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Research Article

Platelet Proteomics in Chronic Myeloid Leukemia - ②

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ABSTRACT

Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder resulting from the malignant transformation of a hematopoietic stem cell and is characterised by Philadelphia chromosome that generates a BCR-ABL protein with constitutive tyrosine kinase activity. Patients with CML have been reported with high platelet count, platelet dysfunction and thrombohaemorrhagic complications. The mechanism underlying such complications of platelets in CML is not well understood. In order to understand the factors responsible we have used 2DE coupled with MALDI TOF/TOF mass spectrometry based identification and characterization of altered proteins in CML platelet samples to investigate the factors responsible for thrombohemorrhagic complications and the results have been validated by immunoblotting experiments. Our study has revealed elevated levels of enzymes like GST, LDH, calcium binding protein, calreticulin, regulatory protein 14-3-3 ζ , INTEGRIN and HSP60 that points towards presence of activated platelets and increased metabolic activity of platelets in CML patients. The altered levels of chaperones, regulatory and enzyme proteins indicate towards regulation of INTEGRIN binding and platelet activation. This is the first comparative proteomics study of platelets in CML. The results could provide an insight into better understanding of the pathophysiology of the disease.

Keywords: 2D gel electrophoresis; MALDI MS; Platelets; Thrombohaemorrhagic complications

ABBREVIATIONS

ACD: Acid Citrate Dextrose; ADP: Adenosine-5- Phosphate; CML: Chronic Myeloid Leukemia; Integrin: Integrin $\alpha 2\beta 3$; LDH: L-Lactate dehydrogenase; PDGF: Platelet Derived Growth Factor; Ph: Philadelphia; PRP: Platelet Rich Plasma; RBC: Red Blood Cell; TBST: Tris Buffer-Saline Tween 20; WBC: White Blood Cell

HIGHLIGHTS

- First differential platelet proteomics study in chronic myeloid leukemia
- Study enlightens the route to thrombohaemorrhagic complications
- Elevated levels of enzymes like GST, LDH, calcium binding proteins, calreticulin, regulatory protein 14-3-3 ζ found
- Altered levels of chaperones and regulatory proteins indicate towards regulation of INTEGRIN binding and platelet activation

INTRODUCTION

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder resulting from the malignant transformation of a hematopoietic stem cell. It is characterized by the Philadelphia chromosome (Ph) formed by translocation and fusion of the long arms of chromosomes 9 and 22 in a pluripotent hematopoietic progenitor cell that generates a BCR-ABL protein with constitutive tyrosine kinase activity [1,2]. With disease progression, the progenitor cells are dominated by Ph positive cells [3]. It is diagnosed by decreased hemoglobin concentration, increased WBC count, presence of blast cells, increase or decrease in the number of platelets and enlargement of spleen depending on the severity of CML which is classified into 3 groups that help predict outlook and are based mainly on the number of immature white blood cells i.e., myeloblasts (blasts), seen in the blood or bone marrow. Chronic phase patients typically have less than 10% blasts in their peripheral blood or bone marrow samples and are mostly asymptomatic. Most of the diagnosed patients belong to the chronic phase. Patients are considered to be in accelerated phase if the bone marrow or blood samples have more than 10% but less than 19% blasts [4], high blood basophil and white blood cell counts, very high or very low platelet counts that are not caused by treatment and new chromosome changes in the leukemia cells. Patients belonging to blast phase have more than 20% blasts that often spread to tissues and organs beyond the bone marrow.

The BCR-ABL transcript does not require activation by other cellular messaging proteins. In turn, BCR-ABL activates a cascade of proteins that control the cell cycle, speeding up the cell division. Megakaryocytes are thought to belong to the BCR-ABL+ clone, and abnormal platelet function has been described in CML. Some of the patients with CML have been reported to have high platelet counts that don't function properly and create bruising [5]. One of the most characteristic platelet defects observed in myeloproliferative disorders is a reduced responsiveness to epinephrine, while the response to ADP usually is intact. Thrombocytosis has been reported in about one out of three patients, and platelet dysfunction occurs in one out of six patients. Bleeding is a frequent complication in CML patients, and qualitative platelet abnormalities are frequently observed [6]. Imatinib is widely used in CML treatment for its efficacy. It inhibits BCR-ABL tyrosine kinase activity and induces apoptosis [5]. However, therapeutic doses of imatinib also results in the inhibition of tyrosine kinase activity of platelet-derived growth factor receptor [2]. In CML thrombocytosis is either observed at the onset of presentation or can develop during the different phases of the disease leading to thrombohaemorrhagic complications such as priapism, massive retroperitoneal hematoma and intracranial haemorrhage [7].

