RELATIONSHIP BETWEEN ENDOTHELIAL MICRO-PARTICLES AND ENDOTHELIAL PROGENITOR CELLS IN TYPE2 DIABETES PATIENTS WITH AND WITHOUT PERIPHERAL VASCULAR DISEASE.
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ABSTRACT

Background: Diabetes mellitus is associated with an increased risk for a number of serious and sometimes life-threatening macro-and microvascular complications. Endothelial progenitor cells (EPCs) are circulating immature cells that contribute to vascular homeostasis and compensatory angiogenesis. Endothelial dysfunction refers to an impairment of the ability of the endothelium to properly maintain vascular homeostasis. Elevated glucose level induces a marked release of endothelial microparticles. Aim of the study: to estimate the endothelial micro-particles (EMPs) and the endothelial progenitor cells (EPCs) in diabetes mellitus type-2 and its relation to vascular complication. Method: usage of CD31 and CD42 in the identification and quantification of EMP also quantifying endothelial progenitor cells using CD34 and KDR by flow cytometry technique in type 2 diabetes with and without peripheral vascular disease. Results: There was a highly significant difference p<0.001 between diabetic patient and control regard endothelial progenitor cells and endothelial microparticles, while significant difference in creatinine and ALT level p<0.05. Also endothelial progenitor cell and endothelial microparticles show highly significant difference between type 2 diabetics with and without peripheral vascular disease with p<0.001. The correlation study uncover a highly significant negative correlation between endothelial progenitor cells and endothelial micro-particles in patient without peripheral vascular disease while significant negative correlation in patients with peripheral vascular disease. Conclusion: reduction in endothelial progenitor cells and elevation of endothelial microparticles are predictors for vascular complication, so ways to reverse it, is considered a potential therapeutic for future management of diabetic patients.

Key words: Type 2 diabetes mellitus, peripheral vascular disease, endothelial progenitor cells, and endothelial micro-particles.

INTRODUCTION

Diabetes mellitus is associated with an increased risk for a number of serious and sometimes life-threatening macro-and microvascular complications. Macrovascular disease is the leading cause of mortality in people with diabetes. Diabetic patients carry an increased risk two to four times greater for heart attack; stroke and other complications related to poor circulation, the majority of deaths are due to coronary heart disease (1).

Endothelial progenitor cells (EPCs) are circulating immature cells that contribute to vascular homeostasis and compensatory angiogenesis, they constitute a circulating pool of cells able to form a cellular patch at sites of endothelial injury, thus contributing directly to the homeostasis and repair of the endothelial layer, suggesting that EPCs have a major role in cardiovascular biology; in fact, the extent of the circulating EPC pool is considered a mirror of cardiovascular health. Virtually all risk factors for atherosclerosis have been associated with decrease and/or dysfunction of circulating EPCs (2).

Alterations in EPCs may have an important causative role in the development and progression of virtually all diabetes complications (3).

Vascular endothelial cells, like most cells, release different types of membrane vesicles, including microparticles (MP) and exosomes, in response to cellular activation or apoptosis. Microparticles are membrane encapsulated vesicles of 0.2-1µm in diameter. These submicron vesicles are released into circulation, carrying with them an array of surface markers, used to identify their cellular source (4).

Circulating endothelial microparticles, generated from the surface of endothelium have received considerable attention as a surrogate marker for endothelial dysfunction. Because of disease-specific changes and associated biological activity, these vesicles are considered active paracrine agents that aggravate further vascular dysfunction and promote inflammation (5).

In type-2 diabetes, plasma MPs levels, particularly those of endothelial origin, reflects cellular injury and appear as a surrogate marker of vascular dysfunction. MPs are also biologically active and stimulate pro-inflammatory responses in target cells, MPs have a prognostic potential in type 2 diabetes,
particularly in the early detection of vascular complications in this disease (6).

A major function of MPs is their pro-coagulant activity, inhibition of nitric oxide derived vaso-relaxation, however, some MPs have been shown to serve anticoagulant function, and MPs are also known to be carriers of biologically active factors and have been demonstrated to interact with other cells. (7).

SUBJECTS AND METHODS

SUBJECTS:

This study was carried out on 40 patients with type-2 diabetes mellitus chosen from inpatients who were admitted to Internal Medicine, Endocrinology and Vascular Surgery Departments in Al-Zahraa Hospital. They were 18 males (40%) and 22 females (60%) with an age ranging from 38-70 years old. Informed consent was obtained as well as IRB approval from the ethical committee.

