WORK REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1.	Name and designation of ICMR- IF	DR. Prashant Prakash Warang, Technical Officer
2.	Address	ICMR-National Institute of Immunohaematology (NIIH), 13th Floor, New M. S. Building, KEM Hospital Campus, Parel, Mumbai: 400 012.
3.	Frontline area of research in which training/research was carried out	Red cell research (Sickle cell disease)
4.	Name & address of Professor and host institute	Prof. Anna Bogdanova, Institute of Veterinary Physiology, University of Zurich, Switzerland.
5.	Duration of fellowship with exact date	04/ 02/ 2023 to 03/12/2023 (10 months)
6.	Highlights of work conducted	
	I) Technique/ expertise acquired	ANNEXURE I
	II) Research results, including any papers, prepared/ submitted for publication	ANNEXURE II
	III) Proposed utilization of the experience in India	ANNEXURE III

P.P.WARANG

Signature of ICMR-IF

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Technique/ expertise acquired: [ANNEXURE I]

This is the work report of my long term ICMR-DHR international fellowship for Young biomedical scientist for the year 2022-23. For this fellowship/training, I had visited a Red Blood Cell Research Group headed by Prof. Anna Bogdanova Laboratory, Institute of Veterinary Physiology, University of Zurich, Switzerland, for a period of 10 months from 04/02/2023 to 03/12/ 2023. During my fellowship, the following blood analysis techniques have been learned and acquired by me from those practiced at the Group of Prof. Bogdanova.

1. Red blood cell (RBC) deformability and membrane flexibility using Osmotic gradient ektacytometry.
2. RBC density and heterogenicity using Percoll density gradient experiment.
3. Regulation of RBC hydration using flow cytometric study.
4. Detection of intracellular free Ca^{2+} levels and its compartmentalization, as well as the movement of Ca^{2+} across the RBC plasma membrane mediated by particular ion transporters.
    i) Uptake of Ca^{2+} in response to mechanical stimulation by PIEZO1 channels and NMDA receptors (osmotic swelling) using Fluo 4-AM fluorescence dye.
    ii) Determination of mechanosensitive ion channel- Piezo 1 and Plasma membrane Ca^{2+} ATPase (PMCA) activity as well as functional study using activator and inhibitor of this channels.
5. Assessment of hydrolytic activity of PMCA by spectrophotometry
6. Detection of intracellular Na^{+} levels using CoroNaTM Green AM dye
7. Monitoring of the activity of Ca^{2+}-dependent Gardos channel (morphological examination of Ca^{2+}-inducible shrinkage)
8. Detection of Na,K-ATPase hydrolytic activity (photometry)
9. Determination of intracellular Na^{+} and K^{+} ions by flame photometry
10. Red cell redox status and RBC turnover by following methods.
    i) Determination of total reduced thiols using Monobromobimane (MBBR) by flow cytometry.
    ii) Determination of Intracellular GSH level using DTNB reagents.
11. Assessment of RBC turnover (reticulocyte count) and average RBC age using deamidation of the band 4.1 protein (band 4.1 a:b ratio) using SDS PAGE.

12. Detection of the redox state, intracellular Ca2+ levels and phosphatidylserine exposure in early and mature reticulocytes using co-staining for RNA, CD71, bulk reduced thiols and Annexin V.
13. Microscopic examination of Ca^{2+} uptake (microfluorescent live imaging); Baseline measurement and visualization of intracellular Ca^{2+} stores (intracellular inside out vesicles) and red cell morphological study. Visualization of Ca^{2+} uptake kinetics using live imaging.
14. Western blot analysis for PMCA and Hemoglobin glutathionylation.

Research results, including any papers, prepared/ submitted for publication

[ANNEXURE I I]

Technology transfer was a primary objective for this Followship. Focus was made on the functional tests that are quite essential for assessment of the rheological properties of cells such as deformability and membrane flexibility (Osmotic gradient ektacytometry), RBC density and heterogenicity (Percoll density gradient experiment), regulation of RBC hydration (Cation channels and water handling), intracellular Ca^{2+} level and transport across the membrane as well as red cell redox state and RBC turnover. Hemolytic activity in turn is defined by RBC membrane stability, oxidative stress as well as hemolysis by using a combination of basic and advanced technology.

