

PLATELET PROTEINS FROM DIABETIC SUBJECTS CAUSE PHENOTYPIC CHANGES IN ENDOTHELIAL CELLS IN-VITRO

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ABSTRACT

Diabetes is a chronic disease which is associated with platelet activation and endothelial dysfunction. Platelet endothelial interaction is the key regulator of the progression of cardiovascular diseases in diabetic subjects. Upon stimulation, platelets get activated and synthesize/secreted numerous proteins which are otherwise absent in the quiescent platelets. These proteins once in circulation may come in contact with underlying endothelial cells. Though there are several studies being conducted to understand the mechanism of endothelium-platelet interaction in diabetes, effect of platelet releasates on endothelium remains untouched. In the present study we tried to explore the effect of platelet releasates on the endothelial cells by analyzing the cell viability, antithrombotic and prothrombotic markers *in-vitro*. The study was conducted with IEC approval. Eight diabetic subjects (test) were selected based on IDA definition and equal numbers of non diabetic controls were included in the study. Ten ml blood was collected from each subject. Activation status of platelets was studied by aggregometry, Scanning electron microscope and flowcytometry. Platelet releasates were incorporated into the fibrin growth factor composite matrix on which ECs were cultured. Apoptosis assay, morphological analysis, prothrombotic marker (von Willibrand Factor (vWF) and antithrombotic marker (endothelial nitric oxide synthase (eNOS), tissue plasminogen activator (tPA) were studied in cells exposed to test and control proteins. Data clearly indicated the hyperactivation of platelets in test group when compared to the control. vWF was found to be upregulated whereas t-PA and eNOS were down regulated in test group thus confirming the phenotypic alteration of ECs towards prothrombotic. Diabetes is a major individual risk factor for the cardiovascular diseases and the two are bridged by platelet activation and endothelial dysfunction. This study showed the effect of platelet releasates to convert antithrombotic ECs to prothrombotic in nature which in-turn lead to the development of CVD in diabetic subjects.

Key Words: Platelets, Diabetes, Endothelial cells, Platelet activation

INTRODUCTION

Diabetes, a chronic metabolic disorder, which is characterized by the presence of elevated glucose concentration in blood, increases atherothrombotic risk due to many factors like high levels of fibrinogen¹, accumulation of advanced glycation end products², over expression of adhesion molecules, platelet activation³ and dysfunctional endothelium⁴ etc. An early event in diabetes is endothelium dysfunction and platelet activation, which could attribute for the accelerated

progression of cardiovascular diseases (CVD) in diabetic subjects. The Endothelial Cells (ECs) are the chief regulator of vascular haemostasis. In normal physiological condition endothelium remains non-adhesive and antithrombotic due to the release of nitric oxide (eNOS), prostaglandin I2 (PGI2) and tissue plasminogen activator (tPA). eNOS and PGI2 prevents the aggregation of platelets whereas tPA converts plasminogen to plasmin which degrades the thrombi by digesting the fibrin mesh. ECs also secrete prothrombotic markers like platelet activation factor (PAF) and

vWF to maintain the haemostasis. In normal physiology the ratio of antithrombotic/prothrombotic molecules always favors antithrombotic nature of ECs. This ratio may be disrupted by hyperactive platelets. Activated platelets express various membrane receptors which are instrumental for platelet-platelet, platelet leukocyte and platelet-endothelial interactions⁵. Platelet endothelium interaction takes place via several molecules like P-selectin, CD40L etc. Role of these molecules is long being investigated in metabolic condition like diabetes to understand the endothelium-platelet interplay and the molecular mechanism behind^{6,7}. In addition, activated platelets also release cytokines and growth factors which promote the process of inflammation and vascular changes. Syntheses of specific protein are remarkable enhanced in response to activation leading to the release of hundreds of proteins which are not present in the normal vasculature⁸. These secreted proteins may have a role in altering chemotactic, adhesive and proteolytic properties of Endothelial Cells (ECs). These proteins may interact with underlying endothelium much prior to the direct platelet endothelium interaction and may cause alterations in the endothelium, which favors progression of CVD in diabetes. Activated stage of platelets may be studies by aggregation, p-selectin expression and adhesive properties of platelets on fibrinogen matrix. P-selectin is membrane receptor, which is exposed on the activated platelets and plays an important role in its interaction with ECs. Activated platelets also become adhesive in nature by exposing its internal pool of glycoproteins which binds to fibrinogen in order to aggregate and the process of activation cause degranulation and release of proteins from the platelets. In the present study we have analyzed the activation status of platelets in diabetic subjects and the effect of platelet releasates which were isolated from these subjects, on the endothelial cells *in-vitro*. Cells were exposed to platelet releasates and expression analyses of antithrombotic, prothrombotic markers were carried out. Effect of these proteins on ECs viability was also analyzed by live dead assay and morphologically.