Patients were divided into two groups:

A- Diabetic patients with peripheral vascular disease (PVD +ve).

They were 20 patients; 8 males (40%) and 12 females (60%) with age ranging from 38-70 years (mean±SD= 51.3±7.4).

B- Diabetic patients without peripheral vascular disease (PVD -ve):

They were 20 patients; 10 males (50%) and 10 females (50%) with age ranging from between 39-62 years (mean±SD= 54±5.7).

Lower extremity vascular disease was diagnosed by a history of claudication or rest pain, bilateral pulse examination (dorsal pedal, posterior tibial, popliteal and femoral arteries), ultrasonography performed bilaterally at levels of femoral and popliteal arteries.

The study also included 20 apparently healthy subjects as a control group. They were 9 males (45%) and 11 females (55%) with age ranging from 30-55 years (mean±SD= 45.5±5.7), having no prior or current history of diabetes or any type of cardiovascular related medication for hyperlipidemia, hypertension or diabetes.

Methods:

Patients were subjected to:

1- Through history taking and clinical examination with stress on duration of disease and presence of complications. Doppler ultrasonography performed bilaterally at levels of femoral and popliteal arteries.Routine laboratory investigations as Fasting and post prandial blood glucose levels, Immunophenotyping of Endothelial Microparticles (EMPs): using CD31 and CD42b.

Immunophenotyping of Endothelial Progenitor Cells (EPCs): using CD34 and vascular endothelial growth factor receptor-2 (VEGF-R2)(KDR).

Sample Collection:

All cases were 12 hours fasting, after resting for 10 minutes in semi-recumbent position. About 15 ml of venous blood were withdrawn under a septic condition in a plastic syringe. The blood sample was divided into three parts. The first part 10 ml of blood anticoagulated with potassium salt of ethylene diamine tetra acetic acid (K3 – EDTA) at concentration of (1.2 mg/ml). For complete blood count and estimation of endothelial progenitor cells. The second part 2 ml of blood was left to clot and serum was separated to measure routine laboratory tests (Fasting blood glucose level, lipid profile, liver function and kidney function tests). The third part of blood sample 2.7 ml was added to 3.2% trisodium citrate (32 g/L) in a concentration of (1 volume of trisodium citrate to 9 volume of blood) to estimate endothelial microparticles. Another sample 2 ml for post prandial glucose levels were measured 2 hours after breakfast.

Preparation of platelet poor plasma (PPP):

Plasma was prepared within 30 minutes after blood collection by centrifugation of citrated blood sample for 20 minutes at 160 g. The top ¾ of plasma was removed using a transfer pipet and plasma was placed in a plastic centrifuge tube with cap. using a wooden applicator stick, the cells remaining in 1st tube was checked for clot (if found the sample is discarded). Plasma was centrifuged for another 6 minutes at 1500-2000 g to form (PPP) and the top ¾ of plasma was removed by transfer pipet into plastic tube.

Staining:

50 Microliter of platelet poor plasma were dispensed in to tubes that were pre-loaded with fluorescent tru-count™ bead lyophilized pellets the total number of beads per tube was supplied by the manufacturer and varies among lot numbers and labeled with the patient’s name and the monoclonal antibodies to be used. 5µl of each monoclonal antibody (CD31, PE & CD42b FITC) were added to the appropriately labeled tubes. Tube were caped and vortex gently to mix. Then were incubated in the dark at room temperature for 15 minutes. After staining, samples were diluted by 300 µl of PBS.Isotype control tubewas prepared by addition 10 µl of
each (IgG2a FITC, IgG1 PE and IgG1 FITC, IgG3a PE) to 50 μl of PPP in a tube labeled with patient name and isotype control.

**Acquisition:**
Using FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) instrument setting adjusted according to manufacture advice using calibrite beads. Using Quest Software, acquired events adjusted to 5000 events, during acquisition operation the machine adjusted at low flow-rate setting with log gain on light scatter.

**Interpretation of Results:**
Quadrants were adjusted by isotype control dot plot. Events with 0.2 to 1.0 μm size on FS-SS graph were gated as EMP. The distinction between platelet micro-particles (PMPs) from EMPs was made by: EMPs were defined as CD31+ / CD42b- while PMPs were defined as CD31+/ CD42b+ using Cell Quest Soft Ware. The absolute number of EMPs was enumerated from the appropriate dot plot values entered into the following formula:

\[ \text{EMP} \text{/ml} = \frac{\text{No. of events in EMP region (R3)} \times \text{total no. of beads per tube}}{50 \mu L} \]

The total number of beads per tube is supplied by the manufacturer and varies among lot numbers.