In the field of hematology, proteomics has enabled the investigation of proteome modifications in different hematological neoplasms and identified therapy related proteomes that has helped in understanding the clinical behaviour in leukemia [8-10]. Earlier proteomic studies in CML had explained imatinib resistance in CML using iTRAQ coupled with nano-LC tandem MS. CML cell proteome showed expression of various potential biomarkers like HSP10 and peroxiredoxin3 in response to imatinib [11]. Comparative proteomics studies of CML cell lines revealed differential expressions of different functional classes of proteins and has highlighted the role of over-expressed HSP70 [12,13]. However, not much has been studied on platelets from CML samples. Anucleate platelet activities are regulated by translocation and post translational modification of proteins [14]. Recent studies on phosphoproteome and glycoproteome in platelet have been reported by Di Michele and coworkers [15].

The mechanism underlying a huge platelet turnover and thrombohaemorrhagic complications in CML cases (mainly chronic phase have been considered) are still unclear. One way to apprehend the various reasons behind the alteration in platelets in CML subjects is to study the platelet proteome.



MATERIAL AND METHODS

Materials

Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA, USA) and α -cyano-4-hydroxycinnamic acid (CHCA) Matrix Assisted Laser Desorption Ionization (MALDI) matrix were obtained from Sigma (St Louis, MO, USA). Sequencing grade trypsin was purchased from Promega (Madison, WI, USA). In-gel tryptic digestion reagents were obtained from Pierce Biotechnologies (Bedford, MA, USA). Sypro Ruby was obtained from Molecular Probes (Invitrogen, Eugene, OR, USA). PVDF membrane was purchased from Millipore (Billerica, MA, USA). Antibodies were obtained from Abcam (Cambridge, MA, USA) (INTEGRIN), cell signaling technology (Boston, MA) (HSP60). All other reagents, if not mentioned otherwise, were purchased locally and were of analytical grade.

Sample collection

Fresh blood samples of Chronic Myeloid Leukemia (CML) donors were collected in 0.38% sodium citrate from Ramakrishna Mission Seva Pratisthan, and Clinical Hematology Service, Kolkata, India with their informed written consents (guardians in case of minors below 20 years of age), following the guidelines of the Institutional Ethical Committee of Ramakrishna Mission Seva Pratisthan and Institutional Animal and Bio ethics committee of Saha Institute of Nuclear Physics. The whole experimental procedure was approved by the institutional ethical review board. Normal blood was collected from healthy volunteers within the institute with respective consents. The healthy control population didn't suffer from any anemia or hematological disorder. The clinical features of the individual patients are summarized in table 1.

Isolation of platelets

Peripheral blood samples were centrifuged at 200×g for 15 minutes at 23°C. Platelet rich plasma (PRP) was separated leaving 1-2ml above the layer of RBC and WBC to avoid the contamination with RBCs and WBCs. PRP was centrifuged at 1000×g for 15min at 23°C to obtain the platelet pellet and washed thrice with ACD (Trisodium citrate dihydrate 75mM, Citric acid monohydrate 42mM, Dextrose 139mM, pH-5.4). The isolated platelets were checked for purity using flow cytometry [16-29] and have been shown in Supplementary Information (Figure S1).

2D Gel electrophoresis and image analysis

Purified platelets were lysed with a detergent based lysis buffer containing 20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, protease inhibitor from Roche Diagnostics and phosphatase inhibitor from Thermo Fisher Scientific (Rockford, USA). The protein concentrations of the samples were estimated using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad). 500-600µg of protein was used for silver staining, 1.2 mg for sypro ruby staining, 1.8 mg for colloidal coomassie staining, and 2 mg for Coomassie Brilliant Blue (CBB) staining respectively. Acetone precipitated protein lysate was dissolved in 2D rehydration buffer containing 7M urea, 2M thiourea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% Bio-lyte 3-10 ampholyte (Bio-Rad, Hercules, CA) and protease inhibitor (Roche Diagnostics). 17cm pH 4-7 IPG strip (Bio-Rad) were passively rehydrated with solubilised samples and IEF was carried out in a Protean IEF cell (Bio-Rad), stepwise up to 120000 Volt-Hours. Equilibration of the

Table 1: Clinical characteristics of normal and CML samples.

