EX-VIVO EFFECTS OF WASHING AND LEUKOREDUCTION ON IRON PROFILE, INCLUDING "*NON-TRANSFERRIN BOUND IRON*" (NTBI) AND STORAGE LESIONS IN NON-IRRADIATED AND IRRADIATED PACKED RED BLOOD CELL (PRBC) UNITS

Thesis

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In partial fulfilment of the requirement for the degree of

Doctor of Medicine (MD)

(Transfusion Medicine and Blood Bank)

June, 2022 **Dr. Richa Mishra**

AIIMS, Jodhpur

DECLARATION

I hereby declare that the thesis titled "Ex-vivo effects of washing and leukoreduction on iron profile, including non-transferrin-bound-iron (NTBI) and storage lesions in non-irradiated and irradiated packed red blood cell (PRBC) units" embodies the original work carried out by the undersigned in All India Institute of Medical Sciences, Jodhpur.

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CERTIFICATE

This is to certify that the thesis titled "Ex-vivo effects of washing and leukoreduction on iron profile, including non-transferrin-bound-iron (NTBI) and storage lesions in non-irradiated and irradiated packed red blood cell (PRBC) units" is the bonafide work of Dr. Richa Mishra carried out under our guidance and supervision, in the Department of Transfusion Medicine and Blood Bank, in collaboration with Department of Pathology, Department of Biochemistry, Department of Anaesthesiology, Department of General Medicine and Department of Pharmacology, All India Institute of Medical Sciences, Jodhpur.

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"Alone we can do so little, together we can do so much."

--Helen Keller

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Dr. Richa Mishra

Dedicated to My Family

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LIST OF ABBREVIATIONS

2,3-DPG	: 2,3-Diphosphoglyceric Acid
AAS	: Atomic Absorption Spectroscopy
AGLT	: Acidified glycerol lysis test
AS1	: Additive Solution 1
AS5	: Additive Solution 5
ATP	: Adenosine triphosphate
C3	: Complement Component 3
CBC	: Complete Blood Count
CPD	: Citrate Phosphate Dextrose
CPDA1	: Citrate Phosphate Dextrose Adenine
CRP	: C Reactive Protein
DAMP	: Damage Associate Molecular Patterns
DAT	: Direct Antiglobulin Test
DEHP	: Diethylhexyl Phthalate
DMSO	: Dimethyl Sulphoxide
DTPA	: Diethylenetriamine pentaacetate
EBV	: Ebstein barr Virus
ELISA	: Enzyme Linked Immuno Sorbent Assay
ESR	: Erythrocytic Sedimentation Rate
FNHTR	: Febrile Non-hemolytic Transfusion Reaction

HPLC : High Performance Liquid Chromatography

HTLV : Human T-Lymphotropic Virus

IAT : Indirect Antiglobulin Test

LDH : Lactate Dehydrogenase

LFT : Liver Function Test

LPI : Labile Plasma Iron

LR : LeukoReduced packed red cells

LORCA : Laser assisted Optical Rotational Red Cell Analyzer

MAC : Membrane Attack Complex

MCHC : Mean Cell Hemoglobin Concentration

MCV : Mean Cell Volume

MDA : Malondialdehyde

NAT : Nucleic Acid Testing

Nitroso-PSAP : 2-Nitroso-5-[N-n-propyl-N-(3-sulfopropyl) amino] phenol

NLR : Non Leukoreduced Packed red cells

NO : Nitric Oxide

NTA : Nitrilotriacetic Acid

NTBI : Non-Transferrin Bound Iron

OD : Optical Density

PRBC : Packed Red Blood Cells

PVC : Polyvinyl Chloride

RBC	: Red blood Corpuscles
RDW	: Red Cell Distribution Width
SAGM	: Saline Adenine Glucose and Mannitol
SLT	: Sucrose Lysis Test
TA-GVHD	: Transfusion-associated graft-versus-host disease
TCA	: Trichloroacetic Acid
TIBC	: Total Iron Binding Capacity
TRALI	: Transfusion Related Acute Lung Injury
TTI	: Transfusion Transmitted Infection
UIBC	: Unsaturated Iron Binding Capacity
VBG	: Venous Blood Gas
WB	: Whole Blood
WBC	: White Blood Corpuscles

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SUMMARY

SUMMARY

Background:

Safety is a critical concern in Transfusion Medicine. "Old" blood has been suspected to be inflammatory, but there is not enough evidence in human adults for harm from transfusion of "old" units. Storage of blood, in the un-physiological blood banking conditions, causes accumulation of various "storage lesions" that include myriad biochemical, morphological, and functional changes. Potassium & % Hemolysis are acutely of safety concern as part of storage lesions. The morphologic deterioration of the RBCs, decay of 2,3-DPG and ATP level etc. are functionally important but are largely reversible after transfusion.

One of the strongest candidates for the biological mediator of harm from storage lesions is Non-Transferrin Bound Iron (NTBI). The redox active fraction of NTBI is called Labile Plasma Iron (LPI) which may be directly pathogenic. NTBI is known to increase in vivo after transfusion, especially of old units. Very few papers have looked at NTBI ex vivo, i.e., in the unit itself. The scant data available suggest that NTBI might increase with the age of blood bag. There is no published information so far on what happens to NTBI when manipulations such as irradiation and leukoreduction are done on the blood bag.

Gamma irradiation is a necessary modification of cellular blood components if the patient is at risk of TA-GVHD (Transfusion Associated Graft vs Host Disease) which is a nearly 100% fatal late complication of transfusion. The shelf life of blood is significantly decreased after irradiation due to aggravated storage lesions and reduced in vivo recovery after more than 28 days of post-irradiation-storage. Recovery can be indirectly predicted by decay in ATP level.

Washing of the red cell units can decrease the toxic substances built up in stored/irradiated blood and can aid in ensuring short term safety. However, washing is a complex process, usually involving multiple rounds of processing and unavoidably shows a lot of variability. Thus, washing needs extensive local validation as well as Quality Control. Washing, if done by a closed method with nutrient containing additive solution, allows the unit to be stored for 1-2 weeks post washing.

The potassium & haemoglobin leak and accumulation of other potentially harmful substances e.g., NTBI aggravated by irradiation may be temporarily overcome by washing and precious

units can possibly be salvaged to avail the full legal viability window— a possibility that we wanted to explore.

Objectives:

1. To measure Iron profile including NTBI, and storage lesion markers i.e., ATP, free Hb, CBC, VBG, LDH, Total Protein, AST, Bilirubin, Morphometry, Osmotic Fragility, Complement Fixation potential in Irradiated and Non-Irradiated PRBC units.
2. To explore correlations between the measured parameters and test whether Leukoreduction and/or Washing can significantly reverse storage lesions in Irradiated and Non-Irradiated units.

Method:

In this study, 56 PRBC units were allocated to two groups: NLR (Non-Leukoreduced) and LR (Leukoreduced), 28 units each, further divided into 4 Sub-groups having 7 bags each, subjected to different combinations of irradiation and/or washing. The following parameters are measured: Routine Iron parameters (Serum Iron, TIBC), a Lab developed NTBI assay, and storage lesion markers i.e., ATP, free Hb, CBC, VBG, LDH, Total Protein, AST, Bilirubin, Morphometry, Osmotic Fragility, Complement Fixation potential. were serially assessed every week from each bag/split unit. Pink test for Osmotic Fragility and sucrose lysis test for complement sensitivity were also performed. All modifications in this study were as per international accreditation/licensing standards for blood banking and were further characterized and validated in the local setting.

Modification and observation of blood bags and samples were carried out by the Department of Transfusion medicine and Blood bank, in collaboration with the Departments of Pathology, Biochemistry and Anaesthesiology, AIIMS, Jodhpur.

Result:

Free Hb levels in early storage was found to be higher in LR units, which though counter intuitive, is consistent with literature.

Washing and resuspension with various combinations of NS and SAGM were tested and the sequence of progressively better quality was found to be NS + SAGM < NS + NS < SAGM +

SAGM. We standardized a double washing by manual closed system handling with SAGM as our preferred method for washing.

Our Lab-developed NTBI assay gave results corroborating with published studies regarding NTBI which are themselves very few in number and all performed abroad. NTBI levels were found to be higher in LR units than NLR units. Our study showed that in LR units NTBI levels remained acceptable (below zero) up to 2 weeks. However, in NLR units NTBI levels may counter-intuitively remain acceptable up to 3 weeks. Variation in NTBI levels between these groups almost parallel free Hb levels.

Conclusion:

We successfully developed and standardized a fully automated homogenous colorimetric lab-developed assay for NTBI with imported reagents.

This is the first ever study in the world investigating NTBI ex-vivo in relation to blood unit irradiation and leuko-reduction. The closed system washing & assessment of its effectiveness in this study might help extend the utilization window of precious antigen typed units for thalassemia patients beyond 2 weeks from collection especially for NLR units. It will also help extend the safety-window of post-irradiation storage. Despite legal acceptance of viability, irradiated units are rarely used in the later part of the legal time window for many patients because validation data for washing such units are unavailable.

In the western countries transfusion dependent Thalassaemic patients as well as all chronic transfusion dependent patients are always given LR units. Moreover, in the west majority of the countries have been practicing universal Leuko-reduction for over 20 years. Though leukoreduction reduces true hemolysis as evidenced by LDH and AST, our study shows that during the early storage (up to 4 weeks) Potassium leak and % hemolysis calculated from free Hb (includes Hb in vesicles) remain higher in the LR units. and NTBI correlates significantly more with free Hb than with LDH or AST. Despite the benefit of reducing the chances of inducing HLA allo-immunisation after transfusion, leuko-reduction seems to have this relatively less known disadvantage of higher early apparent hemolysis (% hemolysis calculated from plasma Hb) which translates to higher NTBI regarding which, to the best of our knowledge, there is no published data in the searchable domain.

Irradiation does seem to increase hemolysis as well as NTBI. During early storage, since NTBI level is apparently "below zero", Washing does not further reduce NTBI, rather it might induce mild hemolysis and NTBI rise, which is statistically insignificant. However, in late storage when NTBI is detectable at positive levels or aggravated post irradiation, washing is able to reduce the NTBI level to below zero. Similar result has been shown in canine blood by Cortes-Puch 2014[1] but we show it for the first time in human stored blood.