As I have learned the basics of all these techniques, I had a chance to apply them working on a small pilot study entitled "Effect of Inflammation on RBCs of dogs". In this study, 23 patient dogs and 04 healthy dogs samples were investigated. Patient dogs had a history of high CRP level (due to sepsis, gastroenteritis, or kidney diseases). Some of the dogs were presented with anemia of inflammation or due to kidney failure. The project aimed to unravel the possible links between inflammation and RBC properties such as deformability, abnormal Ca^{2+} homeostasis and oxidative stress. These parameters were then related to the RBC turnover and oxidative markers. RBCs deformability and hydration state were assessed by ektacytometry in patients and healthy dogs. We observed two patient dogs presented with anemia showed reduced RBC deformability which was most likely associated with overhydration. RBCs density and heterogeneity were assessed by Percoll density gradient assay in all dog samples. Percoll density gradient separation revealed a clearly lower density in one dog with overhydrated RBC phenotype compared to the healthy dog RBCs and higher heterogeneity in

in RBC density of another dog in blood of which both overhydrated as well as dehydrated RBCs were present. This finding was verified using microfluorescent life imaging. Both dogs with abnormal rheological properties and hydration were unable to effectively preserve transmembrane Ca^{2+} gradient and multiple RBCs were showing Ca2+ overload associated with stomatocytosis and swollen RBC phenotype typical for overhydration. Thus, microscopic analysis was supporting the findings revealed by fractionation of RBC in Percoll density gradient as well as by Osmoscan Ektacytometry.

We have further attempted to modulate the intracellular Ca2+ levels in canine RBC using the inhibitor of Na+/$Ca2^{+}$ exchanger (NCX) inhibitor SEA 0400. This transporter has been found to be essential for Ca2+ uptake by RBCs of dogs where primary tranmembrane Ca2+ gradients are used to control Na+ transport out of the cells (exchanging 3 Na+ ions which are extruded from the cell to one Ca2+ taken into the cell). To explore the impact of NCX in Ca2+ we used fluo-4 as a marker of intracellular Ca2+ levels and monitored the changes in fluorescence intensity of this dye using flow cytometry. We have found that treatment of RBCs of dog patients and those of healthy dogs with NCX inhibitor caused a decrease in the intracellular Ca^{2+} levels. Reticulocyte counts and redox status was not significantly changed in the RBCs of patient-dogs. There was no change in the shape of RBCs observed by flow cytometry.

Finally, we correlated all the parameters finding such as RBC deformability (Osmoscan ektacytometry), red cell density and heterogeneity (Percoll density gradient experiment), intracellular Ca^{2+} levels and Ca^{2+} transport across the membrane as well as red cell redox status to confirm the diagnosed or severity of the disease. High Ca2+ levels was shown to be associated with lower deformability of dog RBCs which in turn resulted in faster RBC turnover (reticulocytosis and anemia).

I was also involved in study of Terminal density reversal in human red blood cells: Role of Plasma membrane Ca^{2+} ATPase & Piezo1. In this study, we have identified senescent-like high Ca^{2+} (SLHC) cells present in low density RBCs fractions. This SLHC cells are Na^{+} as well as Ca^{2+} overloaded. SLHC cells have hyperactivated Piezo1 and inhibited PMCA activity. Pieoz1 is playing an important role in mediating Na^{+} and Ca^{2+} influx in SLHC cells.

Proposed utilization of the experience in India

[ANNEXURE I I I]

Our Institute is one of the prime centers in the country which working extensively on inherited hemolytic anemias associated with hemoglobinopathies, enzymopathies and membranopathies where patients are referred from all over the country for diagnosis. Among the hemoglobinopathies, thalassemia syndromes and sickle cell disease (SCD) are well investigated at our institute.

Recently our institute has established a Centre for Research, Management and Control of Haemoglobinopathies at Chandrapur, Maharashtra [ICMR-CRMCH (NIIH), Chandrapur, Maharashtra]. Over the years we have made a cohort of patients with variable clinical presentations. To the best of our knowledge, the role of ion channelopathies or red cell permeability in the SCD and thalassemia patients has not been investigating well in India.

I will utilize this fellowship training experience or knowledge for the following research activity.

1. To standardize all these techniques at ICMR-NIIH, Mumbai and perform them on red blood cells from patients with hereditary hemolytic anemia mainly Sickle cell diseases, Thalassemia, and red cell membranopathies mainly hereditary spherocytosis and Hereditary xerocytosis.
2. To study the effect of drugs on different properties of red cells such as red cell deformability and flexibility, density, membrane integrity and stability.