Clinical parameters	Normal	CML	p-value
Sample size (n), (n = number)	8	8	-
Male / Female, n	4/4	6/2	-
Age (mean) ± S.D	27 ± 1.3	55 ± 15.69	0.0002
Platelet count (mean ± S.D) ×10 ⁹ /mm ³	257.1 ± 51.4	560.44 ± 252.6	0.005
Red Blood Cell count (mean ± S.D) ×10 ⁹ /µL	4.5 ± 4.6	3.18 ± 0.5	0.4332
Hemoglobin (g/dL) (mean ± S.D)	13.2 ± 2.1	9.5 ± 3.54	0.0235
Leucocyte count (mean ± S.D) ×10 ³ /mm ³	7 ± 2.5	160.67 ± 54.4	0.0001
Blast cells	-	≤ 5%	-
Spleen size	Normal	Enlarged	-
Bleeding symptoms	Absent	Absent	-

Note: The CML samples were diagnosed with bone marrow biopsy, karyotyping study on bone marrow aspirate and BCR-ABL detection by RT-PCR of peripheral blood. On cytogenetic study the samples were found to be BCR-ABL and Ph chromosome positive and samples were at chronic phase as per WHO classification.

strips post IEF was performed following published protocol [17]. The second dimension was run on 8-16% polyacrylamide gradient gels in a Protean II XL electrophoresis module (Bio-Rad). The gels were stained with CBB and sypro ruby (Invitrogen) according to manufacturer's instructions, or silver staining following the method of Rabilloud. Image captures and analyses were done on Versa Doc series 3000 imaging system using PDQuest software (version 7.1, Bio-Rad). Densitometry analysis of the gel spots of interest was performed using the density tool of PDQuest. Spot volume (intensity) of the desired spot(s) was normalized as parts per million (ppm) of the total spot volume using the spots that were present in all gels, to calculate the relative abundance of a spot in a sample. Although different stains with appropriate sensitivities were used to visualize platelet proteomic profiles of CML and normal samples depending on the sample volumes available, all the densitometric comparisons were done with similarly stained gels with similar protein loads [18].

Statistical analysis

Densitometric analysis of 2D gel of platelets from CML and normal samples and immunoblots were subjected to unpaired two-tail Student's t-test to determine the significance of the changes between CML and normal platelets. Densitometry data are given in Supplementary Information (Table S1) which shows SEM values to be within 10-15% of the mean value with p-values well below 0.05. We have calculated the p-values in the Microsoft Office Excel software (T-test function, Tail 2, Type 3). We have also used GraphPad software for crosschecking.

In-Gel tryptic digestion and mass spectrometry

Sequencing grade trypsin was purchased from Promega (Madison, WI). All other reagents were purchased from Pierce (Rockford, USA). The protein spots from CBB, sypro ruby and silver stained 2D gels of normal and diseased platelet samples were excised using a robotic spot-cutter (Bio-Rad). The gel pieces were de-stained with 50% acetonitrile, 25 mM ammonium bicarbonate. Subsequent in-gel tryptic digestion, peptide elution with 50% acetonitrile and 0.1% TFA, acquisition of MS and MS/MS spectra and database searches were done following our published protocol. Recrystallized



