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PROJECT COMPLETION REPORT

1. **Project Number** : 8089

2. **Title of the Project** : **"Do platelets in patients with type II diabetes release proteins that can activate aortic endothelial cells"**

3. **Funding Agency Name** : KSCSTE

4. **Project Reference Number provided by the Funding Agency**:
3228/C3/14/KSCSTE

5. **Principal Investigator (Name & Address) : Dr. Anugya Bhatt** Dr. Anugya Bhatt, Div of Thrombosis Research, Biomedical technology Wing, SCTIMST, poojappura, Trivandrum 695012

6. **Co-Investigators (Name & Address): Dr. Harikrishnan S**
   i. Dr. Harikrishnan S, Additional Professor, Department of Cardiology, SCTIMST
   ii.
   iii.
   iv.

7. Implementing Institution : SCTIMST

8. Collaborating Institutions : NA

9. Date of Commencement : 04.04.2014

10. Duration : 3 Years

11. Date of Completion : Dec 2017

12. Objectives as approved :

- To understand the activation status of circulating platelets in the study subjects.
- To distinguish proteome profile of diabetes Vs healthy subjects using 2D gel electrophoresis.
- To identify the inflammatory proteins (like secretogranin/cyclophilin) by western blot analysis.
- MS-MS analysis to identify difference of proteome profile between diabetes and control (healthy) group.
- To identify effect of these proteins on EC phenotypic variation, with special emphasis on up regulation of inflammatory markers

13. Deviation made from original objectives if any, while implementing the project and reasons thereof : NIL

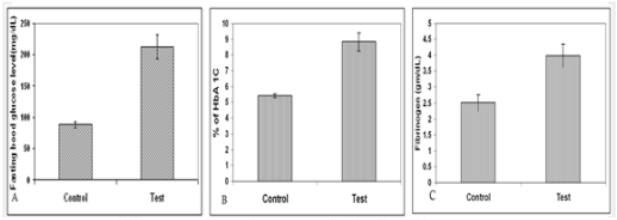
14. Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs : Not Applicable

15. Detailed anaylsis of 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.

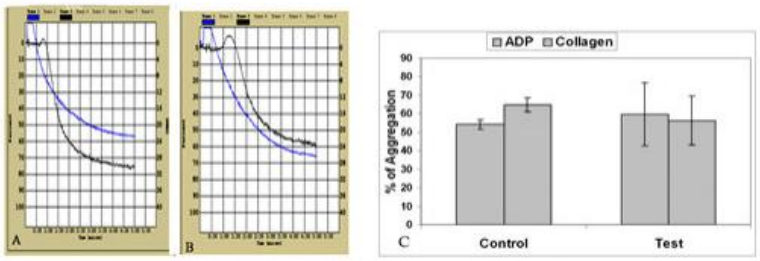


All data are presented as Mean ±SD (n=8), P≤0.05.

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

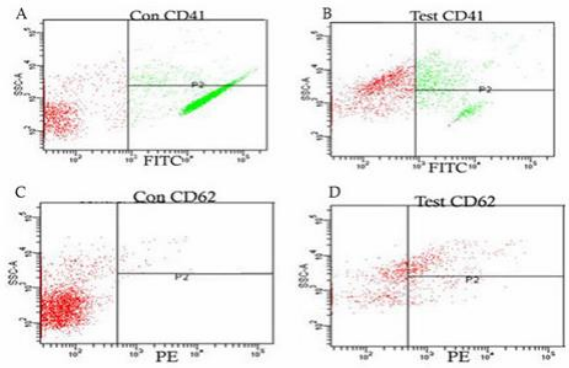


Mean ±SD (n=8). No significant change is observed

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 (≈6.3% in test compared to ≈0.54% in control), whereas CD41 was found to be decreased significantly in test samples compared to control.

Figure 3

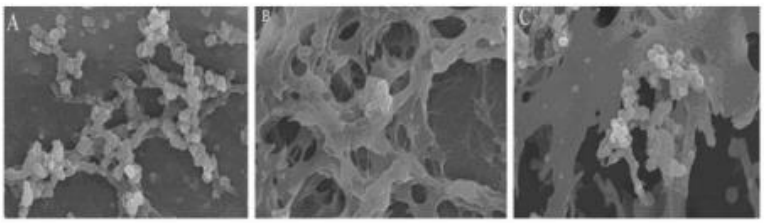
Flowcytometry estimation of p-selectin (CD62) and CD41



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.