MATERIALS AND METHODS

Experimental Design

Experiments were designed in following steps.

- Platelets were isolated from study subjects and were analyzed for the platelet activity by platelet

aggregation, p-selectin expression and adhesiveness on fibrinogen.

- Platelet releasates were separated and quantified. These releasates were incorporated into fibrin growth factor composite matrix.
- HUVEC were cultured on to these platelet releasates incorporated matrix and live dead assay, cell viability, antithrombotic and prothrombotic markers (t-PA, vWF and eNOS) were analyzed.

Study Participants

The study was approved by Ethical Committee Jyothidev's Diabetes and Research Centre, Trivandrum and was conducted as per the ethical guidelines. Eight subjects between age group 45-65 with fasting blood glucose level more than 120mg/dl and HbA1 c level more than 6.5% were included as test group where as subject having fasting blood glucose level below 120mg/dl and HbA1C below 6 were grouped as control. Individuals below the age of 45 and subjects pertaining to the age group of the study if suffering from cardiovascular complication or on anti-platelet drugs were excluded. Clottable fibrinogen in platelet poor plasma (PPP) of test and control was also estimated by clotting assay using commercially (Diagnostica Stago, France) available reagent Fibriprest on Start 4 semi- automated coagulation analyzer (Diagnostica Stago, France). Standard assayed reference plasma (SARP) was used for calibration and the curve generated using diluted plasma was used for automated calculation of fibrinogen by the equipment software.

Preparation of Platelet Rich Plasma (PRP), Washed Platelets and Platelet Poor Plasma (PPP)

Twenty ml blood was collected from control and test group in anticoagulant ACD and PRP was separated after centrifuging blood at 750xg for 5 min. using a table-top centrifuge (Heraeus Labofuge 300, UK). Platelets were isolated from PRP by the standard protocol described by Resmi *et al.*, 2004⁹ with slight modification. In brief, PRP was washed with 1% ACD at 200xg for 10 minutes to pellet down RBC and WBC. The supernatant was further washed in ACD: Tyrode's Buffer in the ratio of 1:9 at 1200xg for 10 minutes. Washed platelets were obtained after washing the pellet thrice in the same buffer at 1200xg for 10 minutes. PPP was prepared by centrifuging the remaining blood at 1000xg for 15 minutes.

Platelet Activation Status in Control and Test

Aggregation Studies

Response of platelets to the agonist ADP (10 μ M) and collagen (1 μ g) was estimated by optical method using whole blood aggregometer (Chronolog-700 aggregometer, USA). Test was performed as per the standard protocol. In brief, 450 μ l of count adjusted (2.5 X 10⁸ /ml) platelet rich plasma (PRP) from each samples were collected and agonist was added after adjusting the baseline with platelet poor plasma (PPP). Test was allowed to run for 5 minutes and then stopped, as per the established standard procedure. Slope and amplitude were determined using the calculate option in AGGRO/LINK8.

Estimation of Activation Markers

To analyze the activation status of platelets, surface expression of platelet activation markers p-selectin (CD62) and GPII b (CD41) was analyzed. Unlabelled resting platelets were used for adjusting the gate position based on FSC and SSC. Staining was done for CD62 using fluorochrome-labeled antibodies obtained from Beckman Coulter (USA). Protocol was developed for analysis by FSC and SSC analysis of unstained platelets and by selecting the negative gate for fluorescence to detect positive cells. In brief, platelet rich plasma was collected from blood of test and control. Platelet count was adjusted to get between 200– 250 \times 10⁴/ ml. To 100 μ l of PRP, 2 μ l of CD 62/CD41 antibody were added and kept for 1 hour incubation in dark. Samples were then fixed with 3.7% formaldehyde and made up to 1ml with PBS. The data was then analyzed using a BD FACS Aria, equipped with BD DIVA software (BD Biosciences, San Jose, CA, USA).

Platelet Adhesion to Fibrinogen by SEM Analysis

Platelet adhesion to the fibrinogen was estimated by Scanning Electron Microscopy analysis. Slides were coated with a fibrinogen concentration of 60 μ g. Once dried, platelets were smeared in such a way so as to form a uniform layer. This was followed by successive dehydration steps involving washing with different concentrations of alcohol (30%, 50%, 70%, 90% and 100%) for 15 minutes each. Samples were lyophilized and platinum coating was done. The morphological changes were observed through SEM (Hitachi model S-240, Japan) with a magnification of 10000X.