CHCA (Sigma) was used as matrix. MS of the digested peptides was done in positive reflector mode in a MALDI-TOF/TOF tandem mass spectrometer (ABSciex, AB 4700). Autolytic and common keratin peaks were validated and subsequently excluded from MS/MS analysis. Twelve most intense peptides from each spot were subjected to MS/MS analysis. Peak lists were prepared from MS and MS/MS data using GPS explorer V3.6 (ABSciex) software and noise reduction and de-isotoping were performed using default settings. Resulting PMF and MS/MS data were searched against human 6 MSDB and Swiss-Prot databases using in-house MASCOT V2.1 (Matrix Science, UK) server and MOWSE score (with $p < 0.05$) was considered to determine significant hits. For homologous proteins having similar MOWSE scores, preference was given to the protein with best match between theoretical and experimental molecular weight and pI. All MS experiments were repeated at least thrice, with spots excised from three separate gels. The database search parameters included one missed cleavage, error tolerance of ± 100 ppm for PMF and ± 1.2 Da for MS/MS ion search and variable modifications like carbamidomethyl cysteine, methionine oxidation, and N-terminal Acetylation [18]. The peak lists prepared from MS and MS/MS data have been given in Supplementary Information.

Immunoblotting

Platelet protein samples (30 μ g) were re-suspended in 30 μ l SDS-PAGE buffer (2% mercaptoethanol (v/v), 1% SDS, 12% glycerol, 50 mM Tris-HCl with a trace amount of bromophenol blue and were heated at 95°C for 5 min, cooled and loaded directly onto 12% gel. 1D-SDS-PAGE was performed in a Mini Protean III-cell (Bio-Rad) using Tris-glycine with 0.1% SDS, following manufacturer's instructions. Proteins separated on gel were blotted onto PVDF membranes and subsequently blocked with Tris buffer- saline (TBS), 5% non fat dry milk for 2h at room temperature. Primary antibodies HSP60 (Cell Signaling Technology), and integrin $\alpha 2\beta 3$ (Abcam) were diluted in TBS/0.1% Tween (TBST) following manufacturer's protocol. Anti-rabbit or anti-mouse HRP-conjugated IgGs were used as secondary antibodies (Abcam). Membranes were washed with TBST and detected by ECL (Pierce) with either the Versa Doc imager or on X-ray film after development. Due to absence of proper loading control whole blots were stained with Ponceau S to ensure equal protein loads in different lanes for different samples.

RESULTS

Differential proteomic profiling of platelets between normal and CML

A total of 8 normal and 8 CML samples were examined for a comparative proteomic profiling of platelets proteins. CML samples were obtained right at the time of first diagnosis with no

history of transfusion. The representative 2D gel profiles of platelets from normal and CML samples have been shown in the figure 1A, 1B. The mass spectrometric details of the differential proteins are summarized in table 2. The densitometric data of the 2D gels from normal and CML samples along with their p-value and standard error of mean have been provided in Supplementary table S1. On careful densitometric analysis the expression levels of six proteins have been found to be significantly altered in CML samples when compared to the normal. This includes elevated levels of enzymatic proteins such as glutathione-s-transferase (GST) and L-lactate dehydrogenase (LDH), calcium binding protein calreticulin, regulatory protein14-3-3 zeta polypeptide (14-3-3 ζ), integrin $\alpha 2\beta 3$ (INTEGRIN) and heat shock protein 60 (HSP60). The differences between the levels of all proteins were tested for statistical significance employing unpaired two-tailed *t*-test and significant differences ($p \leq 0.05$) have been considered. The representative histogram plots of relative spot densities of commonly altered platelet proteins between CML and normal samples are provided in figure 1C.

Validation by immunoblotting

The results obtained from densitometric analysis were validated using immunoblotting. The amounts of differentially regulated proteins in platelets were quantitated from a set of 3 normal and 5 CML samples using immunoblotting. Immunoblot along with the whole blot stained with Ponceau S as loading control has been presented in figure 2A and figure 2B. Representative Histogram plot and relative band intensities of 2 altered proteins have been presented in figure 2C. All data were subjected to unpaired two-tail students' *t*-test. The results obtained from densitometry analysis are clearly supported by the immunoblot experiments showing upregulation of HSP60 and INTEGRIN. The densitometry data of the immunoblots have been provided in Supplementary table S2.

DISCUSSIONS

We have observed significant alterations in the expression of six platelet proteins in CML. GST comprises of a family of enzymes in human which catalyzes the conjugation of reduced glutathione with various hydrophobic substrates. Platelets contain anionic GST that plays a role in detoxification reaction. GST in platelets is also essential for the lipoxygenase activity that ultimately leads to the formation of leukotriene 4 [19]. Thus elevated level of GST in CML platelets signifies the presence of greater amounts of reduced GSH in these samples and production of leukotriene that controls the platelet aggregation in CML patients.