**Flow cytometric estimation of Endothelial Progenitor Cell:**

**Preparation of peripheral blood mononuclear cells (Bp.MNCs):** by a density gradient centrifugation. Using Ficoll-plaque. Gently blood sample (anticoagulated with K3-EDTA) was mixed. Then 10 ml blood was added to conical tube preloaded with 6 ml Ficoll. (N.B tube was tilted and blood was added very slowly on side of tube to avoid penetration of Ficoll). Tubes were covered and were put in the centrifuge buckets. They were spun at (400 G) for (20 min, at 4oc). After spinning, the buckets were carefully removed from centrifuge, were placed inside the biosafety enclosure. Carefully the conical tubes were retrieved from the buckets; four layers should be visible from the bottom (erythrocytes, granulocytes, Ficoll, PBMCs and plasma). The upper layer was aspirated leaving the PBMCs layer (monocytes, lymphocytes and thrombocytes) undisturbed. PB-MNCs were recovered as much as possible using 1000 μl micropipette without taking up too much Ficoll. The recovered PB-MNCs were placed in round bottom tube. The tubes containing the PB-MNCs were topped up with 10 ml sterile PBS (PH 7.4). The tubes were capped, and the PB-MNC suspension was homogenized gently. The tubes were loaded in to the centrifuge buckets, and were spun at 100 g for 10 minutes. Supernatant were discarded directly. The separated PB-MNCs were treated with fetal calf serum (150 μl) for 10 min and the sample were washed with buffer containing phosphate buffered saline. They were spun at 100 g for 10 minutes. Supernatant were discarded directly.

**Staining:** 150 μl of PB-MNCs were incubated with 10 μl of FITC – conjugated anti human CD34 and 10 μl of PE – conjugated anti human KDR followed by incubation at (4℃) for 30 minutes in dark. Control isotype tube was prepared by addition 10 μl of each (IgG2 FITC, IgG1 PE and IgG1 FITC, IgG3a PE) to 100 μl PB-MNCs in a tube labeled with patient name and isotype control.

**Acquisition:**
Using FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), instrument setting adjusted according to manufacture advice using calibrite beads. Using cell Quest Software, acquired events adjusted to 1,000,000 events, during acquisition operation the machine adjusted at high flow-rate setting with lin gain at light scatter.

**Interpretation of finding:**
Quadrants were adjusted using isotype control dot plot by Cell Quest Soft Ware. The frequency of peripheral blood cells positive for CD 34, KDR was determined by a two – dimensional side scatter fluorescence dot plot analysis of the samples. Circulating Progenitor Cells (CPCs) were defined as (CD34+ cells, whereas EPCs were defined as (CD34+ and KDR+ cells). Number of EPCs/ ml was calculated by multiplying the frequency of (CD34+/KDR+) events by the total lymphocyte count (Pirro et al., 2006).

**Statistical Methods:**
Data was analyzed using SPSSwin statistical package version 17 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher’s exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non parametric t-test). Comparison between 3
groups was done using Kruskal-Wallis test (non-parametric ANOVA) followed by the post-Hoc "Schefe test" on rank of the variables for pair-wise comparison. Spearman-rho method was used to test correlation between numerical variables. A p-value < 0.05 was considered significant and <0.001 as highly significant.

RESULTS

Table(1) show the comparison between diabetic and control group regard EMPs and EPCs.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diabetic Patients (n=40) Mean ± SD Range</th>
<th>Control (n=20) Mean ± SD Range</th>
<th>P- value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMPs/ml</td>
<td>4387.9±1976.6 (1808.0-9836.0)</td>
<td>800.6±284.4 (433.0-1359.0)</td>
<td>&gt; 0.001</td>
<td>HS</td>
</tr>
<tr>
<td>EPCs/ml</td>
<td>189.6±87.9 (44.0-360.0)</td>
<td>735.3±299.6 (448.0-1534.0)</td>
<td>&gt; 0.001</td>
<td>HS</td>
</tr>
</tbody>
</table>

Table (1) shows: High significant increase in Endothelial Microparticles (EMPs) and high significant decrease in Endothelial Progenitor Cells (EPCs) in studied diabetic patients type-2 when compared with healthy control (Fig 1).

Fig( 1) shows: comparison between diabetic and control group regard EMP and EPCs.