Isolation and Quantification of Platelet Releasates

Platelets releasates was collected by aggregating washed platelets with 0.8IU thrombin using chrono-

log aggregometer followed by ultracentrifugation 45000Xg for 30min. Supernatant was collected and protein concentration was quantified by lowry's method¹⁰.

Isolation and Characterization of Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as per the method of Prasad *et al.*, 2005¹¹. The cells were seeded on to fibrin growth factor composite matrix (thrombin 5IU/ml, fibrinogen 10mg/ml, gelatin 0.2% and VEGF 50 μ g/ml) coated tissue culture dish in IMDM medium supplemented with 20% FBS and VEGF. After 24h unattached cells were removed and cells were fed with fresh medium. Cells were passage and from 2-4 passage cells were used for further analysis. Cells were characterized using Acetylated Low Density Lipoprotein (labelled with 1,1'-dioctadecyl – 3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) and von Willibrand Factor. Images were captured using fluorescence microscope from Leica Microsystems (DMIRB, Germany) at 10X magnification.

Effect of Platelet Proteins on HUVEC

Coating of platelet releasates in tissue culture dishes

In order to see the effect of platelet proteins on endothelial cells 10ug and 100 ug of platelet proteins from both the groups (control and test) were incorporated into the fibrin growth composites. Proteins were mixed with the fibrin growth factor composites in order to see the effect of slow release of these proteins as in *in-vivo* system platelet proteins comes in contact with EC in a slow and continuous process. The dishes were lyophilized for 3 hours and stored at 4°C. Fibrin growth factor composite matrix coated dishes served as reference.

Apoptosis Assay

Cells at a density of 20,000cells/cm² were seeded on the control and test dishes. Apoptosis assay was carried out at after 48h of seeding using Vybrant Apoptosis Assay Kit from Molecular Probes. Briefly, trypsinized cells were washed with ice cold PBS twice and incubated with annexin and PI for 15 minutes. After the incubation flow cytometry analysis of the samples was carried out using BD FACS ARIA equipped with BD FACS DIVA software (BD Biosciences, San Jose, CA, USA). Dot plots of forward scatter versus side scatter were used to select the desired cell populations by excluding debris and clumps. For flow cytometry

studies 20,000 event were acquired. The compensation was done using single stained annexin and PI cells. Unstained cells were used to gate the population. Gating of the population was done based on the mean fluorescence intensity and the apoptotic cells were gated to quadrant 4. Experiment was done in triplicate and the percentage of dead cells was calculated in control and test group as compared with the reference. Morphological analysis was also done by phase contrast microscope (DMIRB, Leica Microsystems, Germany) Images were captured from all groups at 10 X magnification.

q-PCR Analysis for Antithrombotic and Prothrombotic Marker

The total RNA was extracted from cells exposed to platelet releasates for 48h using trizol (Invitrogen, USA) according to the manufacturer's instructions and quantified using a Qubit RNA assay kit on a

Qubit 2.0 fluorometer (Invitrogen, USA). 1 μ g of RNA was converted to cDNA using the Superscript III reverse transcriptase enzyme (Invitrogen). GAPDH was selected as the house-keeping gene for the study. The primer sequences for specific genes are given in table 1. The experiment was carried out using a Chromo4 system (MJ Research/Biorad, USA). All reactions were carried out in a total volume of 25 μ L containing 12.5 μ L qPCR master mix (Eurogentec, San Diego, CA, USA), 200nM forward primer, 200nM reverse primer and 2 μ L template cDNA for 40 cycle. For each gene, quality and specificity was assessed by examining PCR-melt curves following the reactions. Fold change was calculated after normalization with GAPDH expression using the formula $2^{-\Delta\Delta Ct}$. Molecular weight of product was determined agarose gel electrophoresis (AGE) and the gels were documented using Alpha Imager Gel Documentation System 2000.

Table 1
Details of primers used for PCR analysis

Name of the gene	Forward primer	Reverse Primer	Amplicon Size
GAPDH	5'GCTTGTCTCATCAATGGAAATCCC 3'	5'TCCACACCCATGACGAACATG3'	210bp
eNOS	5'AGCTGTGCTGGCATACAGGA3'	5'ATGGTAACATGCCGCAGAC3'	486bp
tPA	5'ATGGGAAGACATGAATGCAC 3'	5'GAAAGGGAAGGAGACTTGA3'	319bp
vWF	5'CACCATTCTAGCTAAGAGGAGG3'	5'GCCCTGGCAGTAGTGGATA3'	310bp