Calreticulin is calcium binding multifunctional protein present on platelet surface. It is a 46kDa chaperone protein consisting of structurally and functionally distinct domains expressing high and

Table 2: Identification of differentially regulated proteins by MALDI ToF/ToF mass spectrometry.

Spot NO.	Protein name	Accession ID	Mr/ pI	Mascot score	Sequence coverage (%)	Ms/Ms Peaks matched	Fold change of platelet proteins from normal and CML
1	GST	P09211	23.2/ 5.42	207	60	3	+ 2.0
2	CALRETICULIN	P27797	24.3/4.45	146	16	2	+ 2.30
3	LDH	P07864	36.5/5.72	112	66	6	+ 1.74
4	HSP60	P10809	60.9/5.70	135	36	4	+ 1.97
5	14-3-3 ζ	<u>P31946</u>	26.1/4.99	150	50	4	+ 2.66
6	INTEGRIN	B3DIZ6	113.3/5.21	565	37	9	+ 1.95

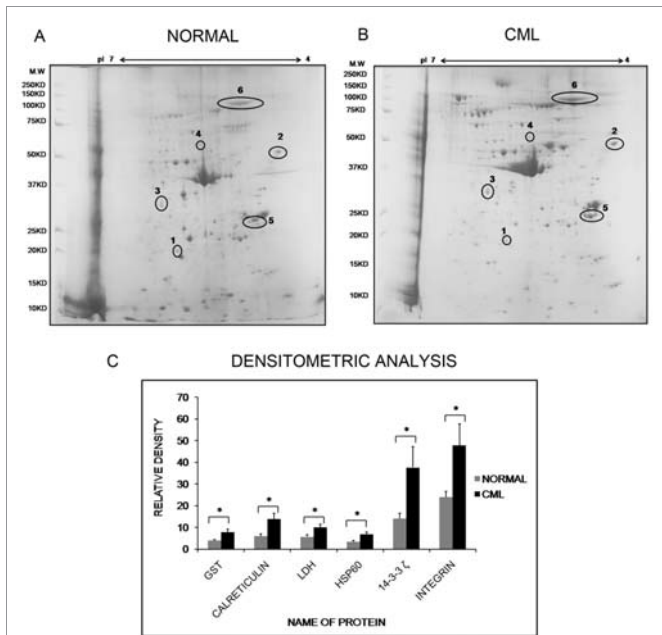


Figure 1: Shows representative 2D gel profiles of platelet proteins from (A) normal and (B) CML blood samples with annotated proteins that are differentially regulated: (1)-GST, (2)-Calreticulin, (3)-LDH, (4)-HSP60 (5)-14-3-3 ζ, and (6)-INTEGRIN. Panel (C) shows the representative histogram plots of relative densities of altered proteins with standard error of mean evaluated from 2D gels of platelet proteins from normal individuals and CML patients.

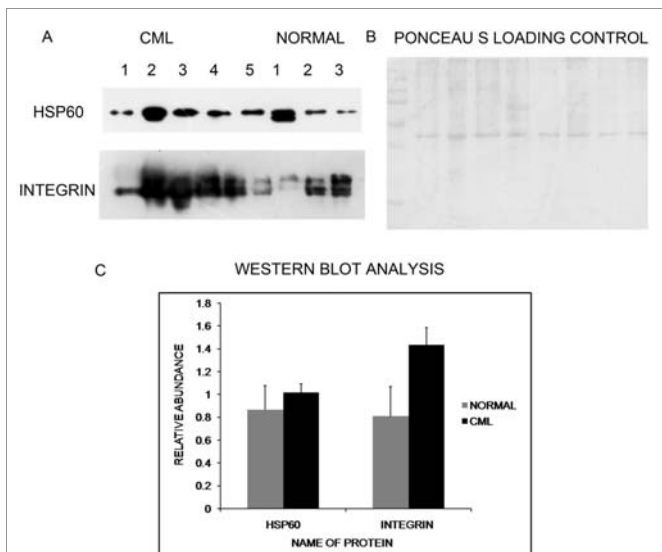


Figure 2: Shows representative (A) immunoblots of HSP60 and INTEGRIN of 5 CML vs 3 normal samples. Panel (B) represents Ponceau S stained whole PVDF membrane used as loading control. Panel (C) shows the representative histogram plot of relative abundances of the differentially altered proteins with standard error mean evaluated from the immunoblots of normal individuals and CML patients in the immunoblot experiments.