INTRODUCTION

INTRODUCTION

At present, half of the science of Transfusion Medicine revolves around safety and quality of "Banking" i.e., storage of blood from healthy voluntary donors. The history of blood banking dates back to World War I where the first blood depot was established in 1917 under the guidance and supervision of Oswald Hope Robertson, an English-born medical scientist who was especially recruited for the purpose of finding successful blood transfusion techniques for the soldiers during the war. The usefulness of blood depots was analysed through the data obtained during the War and eventually the Robertson method was convincingly adopted to develop a greater number of blood depots.

However, the attempt of human-to-human transfusion by a British Obstetrician James Blundell to treat a patient of postpartum haemorrhage was elicited 100 years before the advent of the concept of blood depots and blood banking. The collective idea of bloodletting, its storage and transfusion has faced challenges on the whole ever since their individual conception. One of the first challenges was to prevent clotting of blood before it was transfused to another person. Rous and Turner developed the first red cell storage solution in 1916 which was a mixture of citrate and glucose. In the quest to develop a solution compatible with properties such as longer shelf life, sterility and anticoagulation, solutions like acid citrate dextrose (ACD) (by Mollison and Petit, 1943), Citrate Phosphate dextrose (CPD) and its variants came into use subsequently.

The blood is collected in bags made up of poly vinyl chloride (PVC) plasticized by di-ethyl-hexyl-phthalate (DHEP) and mixed with the primary anticoagulant Citrate-Phosphate-Dextrose with or without adenine (CPD or CPDA-1) present in the bags. In blood banks with component separation facility, whole blood is separated into its components within 6-8 hours of collection. Packed Red Blood Cells (PRBC), the component which is one of the highest in demand, has the same storage duration as whole blood i.e., 21 days (in CPD) or 35 days (in CPDA-1) at 2-6°C, unless mixed with "Additive Solutions" e.g., Saline- Adenine-Glucose-Mannitol (SAGM, AS1, AS5, etc.) which extend shelf life to 42 days.

Storage lesions refer to the array of biochemical, functional and morphological change that occur during storage of blood. Significant biochemical changes include leakage of potassium, LDH, SGOT, cell free hemoglobin (fHb), progressive pH drop, lactate accumulation, increase in non-transferrin bound iron (NTBI) in the supernatant [2]. Functional changes are

potentially due to depletion of intracellular 2,3-diphosphoglycerate (DPG) and ATP over time[3]. DPG is crucial in mediating the release of oxygen at cellular level, it was initially hypothesised that older blood units may not carry out the transport of oxygen effectively. However, studies have not been very successful in establishing any definite correlation between age of the bag and in vivo oxygen carrying capacity of transfused blood, possibly due to rapid replenishment of DPG and ATP which thus lose a part of their significance as a true indicator of quality of storage. Progressive morphological changes include RBC swelling, rounding, spinulation and vesiculation etc. which may be assessed by MCV, RDW etc. in CBC and by morphometric analysis. These morphological changes, again, are reversible upon transfusion until the spherocytes stage, and are thus again not perfect indicators of storage quality.

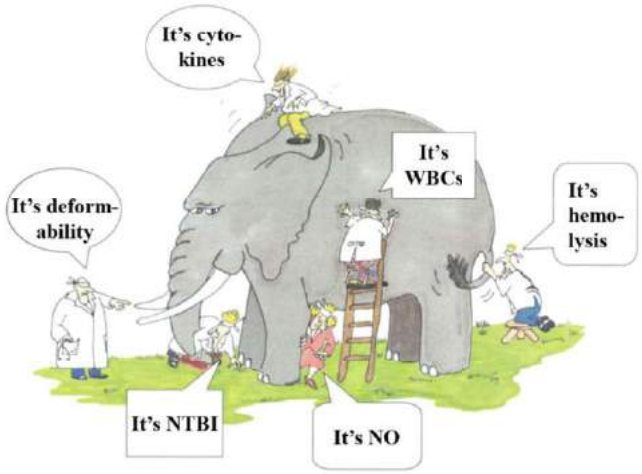


Figure 1: Pictorial depiction of lack of understanding of what is the most important storage lesion akin to 5 blind men looking at an elephant. It is evident that even after decades of research, the understanding of storage lesion is incomplete.

Storage lesions contribute in aggravating the incidence and intensity of transfusion related reactions like febrile non hemolytic transfusion reaction (FNHTR), Transfusion related Acute Lung Injury (TRALI)[4], systemic inflammatory diseases such as infections, multi-organ failures etc. Out of these, TA-GVHD is the most dreaded (nearly 100% fatal) complication of PRBC transfusion in a susceptible host and is caused by clonal expansion of viable T lymphocytes in the transfused blood. The signs and symptoms include fever, diarrhoea, rash, deranged liver function, hypoplasia of bone marrow, all occurring within 3-30 days of

transfusion. Skin and bone marrow biopsy, identification of microchimerism etc are part of the investigations done to confirm the diagnosis.

During storage RBCs remain suspended in an anticoagulant solution in an unphysiologic environment (temperature, pH, matrix, rheology, cellular context etc.). Thus, during storage Blood Cells as well as the supernatant undergo a series of ex-vivo biochemical, physical, morphological and functional changes depending on processing and storage conditions. These varieties of changes have been termed 'storage lesions'. Storage lesions affect the quality of the components and may contribute to transfusion related reactions like febrile non-hemolytic transfusion reaction (FNHTR), Transfusion related Acute Lung Injury (TRALI), aggravate systemic inflammatory diseases including infections, multi-organ failure etc. Research on RBC storage has repeatedly demonstrated that fundamental biology of RBCs is still not well understood. A decrease in pH, glucose, ATP and an increase in lactate content, potassium and some other toxic materials have been repeatedly seen in various studies.

Table 1: Biochemical changes in stored Non-Leukoreduced RBCs

(AABB Technical Manual 2015 edition)

	CPD		CPDA-1				AS-1	AS-3	AS-5
Variable	Whole Blood		Whole Blood	Red Blood Cells	Whole Blood	Red Blood Cells	Red Blood Cells	Red Blood Cells	Red Blood Cells
Days of Storage	0	21	0	0	35	35	42	42	42
% Viable cells (24 hours posttransfusion)	100	80	100	100	79	71	76 (64-85)	84	80
pH (measure at 37 C)	7.20	6.84	7.60	7.55	6.98	6.71	6.6	6.5	6.5
ATP (% of initial value)	100	86	100	100	56 (± 16)	45 (± 12)	60	59	68.5
2,3-DPG (% of initial value)	100	44	100	100	<10	<10	<5	<10	<5
Plasma K+ (mmol/L)	3.9	21	4.20	5.10	27.30	78.50*	50	46	45.6
Plasma hemoglobin	17	191	82	78	461	658.0*	N/A	386	N/A
% Hemolysis	N/A	N/A	N/A	N/A	N/A	N/A	0.5	0.9	0.6

*Values for plasma hemoglobin and potassium concentrations may appear somewhat high in 35-day stored RBC units; the total plasma in these units is only about 70 mL.

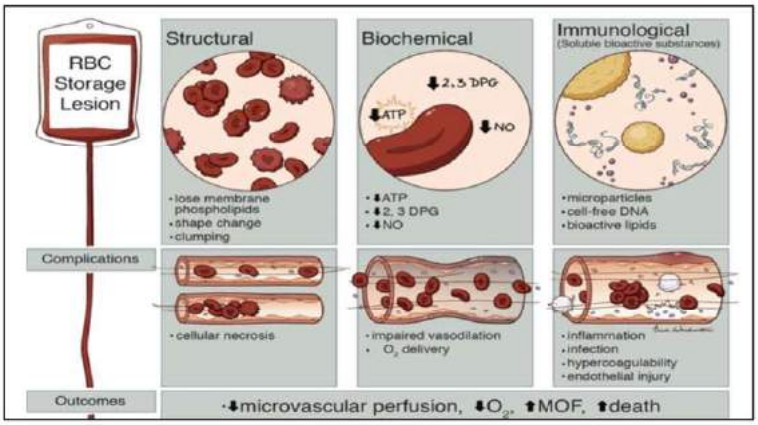


Figure 2: Structural, biochemical and immunological consequences of storage lesion

NTBI

Patients of transfusion dependent thalassemia and a few other hemolytic anaemia and transfusion dependent aplastic anaemia, people of transfusion dependent myelodysplastic syndrome are some examples of patients who undergo regular transfusions. According to a 2016 survey, these patients constitute >20 % of the annual total transfusion demand of India. These patients die early because NTBI in their body mediates organ (liver, heart, endocrine) damage of hemochromatosis in their body which leads to excessive morbidity and mortality & poor quality of life in these which is only partly reversed by chelators. Tx of Old units contribute a large transient in in vivo NTBI levels. However, the contribution of different parts of the unit (red cells and supernatant) in this NTBI surge is still not well researched. According to El ad Hod 2011, it is mostly from the older red ells itself leading of formation of NTBI only after Tx. However, Cortes Puch in 2014 is one the very few publications which have ventured into NTBI during storage and proposed that atleast in a canine model, was able to reduce NTBI in supernatant in older unit. Interestingly, they also tried washing fresher units and found little benefit or rather potential harm and our study found something similar to it in human units.

Ferritin and Transferrin are the prime extracellular proteins which form complexes with Iron in circulation, of which transferrin iron is the most common. Ferritin in the circulation consists mostly of the protein shell with very little iron in it. Majority of the iron in the circulation is bound to Transferrin which is the physiological carrier of iron.

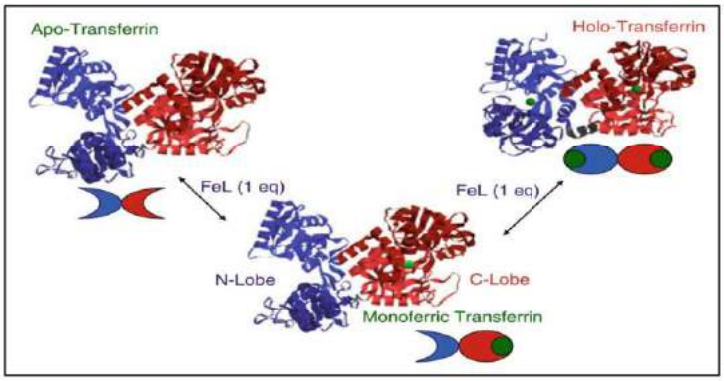


Figure 3: Structure of Transferrin in Apo- Transferrin, Mono- Transferrin and Holo-Transferrin states.