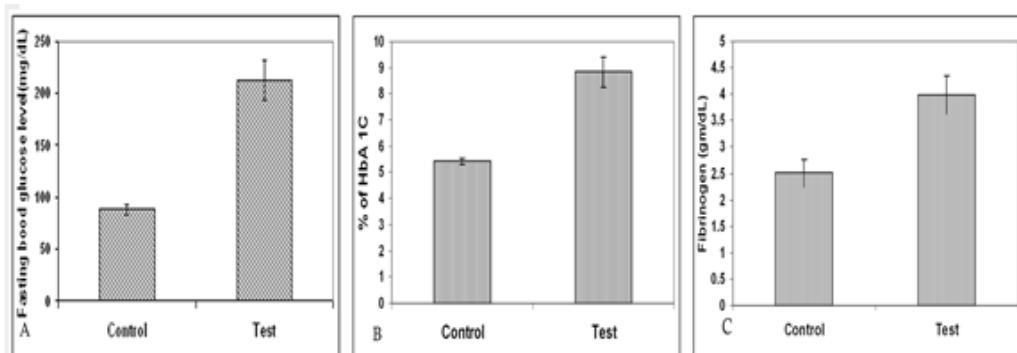
Statistical Analysis

Statistical analysis was done by Student's t- test using Graph Pad online software for all the quantitative parameters to identify significance of difference between the control and test groups. All data are presented as Mean. \pm SD. P value of <0.05 was considered as statistically significant.

RESULTS

Clinical parameters such as blood glucose levels, HbA1C and fibrinogen were estimated and data is shown in Fig 1 (a-c). High level of fibrinogen was observed in the test samples compared to control.

Figure 1
*Parameters for the screening of subjects
(a). HbA1C, (b) Fasting Glucose (c) Fibrinogen.*

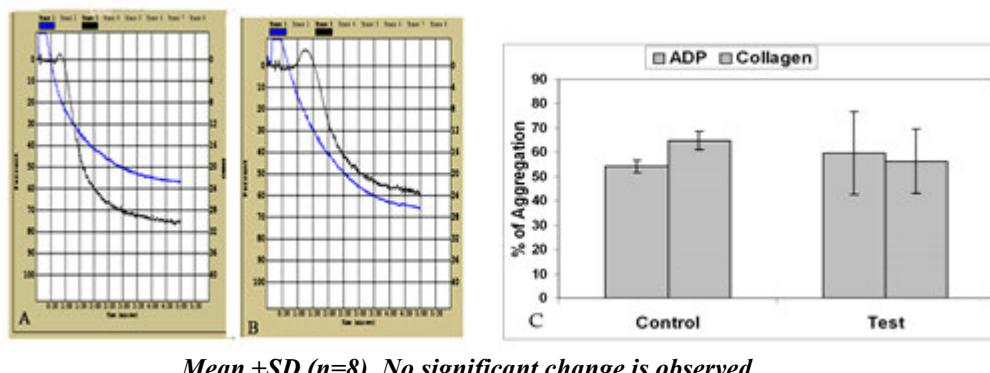


All data are presented as Mean \pm SD (n=8), P \leq 0.05.

Hyperactivity of diabetic platelets was confirmed by platelet functionality, platelet activation marker p-selectin, GPIIb and adhesiveness of platelets on fibrinogen matrix. Figure 2A represents the aggregometry response of test platelets in comparison with control platelets. X-axis shows the time and Y-axis represents the percentage transmittance. As platelet aggregates, % light

transmittance increases due the clearance of light path. It was found that platelet response to the agonists were not similar in test samples. Few test samples showed hyper-reactivity whereas the others showed aggregation comparable to the control (Fig 2). Two samples showed very low response to agonists. This may be related to the progression of diabetes, medication and age of the subjects.

Figure 2
Platelet Aggregation test using agonist ADP and Collagen
(a) Control, (b) test (c) comparative analysis