low affinity calcium binding sites. Calreticulin is a potential regulator of INTEGRIN and also involved in cell migration and adhesion. Upregulation of platelet calreticulin is involved in enhancing the disulfide bond rearrangements of INTEGRIN during elevated levels of cellular Ca^{2+} and subsequent activation of the platelets [20-22]. It has also been reported that calreticulin on platelet surface also

interacts with collagen receptors and modulates platelet collagen interaction [21]. Thus, the rise of calreticulin in platelets along with INTEGRIN in CML indicates towards its regulatory role in platelet activation that might lead to thrombohaemorrhagic complications.

Elevated levels of Lactate Dehydrogenase (LDH) indicate high platelet metabolism, activation which is also supported by the higher levels of GST and calreticulin, and presence of platelet lesion in CML [23]. Platelets contain a narrow distribution pattern for LDH isoenzymes with LDH 3 being the most prevalent one [24].

HSP60 has also been found to be upregulated from the densitometric data and validated from immunoblotting experiments. It has been reported to be present in the mitochondrial plasma membrane and functions diversely in secretion of proteins and their translocation through the plasma membrane. HSP60 is expressed at the membrane surface of platelets undergoing senescence and help in the clearance of platelets by phagocytes. Thus an increased level of expression of HSP60 indicates activation followed by senescence of platelets in CML [25,26].

The 14-3-3 protein family consists of ubiquitous homo or heterodimeric adaptor protein that takes part in signaling pathways of numerous biological responses. 14-3-3 are implicated in the initial steps of the signaling pathways that regulate physiological and pathological events such as development, homeostasis, thrombosis, and the progression of arteriosclerosis, dependent upon INTEGRIN-induced cell motility, spreading, and migration. It has been reported that interaction between GST and 14-3-3 ζ creates a binding site for GPIbα which further regulates INTEGRIN activation and INTEGRIN dependent spreading and cytoskeletal reorganizations [27,28]. Upregulation of 14-3-3 ζ takes place in response to other elevated proteins responsible for activation and plays an essential role in regulating signaling and cytoskeletal reorganization following activation of platelets in CML samples.

INTEGRIN plays an important role in platelet aggregation and hence hemostasis, thrombosis and formation of a hypercoagulable state [29]. This depends on the transition state of INTEGRIN from resting to an activated state which is regulated by the binding of various ligands to its cytoplasmic tails. Alteration of INTEGRIN conformation triggers signaling events and binds fibrinogen and creating platelet plug and more platelet activation [20,30]. INTEGRIN level is also found to be elevated supporting upregulation of other associated proteins leading to platelet activation and ligand binding creating thrombohaemorrhagic complications in CML cases found at the time of diagnosis.

In conclusion, the present work reports the first proteomics study of platelets from CML patients at diagnosis to identify the factors those might be responsible for platelet activation in CML and involved in the pathophysiology and presentation of the disease. On one hand, upregulation of GST, calreticulin and LDH stands for enhanced platelet activation and hence the metabolic activities, on the other hand, increased levels of 14-3-3 ζ and INTEGRIN clearly support such activation and cytoskeletal rearrangements. HSP60 play their roles on being translocated to platelet surface following activation and acting as markers for senescent platelets. Taken together, the present study highlights alteration in certain proteins those could play important role in thrombohaemorrhagic complications of CML patients and could be identified at the time of diagnosis. We have not observed any significant changes in the expression of those proteins



as well as in the platelet parameters with the age and sex. This study has been done with limited number of CML samples, however, the results obtained in this work provides further scope for a detailed study, particularly on platelet signaling and justification for platelet turnover in case of CML.

Conflict of interest and sources of funding statement

The authors declare no conflict of interest. We acknowledge the IBOP project (PIC # R&D-SIN-5.04-0101) of Department of Atomic Energy (DAE), India for funding.

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