Transferrin contains two similar but nonidentical lobes (N-Lobe and C-Lobe). When in the iron-free apo state the two domains of each lobe of transferrin are in an open conformation in which the protein ligands are in contact with the biological fluid. When iron-loaded, the two domains act as a pair of jaws that engorge Fe(III) in a closed conformation, where iron is buried about 10 A° beneath the protein surface which prevents the iron to participate in any redox reaction in the plasma.[5]

Of due concern, is the fraction of iron which is not bound to any of these proteins and circulates "free". This fraction, known as the "Non-Transferrin Bound Iron" (NTBI), has a high propensity to generate reactive oxygen species and has been implicated in the oxidative damage caused, both at the plasma level as well as the intracellular level in various pathological conditions. In historic studies involving computer simulations of metal iron equilibria in bio-fluids provide data suggesting that the main form of NTBI could be iron(III) citrate[6]

During the 1970s, the idea of NTBI originated from the studies on animals by Hershko et al. His experiments have shown that DF (deferoxamine) binds to both parenchymal and RE iron whereas DTPA with an equal efficiency as DF fails to interact with RE iron. But interestingly, iron bound to DTPA and injected IV was recovered almost completely in the urine, which lead to the speculation that urinary excretion of iron following DTPA was not directly derived from iron stores, but most likely from some chelatable extracellular iron pool, that is how the idea of NTBI first came into picture. (Hershko, 2018).

One of the important mechanisms of formation of NTBI is that RBCs are phagocytosed by macrophages and free haemoglobin is released which in turn gives off these iron species[7]. As of now there are some animal models and only a few human models to show the rise of NTBI in stored blood[8], and if transfused, it causes deposition of iron in tissues and triggers inflammation.

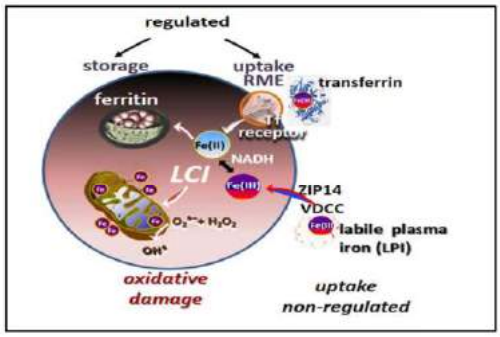


Figure 4: Figure showing mechanism of generation of Labile cell iron (LCI) and labile plasma iron (LPI). Adapted from Cabantchik 2014[9]

IRON HYPOTHESIS: Transfusion of stored, rather than fresh, RBCs causes an immediate bolus of RBCs and RBC-derived iron to be delivered to the monocyte/macrophage system, resulting in oxidative stress and inflammatory cytokine production. Some of the iron absorbed by macrophages is released back into the circulation (i.e., NTBI), where it can induce oxidative damage and promote bacterial growth[10]

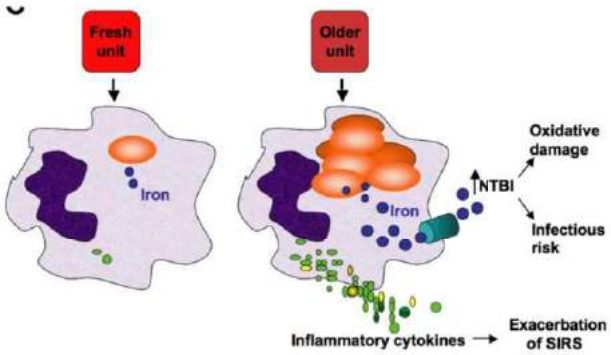


Figure 5: The Iron Hypothesis, Adapted from Hod et al, 2010.

Several attempts have been made to develop a feasible, sensitive assay for determination of NTBI. The methods employed in the estimation of NTBI require only NTBI to be measured, leaving behind the transferrin bound fraction of iron. The methods, in general, involve

trapping of NTBI by a chelator which in turn presents it to a signal generating moiety which determines the concentration of NTBI corresponding to the signal so obtained.

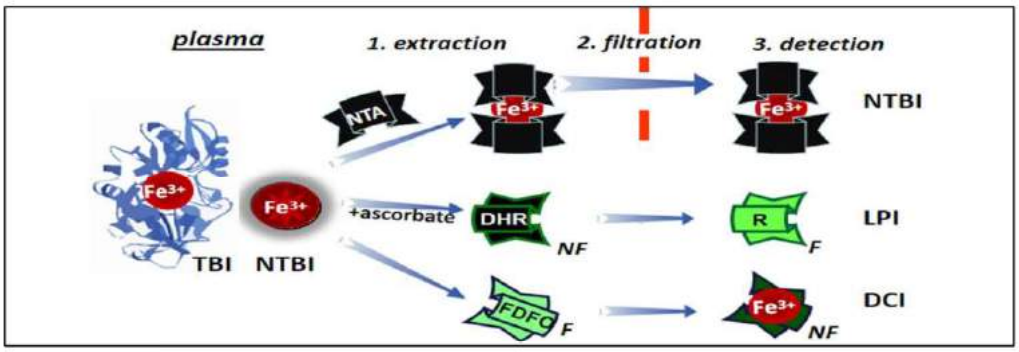


Figure 6: Methods of determination of NTBI. Adapted from Cabantchik, 2014[9]

Figure shows the methods of determination that have been used for various fractions of Non transferrin Bound Iron. Nitrilotriacetic acid in variable concentrations (10-80mM) is used to bind NTBI; the Fe-NTA complex so formed is filtered and then Iron is detected by Colorimetric method or analysed by HPLC. If the serum is exposed to physiological concentrations of ascorbate, the Labile Plasma Iron fraction can be determined using Fluorogenic dihydrorhodamine(DHR). The inhibition of signal change during conversion of non-fluorescent DHR to fluorescent rhodamine by deferrioxamine defines the concentration of LPI. Fluorescent chelators are used to determine the directly chelatable Iron (DCI) component of NTBI. (Cabantchik). Till now, HPLC is considered to be the gold standard of measuring NTBI. However several types of assay including automation[11] and commercial kit[12] have also been developed.

In normal individuals, the NTBI levels are usually undetectable. The level of NTBI is raised in Iron overload conditions such as hemochromatosis and thalassemia, and therefore, NTBI can prove to be an early marker of Iron overload in those patients and help supplement early intervention to prevent organ damage due to iron overload

The **pH** of the stored blood is seen to be decreased with increase in the duration of storage[13]. In storage, the blood cells have to be maintained alive with minimal expenditure of energy, by anaerobic respiration. This results in formation of **lactate**. The pace of glycolysis is slowed down eventually, lactate concentration builds up and gradually the changes in the shape of RBCs become prominent. Only the morphological changes in RBCs reverse once the stored blood is rejuvenated. Rejuvenation refers to the process where depleted

metabolites, particularly 2,3 DPG and ATP are restored. In vivo, this lactate is buffered by kidney but in the absence of any metabolizing agent, this lactate is accumulated and consequential fall in pH occurs without any resistance. Patients receiving these blood units are at risk of devastating effects, especially those receiving multiple units within a short span of time. The normal concentration of potassium is many fold higher in the cells as compared to plasma. The period of storage witnesses cell lysis which causes release of potassium and hemoglobin into the plasma. Blood is stored at 2-6^{0}C which decreases the metabolic rate and energy consumption of the red cells. This causes the Na+-K+ ATPase pump to become inoperative and permit passive movement of **potassium** ions outside the cell and sodium ions into the cell. The increase in potassium concentration extracellularly is approximately 1mEq/L daily. The potassium concentration being 4-6mmol/L increases to 14.3 mmol/L in the second week and then rises exponentially beyond three weeks of storage. Other studies suggest that combined effect of reduction of ATP, increase in lactate and gradual rigidity of the cell membrane is responsible for cell lysis and potassium leak. Blood gas analyser having three modules, that is, for electrolytes, metabolites and cooxymetry is used to monitor and analyse potassium content, pH and lactate in stored blood bags.

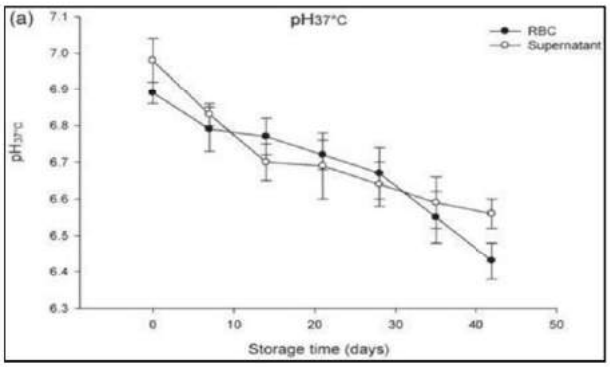


Figure 7: Intra and Extra-Cellular pH vs Storage duration as per Blasi et al 2012[3]

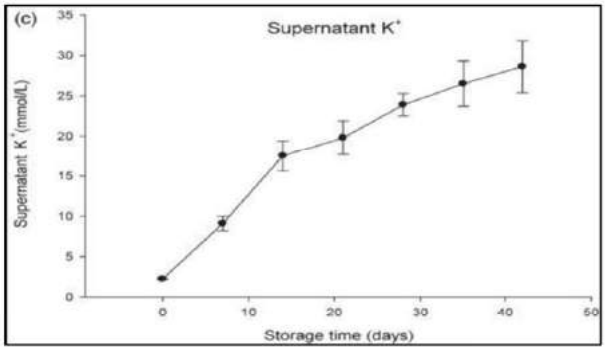


Figure 8: Supernatant K^+ vs Storage duration (Adapted from Blasi et al 2012)[3]

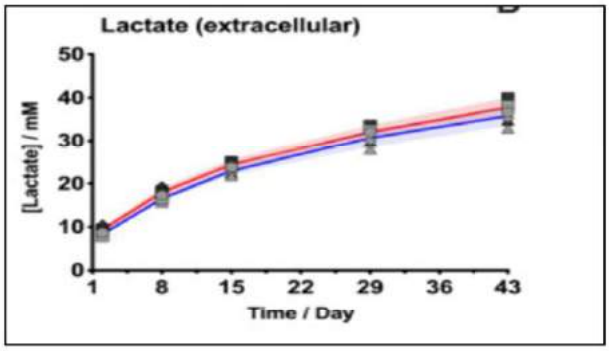


Figure 9: Changes in Lactate concentration in stored blood. (Adapted from Bardyn et al 2020)[14]