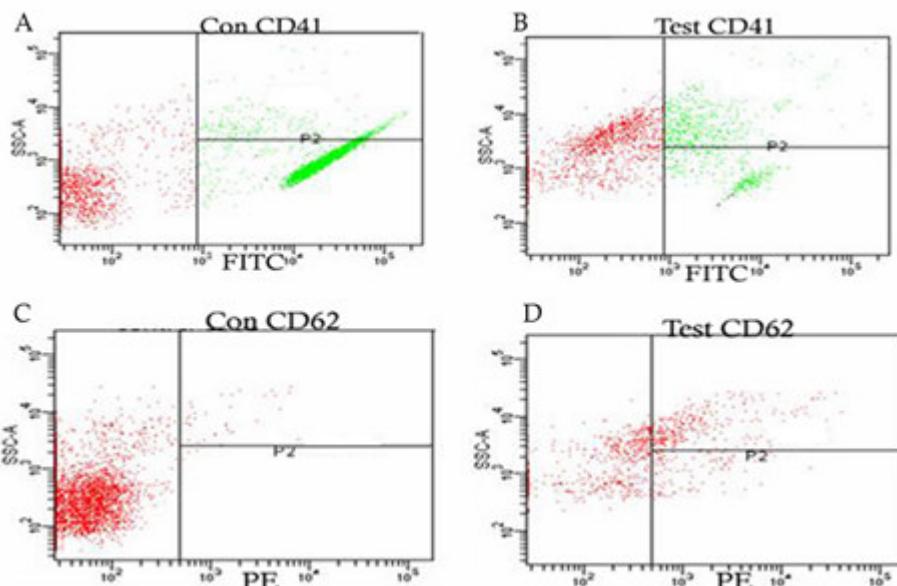


Mean \pm SD (n=8). No significant change is observed

Flowcytometry data is represented in figure 3a & b. X-axis represents the mean fluorescence intensity and Y-axis shows the number of positive cells for p-selectin or CD41. P-selectin positive platelets were significantly high in the test platelets when

compared with control ($\approx 6.3\%$ in test compared to $\approx 0.54\%$ in control), whereas CD41 was found to be decreased significantly in test samples compared to control. A compiled data is shown in fig 3c. Compiled data is shown in table 2.

Figure 3
Flowcytometry estimation of p-selectin (CD62) and CD41



Representative flowcytometry data a & b CD41 Control & Test c & d CD62 Control & Test .
X axis represents the fluorescence intensity whereas Y axis represents the number of positive cells.

Table 2
Compiled data from the control and test group for the flowcytometric analysis of CD62 and CD41

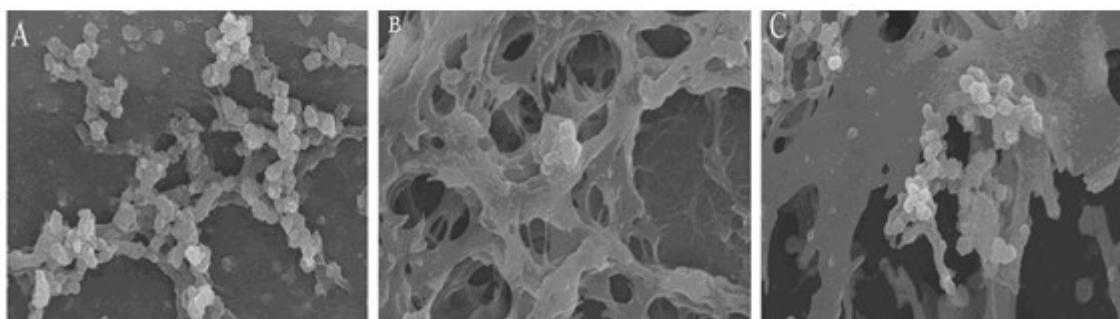
SAMPLE	% of CD62 cells	% of CD41 cells
Control	0.43± 0.28	77.83± 5.61
Test	3.92± 2.38 [#]	43.94± 2.6*

Mean ±SD (n=8), P[#] =0.084, P < 0.0001*

SEM analysis revealed that test platelets are adhesive in nature and form aggregates on the fibrinogen matrix similar to that of thrombin activated platelets, however in control samples such aggregates were not observed (figure 4). Platelet spreading with pseudopodia and aggregation are comparable in test and positive control (thrombin activated) which is evident from fig 4 a-c. In both

cases platelets were plenty with clump formation on fibrinogen coated surface. In the case of the control (healthy subjects) platelets were very rarely found on the surface. The adhered cell was found to be single with no significant pseudopod formation or spreading (Fig 4b). These observations indicate that platelets of diabetic subjects are in activated state.

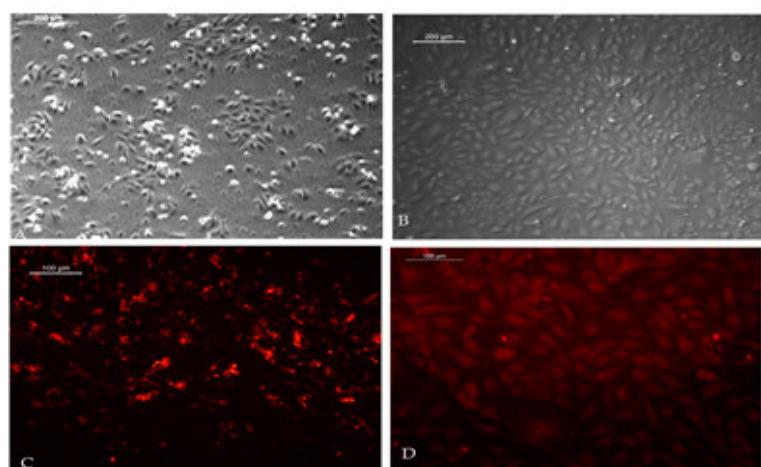
Figure 4
Representative SEM images A. 0.8IU thrombin activated platelets a. positive control b. Control, c. Test.