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.

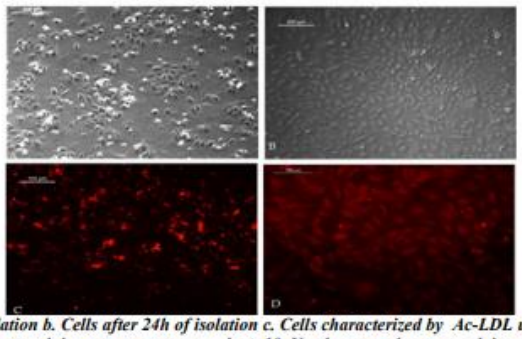
Figure 4
Representative SEM images A. 0.8IU thrombin activated platelets a. positive control b. Control, c. Test.



Effect of releasates on ECs

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

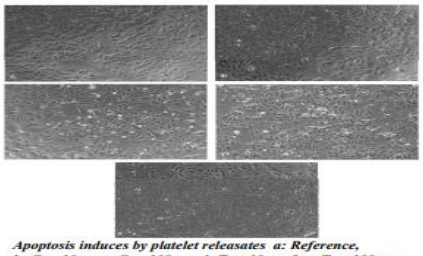
Figure 5
Isolation and characterization of HUVECs



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 Edothelial Cells in-vitro



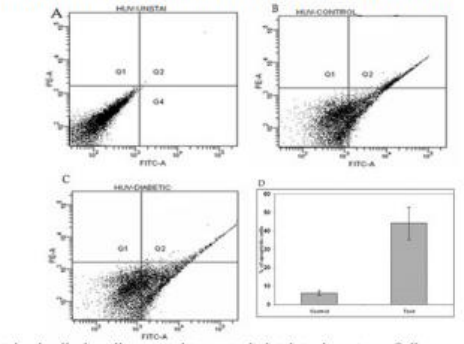
Apoptosis induces by platelet releasates a: Reference, b: Con 10µg, c: Con 100 µg, d: Test 10 µg & e: Test 100 µg

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.

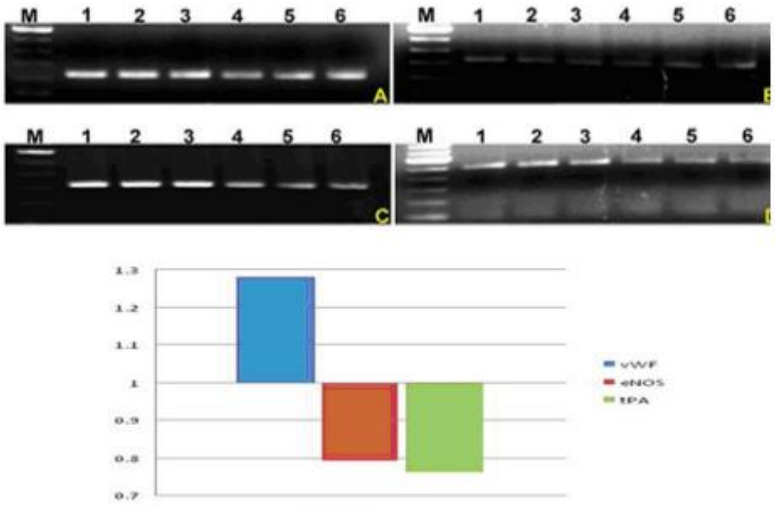


a. Unstained cells, b. cells exposed to control platelet releasates c. Cells exposed to test platelet releasates. d. Compiled data from eight donors. Values are given as Mean ± SD (n=8). P<0.05

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

16. Summary sheet of not more than 2 pages under following heads : (Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential)

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/secretes 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 are 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 where as 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.

Experimental Design Experiments were designed in following steps. a. Platelets were isolated from study subjects and were analyzed for the platelet activity by platelet aggregation, p-selectin expression and adhesiveness on fibrinogen. b. Platelet releasates were separated and quantified. These releasates were incorporated into fibrin growth factor composite matrix. c. HUVEC were cultured on to these platelet releasates incorporated matrix and live dead assay, cell viability, antithrobotic and prothrombotic markers (t-PA, vWF and eNOS) were analyzed.