ATP

ATP plays a pivotal role in normal metabolism of RBC as it helps in maintaining the activity of Na^+-K^+ ATPase, oxidative stress defence mechanism, membrane stability and its phospholipid content and distribution, glucose transport etc. a marked reduction in ATP concentration is observed in banked PRBC with duration of storage. Thus, it can be considered an intracellular source of energy and is required to be in adequate concentration for the smooth functioning of the cellular metabolism. ATP, when released from RBCs, mediates the production of NO (nitric oxide) which causes vasodilation during hypoxic conditions. The fall in ATP content is closely associated with characteristic morphological changes such as formation of echinocytes and spherocytes. A reduced ATP concentration caused by storage leads to the exposure of phosphatidylserine on the outer layer of RBC membrane, acting as a signal for macrophages to engulf the cells[15]. ATP also plays an important role in regulating the interactions between the spectrin network and the RBC membrane[16]. However, it has been shown that ATP concentration is recovered if the red cells are incubated with human serum at 37^0C [17]. Also, the ATP levels and the RBC stiffness caused by its deficiency are normalised soon after transfusion and these deformation changes are reversed. Therefore, it is only considered a surrogate marker of storage lesions. However, Adams 2015 pointed out that the perpetual decrease in ATP content eventually causes irreversible alteration in RBC morphology as echinocytic protrusions start appearing[18]. The asymmetry of phospholipid layer is lost and microvesicles are released.

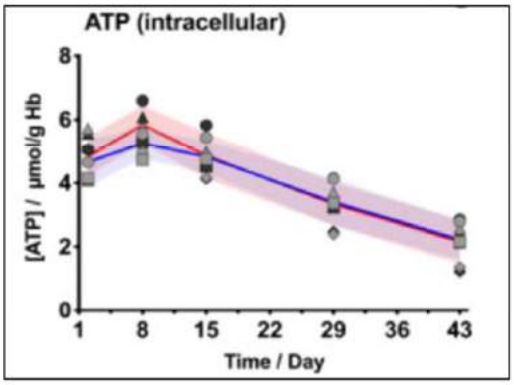


Figure 10: Changes in ATP in relation to storage time (Reproduced as permitted.Adapted from Bardyn et al 2020[14].

MORPHOMETRY

The gradual loss of ATP is associated with morphological changes in RBCs e.g. microparticles/vesicles release and eventually become spherocytes. Discocytes constitute majority of RBC population in the first week of storage. By the end of the second week, the discocyte[19] population is reduced drastically. Also, the fraction of abnormal cells is almost doubled in the second week of storage, majority of them being echinocytes and sphero-echinocytes. Degenerated RBCs and sphero-echinocytes were the majority of the cells present at 42 days of storage (~76%). Deformability of stored RBCs remains normal during the first week of storage. By the end of the second week, deformability decreases substantially and stays low for the subsequent days of storage. This means RBCs become progressively more rigid as the storage duration increases.

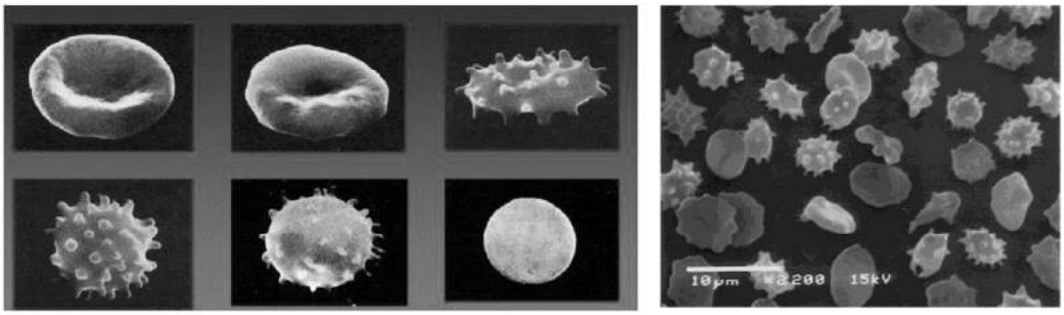


Figure 11: Influence of storage on RBC Rheological Properties (Electron microscope picture of D42 RBC shows spheroechinocytes dominating among irreversibly changed RBC), adapted from Berezina et al. 2002 [20]

As the rigidity of stored RBCs increases, the passage of these RBCs in microcirculation after they are transfused to the patient, becomes difficult. This hampers the tissue oxygenation, dampens the capillary flow thereby leading to multiorgan failure.

CBC

Storage involves a balance of multiple factors, on one hand pinching off vesicles and gradual loss of water and other intracellular ions resulting in cell shrinkage, weakening of cytoskeleton, damage to membrane and depletion of ATP causes a counter balancing swelling force. In normal RBC, MCHC is seen to be increased in older RBCs which have become denser due to increased internal viscosity.

If shrinking forces dominate, this causes an increase in MCHC, however it may flip in phases during storage and the data so far has been controversial.

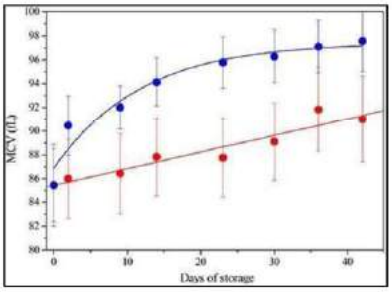


Figure 12: MCV vs Storage duration: Effect of Leukoreduction and Pre-storage Leukodepletion on RBCs, Pertinhez et al, 2016).

The presence of WBCs in the stored whole blood or PRBC as contaminant can pose several harmful effects. Leukocytes may release bio-reactive substances in the stored blood such as elastase, collagenase, cathepsin G, activated neutrophils etc. All these substances are capable of mediating biological reactions which can potentially cause deleterious results. The mechanism of injury mediated by WBCs is yet to be understood. However, some studies have shown that activation of WBCs during storage may result in the release of inflammatory agents known as DAMPs (damage associated molecular patterns)[22].

LEUKOREDUCTION

Leukocytes present in stored blood are responsible for occurrence of a plethora of problems after transfusion. The incidence of these problems such as TRALI, FNHTR

etc. is somewhat decreased by the process of leukofiltration. Leukodepletion or leukoreduction are broad terms and are sometimes used to mean a more than 3-log leukodepletion e.g., by leuko-filtration or it may sometimes be used to cover much less stringent process of Buffy Coat (BC) depletion which causes only a 1-log leukoreduction. However, the starting point of leukocyte number in a bag is so large (about 1 billion i.e. 109), even after a 3-log leukoreduction there would be almost a million leukocytes in a bag sufficient to cause many types of adverse events described above. Leukofiltration is seen to selectively spare healthy and more deformable cells which result in better post transfusion recovery. Removal of WBCs and platelets leads to provision of nutrients to RBCs from the medium for a longer period of time. It is particularly important in reference to the concentration of adenine present in the medium as it becomes a limiting factor in the later period of accepted viability. Pertinez et al, 2016[23] published that the concentration of free amino acids, along with protein content and proteases enzymes, released due to dead red cells, is higher in NLR blood as compared to LR blood. Thus, decreased protein concentration after leukoreduction can further prevent immunological reactions. Postoperative infections and mortality is significantly reduced in the patients of cardiac surgery along with prevention of transfusion associated transmission of EBV and HTLV infections.

IRRADIATION

Gamma-Irradiation (or High dose X ray) on Blood Products is done for the purpose of damaging lymphocytes enough to prevent their proliferation to prevent transfusion-associated graft-versus-host disease (TA-GVHD). In 1970, Graw et al. demonstrated that TA-GVHD from blood components could be prevented by irradiation of the component[24]. Initially it was approved in the US for 15 Gy, however after two cases of fatal TA-GVHD despite 15 Gy radiation led to revision of the US guideline to mandate at least 25 Gy irradiation at the centre of the unit and at least 15 Gy in the periphery [25]. However, gamma-irradiated RBC units demonstrate accelerated in vitro hemolysis, energy imbalance, K+ leakage and diminished post-transfusion recovery in vivo presumably through aggravated RBC storage lesions . Hillyer et al,1991 [26] was one of the pioneers in systematic study of the effects of storage on irradiated red cell units, and concluded that storage for 28 days is acceptable. US FDA also approves 28 day post irradiation storage of blood. However numerous studies done in India (Agarwal 2005, Patidar 2014) [27,28] as well as abroad [29] have shown high rates of potassium leakage that may lead to potential compromise of the safety of

the units at time points shorter than 28 days, which might need to be locally validated. Thus, while AABB (followed by US FDA and Canada) guidelines[30] allows units to be irradiated any time up to 42 days, the European guidelines require expiry by 14 days post-irradiation and no later than 28 days post collection, and British guideline goes one step even further and does not allow units older than 14 days to be irradiated in the first place. Hence the results obtained from the study we performed can potentially contribute to the quality requirement for starting safe transfusion practice for stored irradiated units like in other countries.

SENSITIVITY TO COMPLEMENT

The various changes of RBC membrane in storage, like externalisation of Phosphatidylserine causes an increase in complement activation [31]. As C3 deposition on RBCs increase with storage, their survival is hence decreased[32]. Even after leukofiltration, C3 can be detected on RBC membranes. Also, the levels of soluble MAC (membrane attack complex) increase progressively during storage. All these changes indicate that as storage duration increases, RBCs become more susceptible to damage by complement. Sucrose lysis is a very classical test in textbooks [33,34] however we did it in a scalable microplate format.