Table (2): Comparison between diabetic patients PVD +ve and PVD -ve as regard to EMPs and EPCs.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PVD +ve (n=20) Mean ± SD Range</th>
<th>PVD -ve (n=20) Mean ± SD Range</th>
<th>P- Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMPs/ml</td>
<td>5979.2 ± 1539.5 (4328.0-9836.0)</td>
<td>2796.7 ± 564.5 (1808.0-3507.0)</td>
<td>&lt; 0.001</td>
<td>HS</td>
</tr>
<tr>
<td>EPCs/ml</td>
<td>121.9 ± 44.2 (44.0-252.0)</td>
<td>257.3 ± 65.1 (152.0-360.0)</td>
<td>&lt; 0.001</td>
<td>HS</td>
</tr>
</tbody>
</table>

Table (2) shows: There were high significant increase in EMPs and high significant decrease in EPCs in diabetic patients with PVD when compared with diabetic patients without PVD(Fig 3)
Fig. (2): comparison between diabetic with PVD and without regard EMP and EPCs.

Table (3): Correlations study of EPCs with EMPs in studied diabetic patients type-2.

<table>
<thead>
<tr>
<th>EPCs/ml</th>
<th>Parameters</th>
<th>Diabetic Patients Mean ± SD (n=40)</th>
<th>r</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMPs/ml</td>
<td>4387.9±1976.6</td>
<td>-0.777</td>
<td>&gt; 0.001</td>
<td>HS</td>
<td></td>
</tr>
</tbody>
</table>

Table (3) shows: High significant negative correlations of EPCs and EMPs in studied diabetic patients type -2 (Fig. 3).

Fig. (3): Correlation study of EPCs with EMPs in studied diabetic patientstype-2.

Table (4): Correlation study of EPCs with EMPs in PVD -ve diabetic patients.

<table>
<thead>
<tr>
<th>EPCs/ml</th>
<th>Parameters</th>
<th>PVD -ve Mean ± SD (n=20)</th>
<th>r</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMPs/ml</td>
<td>2796.7 ± 564.5</td>
<td>-0.910</td>
<td>&lt; 0.001</td>
<td>HS</td>
<td></td>
</tr>
</tbody>
</table>

Table (4) shows: There was high significant negative correlation of EPCs with EMPs in diabetic patients without PVD (Fig. 4).
Fig. (4): Correlation study of EPCs with EMPs in diabetic patients PVD –ve.

Table (5): Correlations study of EPCs with EMPs in PVD +ve diabetic patients.

<table>
<thead>
<tr>
<th>EPCs/ml</th>
<th>Parameters</th>
<th>PVD +ve Mean ± SD (n=20)</th>
<th>r</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EMPs/ml</td>
<td>121.9±44.2</td>
<td>0.489</td>
<td>0.029</td>
<td></td>
</tr>
</tbody>
</table>

Table (5) shows: There were significant negative correlations of EPCs with EMPs in diabetic patients with PVD (Fig. 47).

Fig. (5): Correlation study of EPCs with EMPs in diabetic patients PVD +ve.

DISCUSSION

Diabetes mellitus is characterized by endothelial dysfunction and a three- to four fold increase in cardiovascular risk, and diabetic vasculopathy is an important source of morbidity and mortality. Both type-1 and type-2 DM have been associated with low levels and poor function of circulating EPCs (8).

The vascular endothelium has emerged as a critical determinant of cardiovascular health and disease, and improving endothelial function is an important target for therapy. Studies have suggested that, in addition to the surrounding mature endothelial cells, bone marrow derived EPCs also play a critical role in maintaining endothelial function in mature blood vessels by contributing to re-endothelialization and neo-vascularization (9).

Also the study revealed that type-2 DM is associated with increased levels of circulating EMPs (CD31+ and CD42b-) when compared with healthy control. Which is consistent with Tramontano et al., (2010), Feng et al (2010), and Jung et al., (2011). They found increase
EMPs in diabetic patients which was explained by close relation between EMPs and vascular dysfunction.

CD31 is characterized by CAMs integral to the athero-thrombotic process. Patients with the metabolic syndrome have markedly elevated CD31. EMPs. Additionally, CD31 is an efficient signaling molecule related to angiogenesis, platelet function, thrombosis, mechano-sensing of endothelial cell response to fluid shear stress, and regulation of multiple stages of leukocyte migration (13).

Also the study showed high significant increase in EMPs in PVD +ve and PVD -ve diabetic patients when compared with healthy control, and high significant increase in EMPs in PVD +ve diabetic patients when compared with PVD -ve diabetic patients. These results are consistent with Curtis et al., (2010), Tramontano et al., (2010), Feng et al., (2010), and Jung et al., (2011).