Results : 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.

17. Contributions made towards increasing the state of knowledge in the subject : Platelet proteomics and its impact in the progression of cardiac diseases in diabetes subject is not much study. This study provided new circulating targets for future studies

18. Conclusions summarising the achievements and indication of scope for future work : This study provided new circulating targets for future studies

19. Science and Technology benefits accrued :

a. List of research publications with complete details :

S.No	Title of Papers published, Journal Name, Vol. No. & Year	International	National	Impa fact
1	Serene H, Shahna S, Jothydev K , Anugya Bhatt PLATELET PROTEINS FROM DIABETIC SUBJECTS CAUSE PHENOTYPIC CHANGES IN ENDOTHELIAL CELLS IN-VITRO, International J of Life science and pharma Research, **VOL 7/ ISSUE 1/JANUARY 2017**	International		0.9

b. Manpower trained on the project :

i. Research Scientists or Research Fellows : 1

ii.	**No. of PhD's produced**	**:**	**Nil**
iii.	**Other Technical Personnel trained**	**:**	**Nil**
c.	**Patents taken, if any**	**:**	**Nil**
d.	**Products developed, if any**	**:**	**Nil**

20. Abstract: (In 300 words for possible publication in ..... Bulletin)

a. **Background:** According to the WHO report 2002, cardiovascular diseases will be the largest cause of deaths in India by 2020. The increase in number of CVD is mainly because of the increase in the risk factor burden like diabetes and hypertension. Combined effect of risk factors makes the progression of disease worst and lead to the death. Statistically, highest numbers of CVD and associated risk factor patients in India are from Kerala. Diabetes and hypertension are the major individual risk factor for the cardiovascular complications and their mutual effect is also well recognized. Patients from those with such risks are more prone to atherosclerosis and disease progresses with time. Atherosclerosis is otherwise a quiescent disease until the plaque rupture and sometimes results in sudden death. Thus if it can be detected at an early stage in the high risk groups, it could be life-saving.

Endothelial dysfunction is an early event in diabetes and hypertension which aggravates in CVD. Various underlying mechanisms that cause such dysfunction are under study from various angles. Circulating platelets are activated in both diabetes and hypertension and release many different proteins into plasma. Role of platelet proteins are recognized as an important contributor to atherosclerosis progression but the specific effects is not well understood and need more attention.

Atherosclerosis is a multifactorial disease and can be better understood at proteomic level in its early stage. Activated platelet synthesize and secrete number of proteins which are absent in resting platelets. Presences of few of these proteins are reported at plaque site. Though role of these proteins are not studied much, their presence at plaque sites shows that they may be the key regulator for the disease progression. If these proteins can be identified in circulation at early stage, it may serve as a marker for the atherosclerosis in risk factor groups.

b. Materials: Cells, proteins

c. Results: This study aims to understand the platelet activation status in diabetic subjects and effect of released proteins isolated from diabetic subjects, on EC activation with expression of inflammatory markers. The expression of inflammatory marker proteins validated through western blotting and immunocytochemistry and the expression of inflammatory proteins was found more in diabetic subjects compared to control subjects. Also we identified the differently expressed whole platelet proteins of diabetic and control subjects through Liquid Chromatography tandem mass spectrometry (LC-MS/MS). Identified proteins were subjected for pathway analysis and found that the platelet

proteins in diabetic subjects interact with various pathways which may lead to several cardio vascular diseases.

d. Conclusion: Type 2 diabetes is characterized by the presence of elevated fasting glucose concentration in blood. It is associated with systemic insulin resistance which leads to hyperglycemia and dyslipidemia, and it has been proposed that these metabolic abnormalities account for increased cardiovascular risk. Activated platelets released proteins in diabetic subjects play an important role in the development of cardio vascular diseases (CVDs). Platelets get activated and release its content in the circulation. Released proteins will alter the chemotactic, adhesive, and proteolytic properties of endothelial cells (ECs).

21. Procurement/Usage of Equipment: Nil

a. Details of Equipment:

Sl. No.	Name of Equipment	Make/ Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown

b. Suggestions for disposal of equipment(s): Not Applicable

(Name and Signature of PIs with date)

Routing: *Signed copy of "Project completion Report" by PI → root@sctimst.ac.in, rpc@sctimst.ac.in*