OSMOTIC FRAGILITY

Osmotic fragility refers to the fraction of RBCs hemolysed when they are subjected to a hypotonic environment. RBCs become increasingly fragile as storage duration increases. This may be attributed to build up of substances like lactate inside RBCs which make them hyperosmolar, hence more susceptible to osmotic damage[35]. These osmotically fragile RBCs show a decrease in deformability as well. A number of changes in the membrane are seen in these RBCs including increased phosphatidylserine expression and decreased membrane stomatin.[36]. These changes in osmotic fragility and deformability may affect the survival and circulation of the transfused RBCs in-vivo

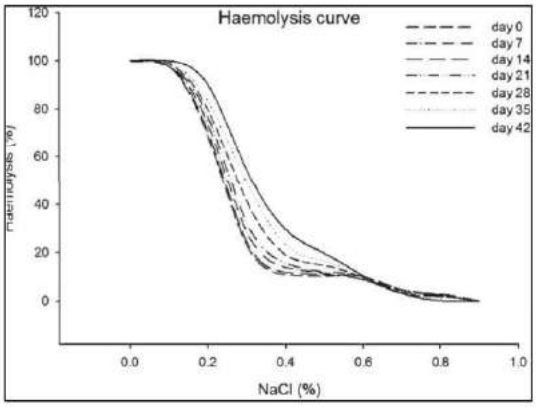


Figure 13: RBC storage and traditional Osmotic Fragility

Adapted from Blasi et al. 2012[3]. (Leftwards shifting of the curve shows higher osmotic stress before hemolysis in comparison to longer stored RBC lyse earlier event at higher concentration of NaCl i.e., rightward shifting of curves)

WASHING

During storage, blood accumulates potentially inflammatory materials for e.g., Cytokines, NTBI etc. and also substances which are harmful at high concentration for example K^+, NH_3 etc, washing involves removal of supernatant followed by resuspension in a safe medium, thus washing is capable of removing above substances or substances to which patient might be allergic to e.g. IgA in case of IgA Deficient patient, antiplatelet or HLA-antibody in case NAIT. [37]

There are several indications of washing which include hyperkalemia, hyperammonemia, anaphylaxis, IgA deficiency, Haptoglobin deficiency, Complement deficiency, allergy to blood preservatives etc. It can be used to remove DMSO from frozen cord blood or stem cells. It can also be employed when use of precious units is required at the end of their storage period.

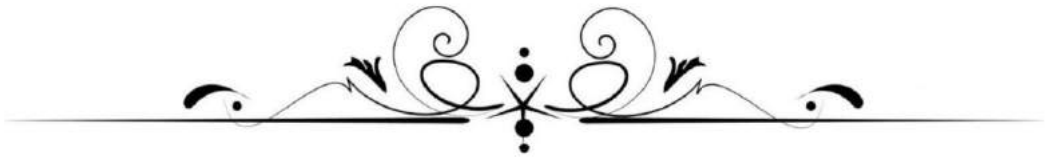
After irradiation, the blood units can be theoretically kept up to 28 days as per US FDA and Indian guidelines or up to 14 days as per UK guidelines or somewhere in between as per European guidelines however, due to irradiation induced exaggerated storage lesions Potassium and other harmful substances start accumulating as a much faster rate, which makes post irradiation storage up to such deadlines impracticable, unless there is a way to remove the supernatant without harming the product.[38–41]. These changes incurred by irradiation and usual storage lesions can be reverted by the process of washing. Washing is

one of the relatively harsh manipulations of a cellular unit which involves centrifugation, separation of supernatant, mixing with a sterile washing fluid which can be normal saline or an additive solution and repetition of the whole process (usually 3 times). A substantial percentage of leucocytes, plasma and platelets is removed. It is indicated where the recipient has undergone repeated transfusion reactions, high serum potassium levels, IgA deficiency etc[42]. The quality of a washed RBC component depends on many factors such as age of RBCs at washing, length of storage after washing, washing method (manual or automated), and the washing and storage solutions used. European guidelines limit the shelf life of use of washed RBCs to 24 hours; however, this may be extended using a "closed" system and storage in an approved additive solution.[42]

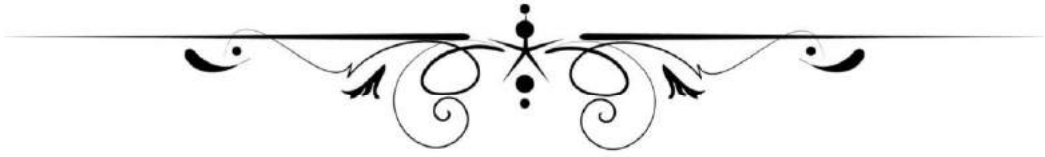
Though, washing apparently could be helpful modification. however, washing is difficult to standardize and depending on methodology of washing (manual vs automated, single vs double vs triple and gentle mixing vs vigorous) and nature of the washing and resuspension fluids (Normal saline and/or glucose solution and/or additive solution) and stage of storage (early vs late) [43] the unit could incur either significant improvement or deterioration due to the washing thus washing needs to be rigorously validated locally to ensure safety and quality.

Many permutation combinations of washing and storage solutions have been used but so far no method of washing has been globally standardised. If open system for washing is used, shelf life is limited to 24 hours but if closed system is used along with appropriate additive solution then shelf life may be increased however is subject to validation.

The potential of NTBI to cause oxidative damage calls for attention towards the time trend of its concentration in stored blood bag. It is important to define correlation of NTBI with various other storage lesions and there effect on each other to mitigate and prevent there harmful effects after transfusion. Thus, establishing a reliable method of NTBI detection and standardising a method of washing which can remove the accumulated products of storage lesions becomes a prime requirement.



REVIEW OF LITERATURE



REVIEW OF LITERATURE

Storage lesions occur due to lipid peroxidation of red cell membrane[44]. Over the storage period, 2,3-DPG decreases, nitric oxide metabolism subdues, deformability is impaired and endothelial adherence is increased. They have a miscellaneous presentation in terms of oxidative and enzymatic injuries, platelet activation, senescence, apoptosis, release and/or eventual release of waste products like particularly from leucocytes. There is pinching off of microvescicles releasing toxic substances which are known to produce inflammation, immunomodulation, and other transfusion reactions like FNHTR, TRALI, increased risk of repeated infections and mortality[4]

Several factors work parallely to affect the quality of stored RBCs such as storage temperature, visible hemolysis, closed sterile system, residual leucocyte count, and conditions of storage. Specific biochemical measurements, such as adenosine 5′-triphosphate (ATP) and 2,3-diphosphoglycerate (DPG) concentrations, intracellular calcium and potassium content or lipid breakdown products, require specialized methods that are not widely available, involve destructive testing and generally reflect only a part of the storage lesion.

Some studies have been conducted in India pertaining to assessment and quantification of the routine storage quality parameters. Mukherjee et al[45] performed serial assessment of supernatant potassium, pH, lactate, haemoglobin, glucose and red cell 2,3 diphosphoglycerate (2,3 DPG) up to 21 days of storage of 25 units each of three types of units: CPDA-1 RBC, SAGM-RBC (CPD 1° anticoagulant) and CPDA-1 whole-blood and found that biochemical changes remained within acceptable limits of safety but CPDA-1 RBCs had the highest degree of these changes. In another study from India, Chaudhary et al[2] performed serial assessment of Hb, Hct, MCV, MCHC and RDW, free Hb, plasma K+ and LDH concentrations and markers of oxidative injury such as malondialdehyde (MDA) levels, Hb-oxidation and osmotic fragility on 30 Buffy Coat depleted red cell blood units preserved in SAGM. Statistically significant ($p<0.001$) progressive increase in mean free Hb, plasma K+, LDH, MDA and Hb-oxidation during storage period of 28 days and direct correlations of MDA and Hb oxidation with free Hb concentration was observed.

Leukodepletion filters date back to 1962 wherein first generation filter was used to prevent febrile transfusion reactions[46] . Some studies have shown that leukodepletion reduces in

vitro hemolysis in blood units[47] but the literature is inconsistent in this regard. While USA still does not need universal leukoreduction, UK and Canada employed universal leukoreduction by 1999. Leukodepletion is widely used as an accepted measure to prevent febrile non hemolytic transfusion reaction (FNHTR) or to reverse transfusion related acute lung injury (TRALI), its significance in affecting benefits in classical TRALI or nosocomial infections in trauma patients is not well established[48] the reason why pulmonary system is the most commonly involved system in transfusion reactions is because pulmonary microcirculation is the first to be exposed to the mediators of storage lesions.

The blood components are irradiated with gamma rays to prevent the proliferation of lymphocytes thereby decreasing the possibility of TAGVHD. Graw et al in 1970, demonstrated that irradiation can be used to destroy lymphocytes. Earlier, USA approved for 15Gy as the limit of irradiation but after occurance of 2 fatal TAGVHD, the limit was increased to 25 Gy in the centre and at least 15 Gy in the periphery[25] as much as it is important in preventing TAGVHD, irradiation itself has dreaded demerits as it causes accelerated hemolysis in vitro, energy imbalance, K+ leakage and attenuated post transfusion recovery in vivo[49]. In a systemic study conducted by Hillyer et al,1991, effect of storage on irradiated units was observed and it was concluded that storage upto 28 days is acceptable[26]. USA FDA has also employed the same guideline. However, several Indian[27,28] and international studies have shown aggravated potassium leakage post irradiation which makes the unit potentially harmful even before the time limit of 28 days. The safety level of irradiated units needs to be locally validated. AABB allows units to be irradiated anytime up to 42 days[30], the European guidelines allow shelf life upto 14 days post irradiation[40] while the British guidelines do not allow units to be irradiated post 14th day of collection[39,50].

Storage lesions are very diverse and include various enzymatic & oxidative injury, leukocyte and platelet activation, senescence and programmed cell death, gradual accumulation of cellular waste products as well as products actively and passively released from cells, especially from leukocytes in the unit. Storage lesions also include morphologic & functional changes, hemolysis, reduced in-vivo recovery, energy and membrane loss, altered oxygen release, reduced ATP, 2-3DPG and NO levels, and shedding of micro-vesicles and other bioactive/toxic products.

For storage of RBCs, they are removed from circulation, separated from plasma and stored in an acidic medium at very low temperature which causes observable metabolic impairments.

Furthermore, acidification occurs due to exposure of RBCs to the plasticizers in the storage bag.

The primary forces leading to storage lesions are seen to stem from: i) accumulation or depletion of metabolites, which has been the concern since the beginning of blood banking and ii) oxidative damages, which have been evidently observed but are yet to be resolved systematically. Oxidized hemoglobin can thus be either reduced back to ferrous state (+II) by enzymes such as methemoglobin-reductase, or denatured/aggregated (Heinz bodies) before removal via vesiculation. The oxidative stress works more in senescent erythrocytes as compared to young erythrocytes. Consecutively, old RBCs are removed more easily from the bloodstream via phagocytosis by reticuloendothelial system macrophages in the liver and spleen[51].