Also the study showed high significant increase in EMPs in PVD +ve and PVD -ve diabetic patients when compared with healthy control, and high significant increase in EMPs in PVD +ve diabetic patients when compared with PVD -ve diabetic patients. These results are consistent with Curtis et al., (2010), Tramontano et al., (2010), Feng et al., (2010), and Jung et al., (2011). The increase in EMPs in patients with macroangiopathy appeared to results from endothelial cell apoptosis rather than activation. Since apoptotic and activated EMPs are closely related to intracranial and extracranial arterial stenosis, respectively, a higher incidence of intracranial arterial stenosis in type-2 DM suggests that apoptotic EMPs are more significant than activated ones (12).

Microparticle shedding is a natural defense of ischemic endothelial cells to prevent phosphatidyserine related activation of the clotting system and binding inflammatory cells. Therefore, the circulating level of EMPs may reflect endothelial ischemic stress due to macroangiopathy and related perfusion defects (14).

Alternatively, these circulating EMPs can be toxic to the vascular wall and therefore induce atherosclerosis. EMPs have been suggested to reduce endothelial vasodilation, which may point to a pro-atherogenic mechanism (15).

Our study showed significant increase in EMPs in hypertensive diabetic patients when compared with normotensive diabetic patients. This finding is consistent with Jung et al., (2011), which was explained by endothelial cell injury being more significant in hypertension.

Endothelial progenitor cells are a subset of bone marrow- derived cells committed to the maintenance and preservation of vascular turnover, remodeling, and homeostasis. EPCs are immature cells, endowed with the capacity to be mobilized from the bone marrow into the blood stream in response to growth factors and cytokines release. EPCs may differentiate into endothelial cells and finally take part in the vascular repair (16).

Endothelial progenitor cells a population coming from mobilization and differentiation of precursors present in the bone marrow (BM) or other tissues. The antigenic phenotype of EPCs in accordance with the term “endothelial progenitors” should include at least one marker of immaturity/stemness (CD34 or CD133) plus at least one marker of the endothelial lineage (KDR, CD31, and vWF) (17).

The physiological function of circulating EPCs is to maintain vascular integrity which is also crucial in the pathogenesis of various diseases with vascular insult. The vasculogenic potential of EPCs is also exploited by tumors by recruiting EPCs to facilitate their growth and metastasis. EPCs are also involved in wound healing, tissue regeneration in ischemia (e.g. myocardial ischemia, limb ischemia), tissue remodeling (Diabetes mellitus and Heart failure) and neovascularization and growth of tumors (18).

The clinical usefulness would stand in that EPCs not only mirror of vascular function and atherosclerotic burden but also reflect the endogenous vasculoregenerative potential, suggesting that measuring EPCs would provide additional information over the classical risk factor analysis (19).

Diabetic patients type-2 either those with or without PVD have high significant reduction in EPCs number when compared with healthy controls. These are consistent with Mohler et al., (2009), Fadini et al., (2010), Curtis et al., (2010), and La Vignera et al., (2012). They found decrease in EPCs in diabetic patients' type II and explained this by bone marrow dysfunction and shortened survival in peripheral blood.

A reduction in EPCs indicates a loss of vascular reparative ability, contributes to endothelial dysfunction, accelerates atherogenic processes, and leads to vascular diseases. Furthermore, EPCs depletion impairs
collateralization and favors complications, such as foot lesions (20,22).

Our study showed high significant decrease in EPCs in diabetic patients with PVD when compared with PVD-ve group. Which is consistent with Fadini et al., (2006) and Avogaro et al., (2011), they explained this decrease in EPCs due to diabetic vasculo-pathy which is associated with EPC impairment: depletion of the EPC pool and defective adhesive capacity is probably one cause of the aggressive diabetes cardiovascular disease in these subjects.

Our study showed a high significant negative correlation between EPCs and EMPs in diabetic patients type-2 and those with PVD,which was consistent with Curtis et al.,2010).

CONCLUSION
Study of EMPs might help to improve in prediction and early diagnosis of vascular complications in type-2 DM. Also alterations of endothelial progenitor cells have a role in the development and progression of virtually all diabetes complication. Therefore, ways to reverse EPC alterations in diabetic patients should be actively pursued. Available data suggest that metabolic intervention by either lifestyle change or glucose lowering is able to improve EPC biology.

REFERENCES