Effect of releases on ECs

Isolated cells were characterized by Ac-LDL and vWF (figure 5). Cells showed a homogenous population of ECs on culture dishes.

Figure 5
Isolation and characterization of HUVECs

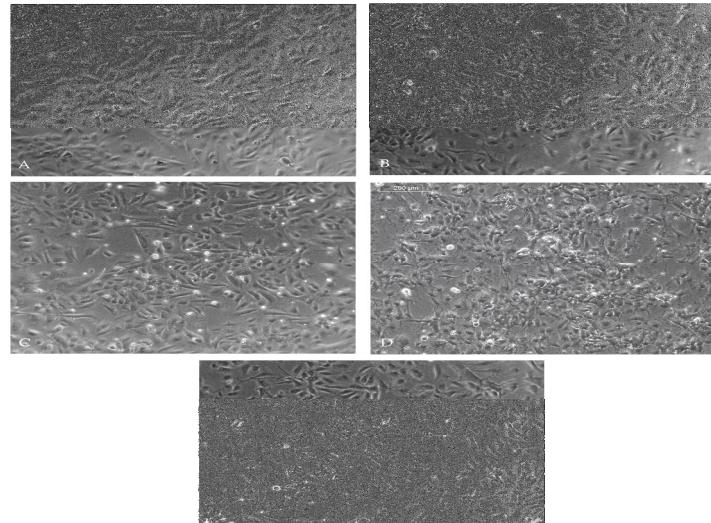


a. HUVECs after 12h of isolation b. Cells after 24h of isolation c. Cells characterized by Ac-LDL uptake d. Cells characterized by vWF expression. Upper panel images were captured at 10 X where as down panel images were captured at 20 X magnification.

These cells were exposed to 10ug and 100ug of platelet releasates incorporated into the fibrin matrix and it was observed that even at lower concentration test platelet releasates induce apoptosis in culture cells. Morphological analysis of HUVEC after 48 hours of exposure to test

platelet releasates showed considerable changes as compared to the reference HUVEC (figure 6 a-c). These changes however were not very prominent in HUVEC cultured with platelet releasates from the control group (6 d & e).

Figure 6
Effect of platelet releasates on Endothelial Cells in-vitro

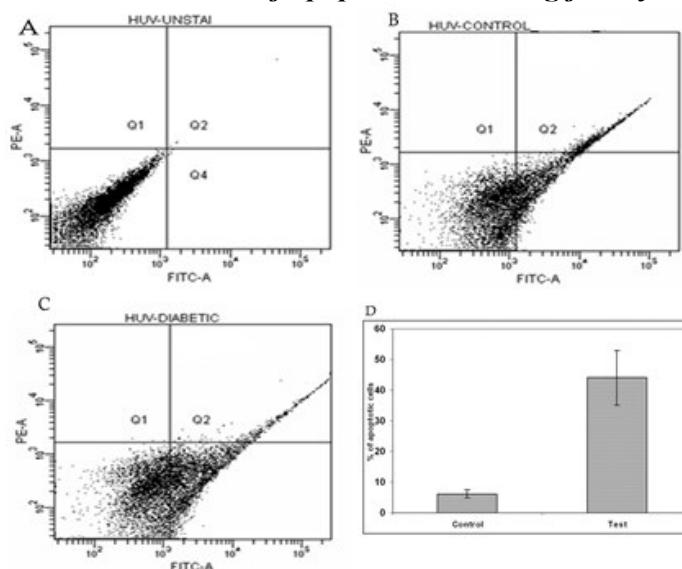


Apoptosis induces by platelet releasates a: Reference, b: Con 10 μ g, c: Con 100 μ g, d: Test 10 μ g & e: Test 100 μ g

The observation in culture co-related with the FACS data since the percentage of apoptotic cells in HUVEC exposed to test platelet releasates was significantly higher when compared to HUVEC cultured in control platelet releasates matrix (figure

7). A compiled data analysis from both the groups is shown in (figure 7d). More than 44% of ECs become apoptotic when exposed to 100ug of platelet releasates from test for 48h, compared to the 8% in control.

