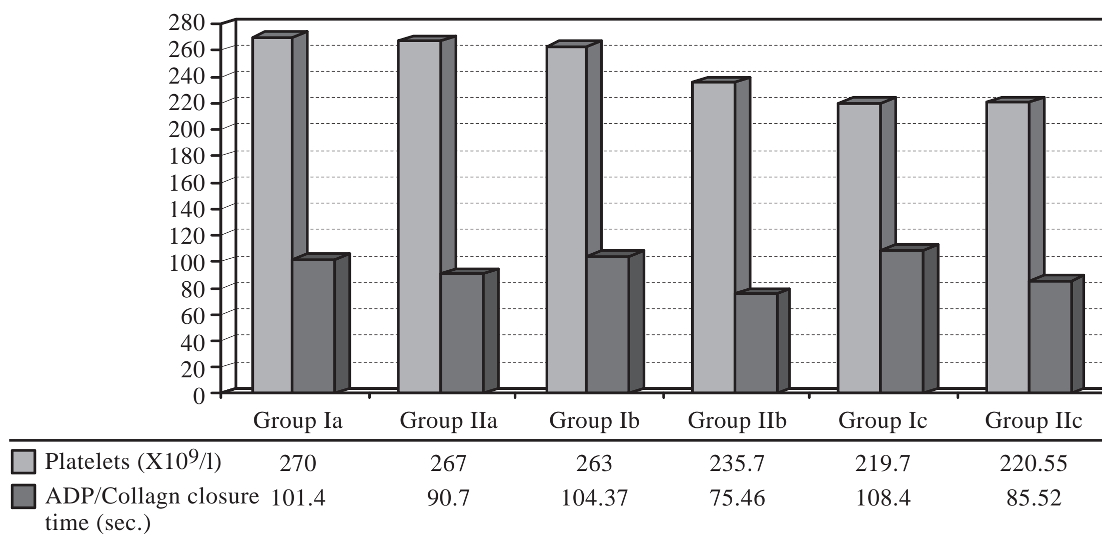

Fig. (1): Platelet count and platelet activation in the studied groups.

DISCUSSION

Thrombotic and bleeding complications of pre-eclampsia are the leading causes of maternal morbidity and mortality with hypofibrinogenaemia and DIC being the most serious complications **[26]**. Early termination of pregnancy leads to fetal loss; however, when the pregnancy is allowed to continue with the usual treatment of pre-eclampsia, intrauterine hypoxia may result into fetomaternal complications as gestational hypertension, IUGR or fetal deaths **[16]**. Thus, though termination of pregnancy would limit maternal complications, the neonatal outcome whether pregnancy was terminated or allowed to continue is in fact limited in this

disease. Therefore, searching for a way to improve placental function in pre-eclamptic mothers seems to be the only way to improve neonatal outcome in such condition. It was shown that when the placental function is improved with healthy functioning endothelium the fetal blood flow increases with possible less complications for mother and fetus [27].

Possible contributing factors for development of pre-eclampsia may be the presence of excessive amounts of free radicals, and a decline in natural body antioxidant enzymes [9], that lead to endothelium dysfunction, with subsequent platelets activation and adhesion and finally the beginning of DIC [14]. Antioxidants, such as vitamin C, vitamin E, selenium and carotenoids, can neutralize free radicals [28]. The current randomized controlled study evaluated the effect of anti-oxidants (vitamin A, C, E and selenium) in pregnant women at risk for pre-eclampsia. We hypothesized that maternal supplementation with these combined anti-oxidants in a group of population at risk for pre-eclampsia might decrease the risk for the development of pre-eclampsia and its complications and improve the neonatal outcomes.