24-hour recovery is an important factor to be taken care of with regards to age of RBC transfused. The ability of transfused RBC to circulate in the patient's blood does not guarantee normal oxygen delivery. Zimring et al, 2015 suggests that oxygen delivery by stored RBCs may not be optimum until they have "recovered" after a period of time circulating in the recipient[52]. This has been attributed to extreme metabolic and biochemical changes.

These products so formed during storage may cause inflammation, FNHTR, increased risk of pulmonary complications such as TRALI, endothelial dysfunction, multi-organ failure, immunomodulation, susceptibility to infections, acute liver injury and mortality[4]. The duration of RBC storage is suspected to impact both transfusion efficacy and the associated risks but studies have so far been inconclusive.

2,3-DPG and ATP

2,3-DPG is an important molecule mediating the peripheral unloading of oxygen. It is markedly decreased during storage and results in left shift in the oxyhemoglobin curve[53]. Intracellular ATP content is more slowly decreased and the apparent change is seen more prominently in blood stored for more than 5 weeks[54]. Both 2,3-DPG and ATP are classical markers of storage lesions but are not routinely used since they are rapidly replenished upon transfusion.

Functional changes during storage are potentially due to depletion of intracellular 2,3-diphosphoglycerate (DPG) and ATP over time. As 2,3-DPG is critical in the release of oxygen in vivo, it was historically postulated that older blood may not transport oxygen efficiently, although studies have failed to find consistent clinical correlation possibly due to rapid replenishment of the depleted 2,3-DPG reserve after transfusion.

ATP is absolutely necessary for a variety of important functions in RBCs, including maintenance of membrane stability and functioning of Na+-K+ ATPase pumps. As ATP levels fall over time during storage, it leads to echinocytic and subsequently spherocytic shape changes in RBCs. However, as ATP levels get replenished post transfusion, it is but only a surrogate marker of storage lesions

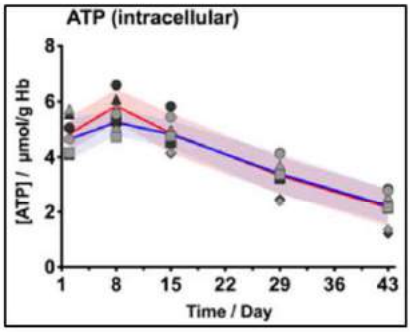


Figure 14: Changes in ATP in relation to storage time, adapted from Bardyn et al 2020[14]

LEUKOREDUCTION

Leukocytes in a collected unit of bag are not only unwanted but often considered a problem contributing to a lot of transfusion reactions and an additional range of complications starting from allergic reactions, HLA sensitization, viral transmission, TA-GVHD etc., to poorly understood increases in morbidities and mortalities. Even after current state of the art leukodepletion, e.g., by 3rd or 4th generation leukofilters or elutriation, the problems like FNHTR, allergic reactions, sensitization, viral transmission, TA-GVHD are not completely eliminated but somewhat reduced[48]. The mechanism of the effects of the leukocytes on the patient as well as on the other cells is still not fully understood. Activation of leukocytes, either slowly during storage or possibly also during manipulations e.g., filtration and irradiation, may lead to release of potentially inflammatory biologically active "damage associated molecular patterns". Leukodepletion or leukoreduction are broad terms and are sometimes mean a leukoreduction more than Log 3, e.g., by leukofiltration or it may

sometimes be used to cover much less stringent process of Buffy Coat (BC) depletion which causes only a 1-log leukoreduction. However, the starting point of leukocyte number in a bag is so large (in the order of 1 billion i.e., 109) that even after a 3-log leukoreduction there would be almost a million leukocytes in a bag sufficient to cause many types of adverse events described above.

IRRADIATION

Gamma-Irradiation (or High dose X ray) of ~25 Gy on blood components damages lymphocytes enough to effectively prevent proliferation and usually eventually kills most of them through induction of active apoptotic signaling. Irradiation is the currently approved licensed method (also recently licensed to AIIMS Jodhpur) to prevent TA-GVHD. However, gamma-irradiated RBC units demonstrate accelerated in vitro hemolysis, energy imbalance, K+ leakage and diminished posttransfusion recovery in vivo presumably through accelerated RBC storage lesions [55]

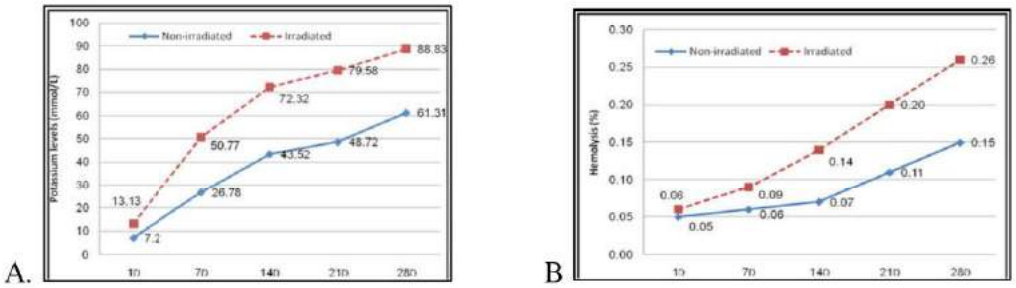


Figure 15: Comparison between changes in supernatant: A. potassium levels. B. Percentage Hemolysis in irradiated and non-irradiated blood units (Adapted from Ibrahim et al 2015[55]

BLOOD GAS ANALYSIS

Biochemical changes during storage include progressive pH drop, lactate accumulation, leakage of K^+ extracellularly, leakage of LDH, SGOT & free hemoglobin (possibly due to trace hemolysis), increase in non-transferrin Bound Iron (NTBI) etc. During storage, the main source of energy for the RBCs comes from anaerobic respiration, which leads to build up of lactate in them. Over time, even the rate of glycolysis decreases. As there is no possible way for lactate to be buffered, pH of stored blood units show a steady decrease as storage time increases.

Potassium homeostasis depends on the functioning of Na+-K+ ATPase pumps on the RBC membrane. In the cold storage temperatures, these pumps become inactive hence leading to easy passive movement of potassium ions out of the RBCs. Also during storage, cell lysis contributes to further leakage of potassium and hemoglobin from RBCs. The rate of potassium leakage in stored units is approximately 1mEq/L/day[56]. Transfusion of units having high concentration of potassium is of major concern in renal failure patients undergoing dialysis[57].

CBC

During storage there is a loss of water and ions from RBCs, causing their shrinkage in size. The overall cell volume decreases whereas the cell density increases. This leads to several changes in the RBC indices.

There is an increase in **MCV** over time during storage, while MCHC decreases. The changes in MCH values are negligible.

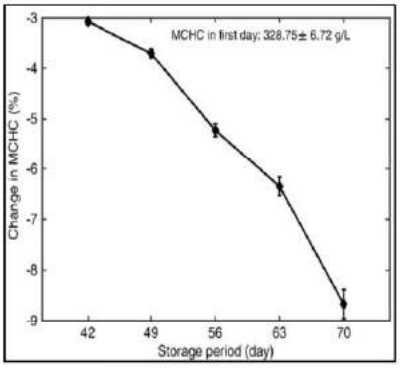


Figure 16: MCHC vs storage time

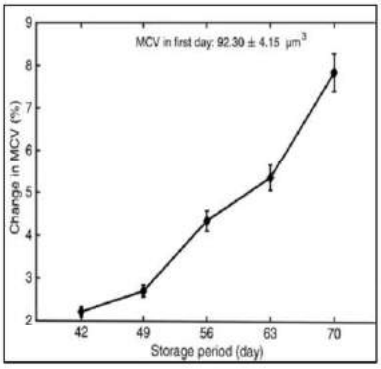


Figure 17: MCV vs storage time

(Adapted from Can et al 2018- Estimation of free Hb concentrations in blood bags[58]

MORPHOMETRY

Typical morphological changes e.g. progressive rounding, along with spinulation followed by loss of micro-vesicles, are seen with prolonged RBC storage and correlate better with ATP levels than most of the other biochemical parameters. Majority of these morphologic changes are reversed with normalization of ATP levels[48]. Importantly, these levels normalize quickly after RBC transfusion and thus are of unclear prognostic significance[3] During the first week of storage of blood units, discocytes dominate the cell population and very few or no irreversibly changed RBC can be seen. By the 14th day of the storage,

discocyte population decreases and the number of reversibly and irreversibly changed RBC increases significantly[3]. As storage period increases to 3rd and 4th weeks, the population of echinocytes and sphero-echinocytes increase drastically. By the end of 6 weeks of storage, along with echinocytes and sphero-echinocytes, there is a significant increase in the number of irreversibly changed RBCs like spherocytes. These morphological changes in RBCs can be attributed to the gradual depletion of ATP over time, which leads to their decreased deformability. Also, oxidative damage during storage leads to changes in shape and deformability of RBC membranes [20]. Hence, after transfusion of such units, there is an expected sluggish capillary flow leading to decreased tissue oxygenation and possible multi-organ failure.

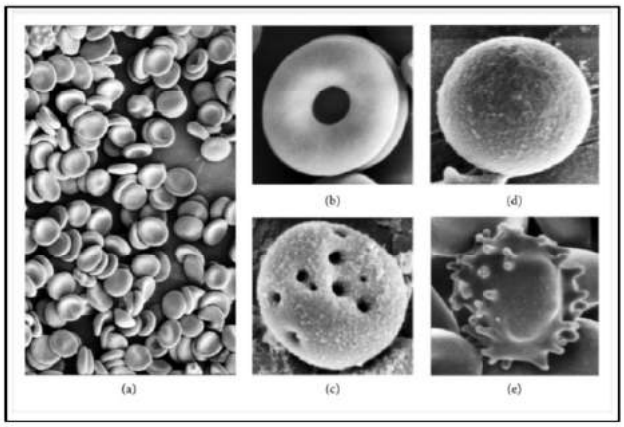


Figure 18: Scanning electron micrographs of RBC with signs of hemolysis. Image (a) shows RBCs with normal biconcave shape on day 0. Image b and c demonstrate tiny holes in the membrane of RBCs. Image d spherocytes. Image e echinocytes, day 28.