Figure 7
Quantitative estimation of apoptotic cells using flowcytometry.

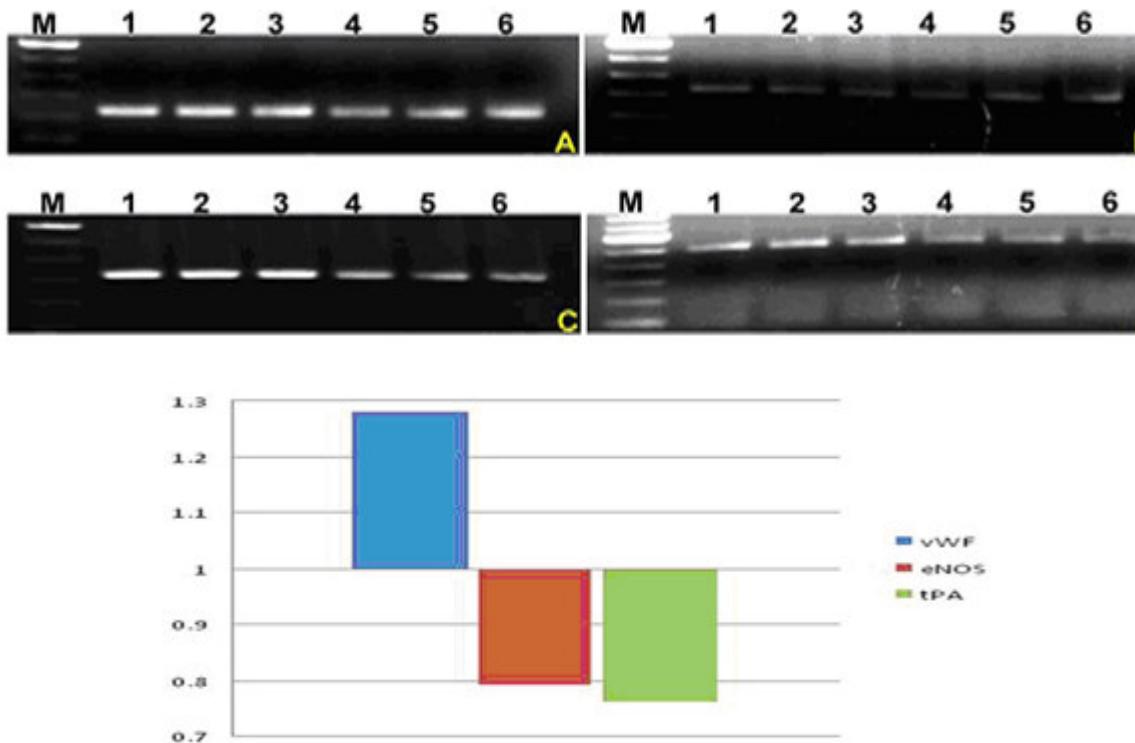


a. Unstained cells, b. cells exposed to control platelet releasates c. Cells exposed to test platelet releasates. d. Compiled data from eight donors. Values are given as Mean ± SD (n=8). P<0.05

Expression analysis of antithrombotic and prothrombotic markers suggest the disturbance of balance which favors the prothrombotic nature of ECs when exposed to platelet releasates. qPCR

analysis showed decrease in eNOS and tPA expression while significant increase in vWF expression (Figure 8a-e).

Figure 8
PCR analysis for the gene expression



a- GAPDH, b- tPA, c- vWF, d- eNOS. Loading order in all gels- M: DNA Ladder, 1-3 test 4-6 Control e. Fold-change in expression of vWF, eNOS and tPA

DISCUSSION

Hyperglycemia is a major independent risk factor for cardiovascular diseases and most of the patients with diabetes have atherosclerosis associated diseases¹². There are reports stating the alteration in the proteome of platelets in the different pathological conditions, as well as upon activation¹³⁻¹⁶. Alteration in the proteins may be associated with the platelet activity as well as with the dysfunction of underlying endothelial cells. There are ample evidences that platelets from diabetic subjects are larger and hyper reactive and cause increased aggregation. But in our study, we observed high donor to donor variation in the platelet response to the agonists from low to high. Previous studies have also revealed no direct correlation between high glucose level and platelet aggregation¹⁷. It is suggested that elevated fibrinogen level may be closely related to the abnormality of platelet functions in diabetic subjects, which may lead to the vascular lesions.