Indeed in this study supplementation of antioxidants, although reduced the severity of pre-eclampsia, it did not prevent it; however, the most pronounced effect was on the neonatal outcome. The neonatal outcome showed marked improvement in the supplemented group. This was reflected as a reduction in number of low birth weight and admissions to neonatal intensive care unit with marked improvement in the incidence of neonatal hyperbilirubinaemia. This agrees with the previous studies that showed an increased neonatal tolerance to stress and improvement of neonatal outcomes if pretreatment with antioxidants such vitamins C, E and carotenoids was given in a model of neonatal sepsis [29]. In animal studies this was shown to be caused by antioxidants ability to correct the endothelium insufficiency with recovery of the fetal circulation [27]. Also, our finding that the incidence of neonatal jaundice was decreased in neonates whose mothers were supplemented with antioxidants during pregnancy suggests that neonatal antioxidant capacity improved through maternal supplementation of antioxidants. This agrees with the previous studies in which antioxidants were shown to correct destruction of erythrocytes-deficient glucose-6-phosphate dehydrogenase enzyme, thus, reducing hemolysis and hyperbilirubinaemia [10].

Our data could not confirm that maternal supplementation with anti-oxidant can reduce the risk of pre-eclampsia when compared to the control group and this result agrees with that of both Rumbold et al. [30] and Spinnato et al. [23]. However, this finding contradicts the findings of Chappell and his colleagues [21], who reported a reduction in pre-eclampsia in high risk women who were supplemented with anti-oxidants. Nevertheless, the risk of severe pre-eclampsia and HELLP syndrome was significantly decreased in the current study. In this regard, our findings are compatible with that of Rumiris et al. [31].

In the present study the supplemented group showed a significant reduction in values of D-dimer, which means a reduction in the degree of coagulopathy. This finding is in agreement with the work of Rumiris et al. [31]. vWF which has a large multimeric structure that increases the adhesive property to the platelets and endothelium and thus acting as a nidus for thrombosis, showed a tendency to decrease in antioxidants supplemented group. This combined with the highly significant reduction in platelets activity in the supplemented group reflects a marked reduction in platelet-endothelial interaction, which is responsible for coagulopathy. Also vWF reduction is a very important indicator for improvement of the inflammatory process associated with the placental endothelium dysfunction, as it is one of the acute phase reactants. These findings are in agreement with the findings of both Rumbold et al. and Sibai et al. [11,23]. Both suggested that antioxidants may improve placental endothelium insufficiency and cause improvement of fetomaternal circulation.

Our study showed a significantly lower fibrinogen level in the supplemented group. This further supports our suggestion that anti-oxidants reduced hypercoagulability state in those patients. Fibrinogen is considered the most important member of the acute phase reactants family and its reduction together with the reduction in the D-dimer level indicates that inflammation is somewhat controlled and thus possibly controlling pathogenesis of DIC. These findings were previously reported [4,9]. In the placebo non supplemented group, the plasma

fibrinogen showed an increase to the hypercoagulation level; this together with presence of high vWF level and activated platelets promote thrombosis. However, it was also noticed in our study that hypofibrinogenaemia occurred in complicated cases regardless of being supplemented or not. This could be explained by the occurrence of DIC, which agrees with other authors **[1,8]**. Our study reported a significant reduction in the platelet count in both supplemented and placebo. This reduction was marked in the complicated cases denoting possible increase in pathogensis of DIC.

The plasma levels of cholesterol and low density lipoprotein were significantly lower in the supplemented group with a significant negative correlation between LDL-C and vitamin C level (p=0.004). This may suggest that placental production of lipid peroxides is abnormally increased in pre-eclampsia. The reason for this is not clear, but if placental antioxidant enzymes were deficient, lipid peroxides would increase unchecked. This explanation was suggested by Chappell et al. **[12]**.

In conclusion supplementing high risk ladies with antioxidants during pregnancy may help to counteract the oxidative stress and control hypercoagulability state that are blamed in the pathogenesis of pre-eclampsia. However, the only maternal clinical benefit of this seems to be reducing the severity of pre-eclampsia rather than preventing the disease. This study suggests potential benefit for gestational antioxidants as regards neonatal outcome in pre-eclampsia that may exceed maternal benefit.

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