IRON

Chemical oxidation of iron in haemoglobin is the chief triggering agent for oxidative stress, the major culprit for the initiation of the storage lesion in stored blood. Reactive ferrous iron present in the heme prosthetic group of haemoglobin is present in red cells along with dissolved oxygen. Iron present in the ferrous state react with the dissolved oxygen and gets converted to ferric iron (methaemoglobin). This causes production of superoxide anion which in turn gets converted to H_2O_2 by superoxide dismutase. H_2O_2 is an important reactive oxygen species which also act as a substrate for hydroxyl radical. The superoxide so formed reduces the ferric iron in heme to form ferrous iron which reacts with H_2O_2 by the Fentons reaction to produce hydroxyl ion by the Haber-Weiss reaction[59]. H_2O_2 is also capable of

forming Ferryl hemoglobin which together with hydroxyl ion, oxidise enzymes and lipids. These effects are distinctly seen after 2 weeks of storage[51].

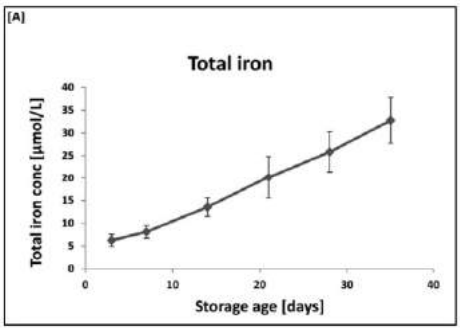


Figure 19: Graph showing time trend of Iron in stored blood supernatant. Adapted from Collard et al, 2013[60].

NTBI

Non transferrin bound iron is the fraction that comes into picture where there is iron overload in the body. In stored blood various chemical changes cause production of NTBI which causes oxidative lysis[7]. NTBI can enter cells, produce free radical species by Fenton's reaction and result in cell and organ damage[11]. Iron is a biologically important element but is equally dangerous in its property of readily undergoing oxidation reduction reactions, changing between Fe2+ and Fe 3+ thereby producing oxidative stress and tissue damaging free radicals [7]. There has been only one study regarding the correlation of iron content, NTBI concentration and age of the stored blood which was carried out on animal(mice) model and it was found that transfused RBCs are rapidly phagocytosed by macrophages, the resultant iron load is deposited in kidney, spleen and liver and also leads to production of plasma NTBI[10]. Comparisons were made among fresh blood, stored RBCs, washed stored blood, supernatant prepared from stored blood and ghosts derived from stored blood and it was noted that increased plasma NTBI was seen 2 hours after transfusion stored RBCs and washed Stored RBCs[10]

One study pertaining to NTBI has been conducted in human population which explored increased morbidities in patients of transfusion dependent states and ascribed it to increased concentration of NTBI in plasma depleted stored blood units[8]. Collard et al followed 10 pediatric bags upto day 35 of storage, sampling at every 7th day.

Various methods have been employed in determining the concentration of NTBI in blood. The most conventionally used is the bleomycin method which binds with iron, the iron-bleomycin complexes quantified by the amount of DNA cleavage products that are formed[61]. Automated methods for quantification of NTBI are being developed wherein chelators like Nitroloacetic acid are used and the result is read through an automated analyser[11,61]. The method of measurement of NTBI involves the following:

A.) a chelator that is capable of extracting iron from weak ligands such as citrate, phosphate etc-eg. Nitrilotriacetic acid, oxalate. At an alkaline pH, these chelators are not capable of extracting iron from transferrin, therefore the concentration of transferrin-bound iron will not interfere with measurement of NTBI. However, studies have shown that at higher concentrations, NTA can take away iron from these ligands and donate it to transferrin which can result in underestimation of NTBI.[62]

B.) an oxidising agent that keeps iron in ferrous state so that it may be transferred to the signal generating moiety.

C.) A signal generating moiety which when combines with iron, gives a unique signal which could be read and concentration of NTBI van be determined.

Table 2: Comparison of the different methods of NTBI measurement.

S no	Year	Author	Method	Type of sample	Volume of sample	Sensitivity	Calibrator
1	1990	Singh et al[63]	HPLC	Serum	900uL (20uL ultrafiltrate)		Iron NTA solution linear curve
2	1994	Evans et al[64]	Bleomycin Assay	Serum	50 uL	0.5-5mM	Fe AAS solution
3	1999	Gosriwatana et al[65]	-HPLC -Colorimetry -ICP-AES	Human serum	900uL	-0.47 uM -0.11 uM -0.1uM/	FeCl3
4	2001	Breuer et al[66]	Fluorescence Assay	Serum/ plasma	10uL	0.3uM	Ferrous ammonium sulphate-NTA
5	2002	Marwah et al[8]	Bleomycin assay	Human/ Canine blood (stored-42 days)	20uL	0.3uM	FeCl3 (AAS)
6	2009	Kolb et al[67]	HPLC	Human serum		0.2	Ferrous ammonium sulphate+ stripped serum
6	2010	Prezelj et al[61]	Automated (colorimetry)	Serum	900uL	0.076uL	Ferrous ammonium sulphate
7	2014	Ito et al[68]	Automated analyser	Serum	15uL	0.34	Ferric citrate
8	2013	Collard[60]	HPLC	Serum	250uL ultrafiltrate		
9	2018	Blaine[12]	Aferrix (kinetic fluorescence)	Serum (Stored-42 days)	20uL	0.2 uM	Internal Controls and Standard
10	2012	Nivedita Gohil[69]	Azotobactin (Fluoroscopy)	Serum	450uL	0.3uM	Standard Iron solution

HPLC: This method, however considered to be very sensitive, cannot be established in resource limited laboratories. This assay utilises the three main drawbacks of this method are its cost, its cumbersome nature, which makes it difficult to set up in non-specialized laboratories, and its relatively low through put efficiency.

Bleomycin Assay: A second method Evans, P.J. and Halliwell, B. (1994)[64] employs the antibiotic bleomycin, which combines with NTBI, but not with transferrin-bound iron, to form highly reactive complexes which generate DNA cleavage products. The relative amount of DNA cleavage products is proportional to the amount of input NTBI and is quantified by the thiobarbituric acid test. The drawback of this method is that it tends to overestimate NTBI and may give false positive results.

Commercial Spectrofluorometric NTBI assay Kit (Aferrix): This commercial kit from Tel Aviv Israel has its own controls and calibrator and is claimed to be highly sensitive, but the cost of the kit is $1400 and it allows only 44 samples to be tested per kit[8].

WASHING

Storage of packed red blood cells causes accumulation of potentially harmful substances eg capable of causing adverse reactions in transfusion recipients. Washing is the method implicated in facilitating removal of these harmful substances. However, till now, no single method has been fully standardised to be used internationally for washing.

Attempts have been made to validate washing in various aspects comparing:

1. Different automated cell washers
2. Automated vs manual washing
3. Different wash solutions for washing

Studies have been conducted on PRBC units with or without irradiation and measured Potassium levels over 24 hours post wash and it has been found that potassium levels increased in both non-irradiated and irradiated units with a greater increase in the latter (Weiskopf, 2005).

Weisbach et al washed pre storage leukodepleted units with different types of wash solutions (saline, SAG-M or 5% albumin). A number of parameters such as ATP, pH, lactate, hemolysis, were followed up for 6 hrs post wash period. The study concluded that SAGM was the best washing and resuspension medium.

Washing is traditionally done with normal saline; however, NS containers require spiking to connect a blood unit which makes the process open and shortens the post washing storage to 24 hours even at 4^{O} C due to risk of bacterial overgrowth after the "open" handling. Even if, the system is not made open normal saline does not contain any nutrient or source of ATP to reduce Potassium leakage thus an alternative to normal saline could be glucose containing solutions or additive solutions which at the advantage of" closed "system handling and longer post-wash storage.

Thalassemia patients are recommended to receive fresh blood units not older than 2 weeks[70] however, the mechanism of the damage due to older units is still controversial some publications suggest the washing of older units could reduce the NTBI load from the supernatant which is a principle worth testing to make precious blood units (Antigen phenotyped) available for longer storage periods[43,60].

Washing is also done with a series of hyperosmolar solutions with decreasing osmolarity for the purpose of thawing and de-glycerolization[71] for frozen red cell units, however, this involves large number of washes, usually requires automated cell washers which are not available in most centres in India. However, manual washing is possible but rarely used in India, which could be utilized for the rest of the indications above.

Our study included a series of washing validation studies including saline wash, SAGM wash, Double wash, closed system SAGM wash etc. we explored closed system SAGM wash as a method for salvaging units for long term post-irradiation storage.

Our novel fully automated NTBI assay was used to explore whether washing could be used to reduce NTBI in stored and/or post irradiation supernatant as potential method for making older units safer for transfusion.

Table 3: Comparison Table of Red Cell Washing.

S no	Year	Author	Sample size	Method (manual Vs automated)	Type of washing fluid	Type of sample (species)	Post-wash follow up	Remarks (conclusion)
1.	2018	Proffitt et al[42]	Non-irradiated = 5 Irradiated = 3	Manual + Automated (ACP 215)	Saline Glucose SAGM	human	Day-5,7,11,14	SAGM> SALINE
2.	2015	Proffitt and cardigan et al [72]	48 Units WB (3 units)	Automated ACP 215	SAGM AS-7	Human	Day-16,21,28,35,42,56	AS-7>SAGM As storage media following washing
3.	2011	O'leary et al[73]	24 units	Automated - CATS, COBE	Normal saline	Human	Hours-0,4,6, 12,24	1.**At 24 hours-** K^+- prewash<post wash 2.**At 12 hrs-** Significant difference between K values of different devices. 3.**HI-** increased immediately after wash on all devices


AIM & OBJECTIVES

AIM AND OBJECTIVES

AIM:

1. Correlation of Iron Profile including **NTBI** in Irradiated and Non-Irradiated PRBC units, with other Storage Lesion markers and effect of **Leukoreduction** and/or **Washing** on the above.

OBJECTIVES:

1. To measure Iron profile including NTBI, and storage lesion markers i.e., ATP, free Hb, CBC, VBG, LDH, Total Protein, AST, Bilirubin, Morphometry, Osmotic Fragility, Complement Fixation potential in Irradiated and Non-Irradiated PRBC units.
2. To explore correlations between the measured parameters and test whether Leukoreduction and/or Washing can significantly reverse storage lesions in Irradiated and Non-Irradiated units.