Our findings also indicated that fibrinogen level was high in test samples which showed hyperaggregation. However, comparable platelet aggregation in diabetic subjects with and without vascular complication is also reported¹⁸. P-selectin and CD41 (GPIIb/IIIa) are considered as platelet activation marker^{19,20}. Our data is well in correlation with earlier finding (Bhatt and Krishnan., 2015)²¹, where it is proven that CD62 increases and CD41 decreases as the platelet activation increases. Decrease in CD41 may be primary due to the binding of CD41 to fibrinogen as CD41 is a receptor for fibrinogen and its affinity to fibrinogen increases upon platelet activation. In the present study test samples showed high levels of fibrinogen, this available fibrinogen will bind to the CD41 and thus decrease in the CD41 antigen-antibody interaction. Clottable fibrinogen is an indicator of risk of cardiovascular complications which may be due to the risk of cardiovascular disease in test subjects. Activated platelets can be procoagulant and may result in conversion of

fibrinogen to a procoagulant state. So, the elevated level of fibrinogen may be due to the activation of platelets by altered metabolism of hyperglycemia. Platelet activation is marked by platelet shape change (discoid shape to round with extended pseudopods and aggregates) and spreading on adhesive substrate. Thus morphological change associated with the activation is studied by spreading of platelets on the fibrinogen matrix. Previous studies have shown that the ultra-structural changes in clot formation occur in patients in whom there are changes in the coagulation system due to, for example, an inflammatory condition. The data from this study also indicated the shape change and pseudopodia elongation in test platelets when spread on a fibrin matrix, whereas control platelets were round and non-adhesive as very few platelets were seen on the matrix. Endothelial dysfunction has been proposed as a fundamental component in the pathophysiology of diabetes. The effect of these circulating platelet proteins were analyzed by coating it on the cell culture dish because this strategy helps in studying the effect through slow release. Slow release of the proteins into the cell culture medium mimics the body system since platelets release the proteins into circulation in a gradual process. The half life of platelet proteins in circulation is short and hence by stabilizing these proteins in a matrix the role of these proteins can be studied more effectively *in-vitro*. ECs are highly sensitive to the changes in the environment which result in functional impairment of these cells. Reports indicate that the development of atherosclerosis can involve apoptotic process in the vasculature²². From the study conducted it is evident that platelet releasates of test subjects can induce apoptosis in endothelial cells which can predispose these vessel walls to initiate plaque development. Though mild cell death was observed in the culture exposed to platelet releasates from control samples compared to reference well, but this was significantly lower to from the test samples. This could be the attribution of the inhibitory proteins present in the platelets. PCR analysis of gene expression was carried out in order to study the effect of circulating platelet proteins in causing endothelial dysfunction. Expression levels of tPA, eNOS and vWF was studied after exposing ECs to plasma proteins for 72 h in culture (figure 7). tPA is a serine protease which converts plasminogen to plasmin to degrade fibrin clot²³ thus favors smooth blood flow. ECs in culture constitutively secrete tPA. The rate of synthesis is affected by many

components like thrombin, histamine, plasmin etc²⁴. vWF is an important marker for endothelial dysfunction²⁵. An increased plasma level of vWF is an already established biomarker for CVD²⁶. Nitric oxide, widely expressed in virtually all vascular cell types, is mostly produced by the endothelial isoform (eNOS). It plays a crucial role in vascular tone and structure regulation. It also exerts an anti-inflammatory influence, inhibits platelet adhesion and aggregation and prevents smooth muscle cell proliferation and migration²⁷. The expression level of vWF was upregulated in ECs upon exposure to platelet proteins from test group. This indicates that ECs were shifting to prothrombotic phenotype. This result correlates with reports which indicates that prothrombotic endothelium up-regulates the expression of vWF. At the same time decrease in the expression of antithrombotic marker tPA and eNOS indicated the phenotypic alteration of ECs. However there are reports stating the increase in eNOS expression upon exposure to diabetic environment due to the adaptive response of ECs. However in this study cells were exposed for 72h which lead to the significant changes in the expression of antithrombotic and prothrombotic markers. It was observed that the balance between the prothrombotic and antithrombotic genes expression is altered such that ECs did become prothrombotic in nature. The data is significant because upregulation of vWF makes ECs prothrombotic which predisposes the diabetic individual to the risk of atherogenesis.

CONCLUSIONS

From the above study we can conclude that diabetic platelets are active in circulation and release its granule content. The proteins from the activated platelets induce apoptosis in the endothelial cells. Gene expression analysis confirms the phenotypic alteration (prothrombotic from antithrombotic) of the endothelial cells *in-vitro*.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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CONFLICT OF INTEREST

Authors have declared that no conflict of interests exists and the research work is entirely for academic purpose and no competing financial interests exist.

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