MATERIALS AND METHODS

MATERIALS AND METHODS

STUDY SETTING

It was an ex-vivo observational study involving parameters from samples obtained by sterile blood bank techniques from stored blood units. The modifications, the units underwent in our blood bank were routine blood bank techniques permitted by regulatory authorities all over the world, including Drug Controller General of India (e.g., Component separation, buffy coat depletion, leukofiltration, irradiation). The materials used for this study (leukofilters, irradiator, blood bags, sterile connecting device) were all licensed products. The effects of these interventions on the quality and storage lesion indicators were followed up rigorously.

Intervention and observation of blood bags and samples were carried out in the Department of Transfusion medicine and Blood bank, in collaboration with Department of Pathology, Department of Biochemistry, and Department of Anaesthesiology, AIIMS, Jodhpur.

STUDY DESIGN

It was a prospective /longitudinal study of iron profile including NTBI along with other markers of storage lesion in red blood cell units receiving various modifications such as leukoreduction, irradiation and washing.

METHOD

- Fifty-six donors of common blood groups (Rh+), such that there was no shortage of supply and no lack of demand, were requested for informed consent.

- After informed consent and transfusion transmitted infection (TTI) screening, donated CPD+SAGM Buffy Coat reduced Packed Red Blood Cell (PRBC) units were made from 450ml bags. Then about 50ml of the product was one time transferred to a transfer bag using licensed sterile device. The primary bags were released back to blood bank pool, while the aliquot bags were subjected to serial sampling and invitro testing and were not released to be issued for patients, thus involving no safety concerns.

- These aliquots were allocated randomly into two major groups as:

1. Group 1: Non-Leukoreduced (NLR Group)
2. Group 2: Leukoreduced (LR Group)

1. Group 1: NLR (Non-Leukoreduced):

Twenty-eight aliquots did not undergo leukoreduction and were monitored in subgroups of 7 upto week 4 as follows:

a. NLR-N (7 aliquots with No further modifications)

b. NLR-W (7 aliquots with Washing on Day 14 of storage)

c. NLR-WW (7 aliquots with early washing on Day 14 & late Washing on Day 28 of storage)

d. NLR-IW (7 aliquots with early Irradiation on Day 14 & late Washing on Day 28 of storage)

2. Group2: LR (Leukoreduced):

Twenty-eight split units underwent leukoreduction and were monitored in subgroups of 7 upto week 4 as follows:

a. LR-N (7 aliquots with No further modifications)

b. LR-W (7 aliquots with Washing on Day 14 of storage)

c. LR-WW ((7 aliquots with early washing on Day 14 & late Washing on Day 28 of storage)

d. LR-IW (7 aliquots with early Irradiation on Day 14 & late Washing on Day 28 of storage)

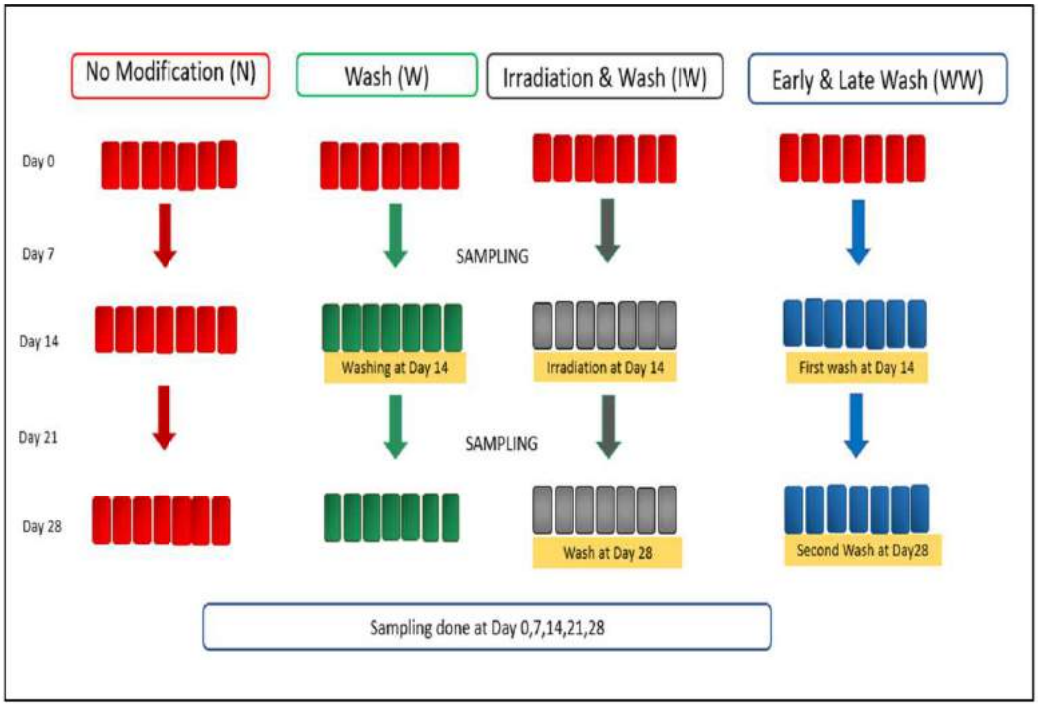


Figure 20: **Scheme of sampling**; Thesis workflow for both LR & NLR groups.

- Bags were sampled on a fixed convenient day of the week on a weekly basis and an additional sample was taken after any major manipulation (leuko-filtration or irradiation or washing).
- All samplings of bags were done through integrally connected tubing using a sterile technique and bags will be hermetically resealed.
- The following Quality parameters were tested serially on each bag at defined time points (weekly and additionally 4 hours after any modification)- CBC, free Hb, RBC morphology, LFT (total protein, AST, Bilirubin), LDH, VBG, Lactate, Osmotic Fragility Test or its equivalent (Pink Test), Sucrose Lysis Test and Iron profile and NTBI.

In all the bags subjected to early washing, blood culture was sent at the end of storage.Washing was done by sterile closed technique using SAGM (Saline Adenine Glucose Mannitol).

The storage quality parameters mentioned above were tested and assessed in the Department of Transfusion Medicine and Blood Bank, Pathology, Biochemistry and Anesthesiology.

STUDY PARTICIPANTS

Donated Blood Bags from 56 adult donors between the age of 18-65 years with common blood groups (Rh +) visiting Department of Transfusion Medicine and Blood Bank, AIIMS, Jodhpur were selected.

INCLUSION CRITERIA

450 ml quadruple blood bags from adult (18-65yr) male or female donors with common blood groups (Rh+) weighing >55Kg.

EXCLUSION CRITERIA

- Bags that do not match the criteria for assessment
- Donors who deny consent
- Donors who test reactive for TTI screen.

SAMPLE SIZE

Total of 56 donors subdivided into two groups (LR&NLR) of 28 each. After splitting of units for further modification and assessment, the total subgroups are 4, with 7 units in each which makes a total of 56 units to be followed.

Total samples taken from bag during storage: 5-7 samples were taken from each bag, including the baseline sample for untreated whole blood sample.

STUDY DURATION

The study was completed in 1 year.

MODIFICATIONS:

1. **Irradiation**:

The blood units were irradiated using BI 2000 which is a Ceasium- 137 based Gamma Irradiator. The irradiator we have is manufactured and quality checked by BRIT (Board of Radiation and Isotope technology) and recently licensed in 2017 by AERB (Atomic Energy Regulatory Board) both of which are under Department of Atomic Energy, Govt of India. The Indian Guidelines (National Blood Transfusion Council) allow a minimum dose of 25 Gy and storage period of 28 days post irradiation or original expiration, whichever is earlier.

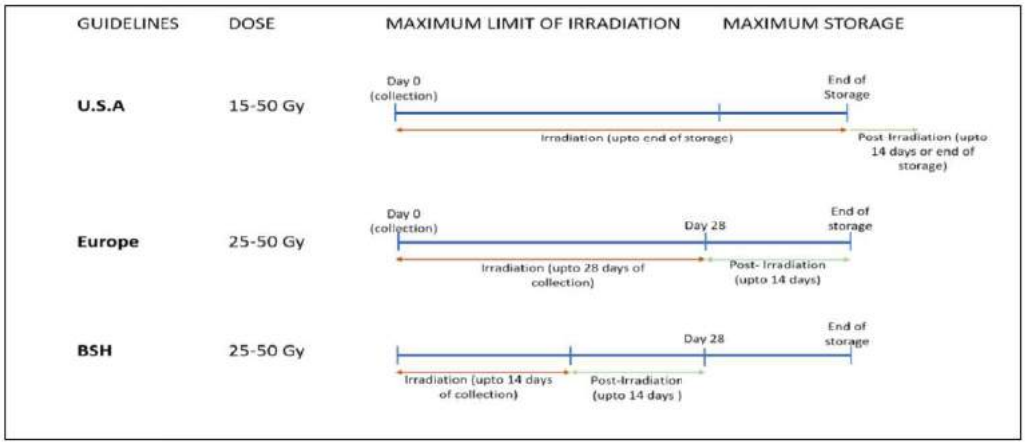


Figure 21: Comparison of Guidelines for irradiation and post irradiation storage period.

The Irradiator should be monitored routinely to make sure that the dose delivered to Blood bags during irradiation is adequate. This dose mapping is done using irradiation-sensitive films or badges for quality control. Therefore, we used RadTag stickers on our bags to ensure adequate irradiation. Such irradiation sensitive labels are necessary in Europe while the US-FDA only requires annual monitoring or after major repairs or after relocation of irradiator

Before Irradiation After Irradiation

Figure 22: RadTag® sticker, before and after irradiation of unit

2. Leucofiltration:

Leucofiltration was done for PRBC bags recruited in Group 2 on the day of collection after component separation. BioR Flex leucofilter bags from Fresenius kabi were used.

Figure 23: Leucofilter Bag (Fresinius Kabi)

1. Washing:

- Usually, institutional protocols are used for washing which are standardised according to the need and availability of resources in the concerned blood centre, therefore we also standardised the same for our centre.

- First of all, a trial of washing was run using different combinations of wash and resuspension solutions to assess which one washed better and caused less insult to the RBCs during storage.
- The following three combinations were used:
    - Washed and resuspended in NS
    - Washed in NS and resuspended in SAGM
    - Washed and resuspended in SAGM
- Two units 25-day old, were taken for the trial
- Three 50 mL aliquot was taken from each unit and subjected to the above washing protocols
- For washing, first a spin of 3200 rpm for 10 mins was given, supernatant was removed and volume of supernatant noted
- Then equal volume of wash solution was added, mixed by inverting the bag 3 timesThe bags were spun at the same speed and time again and supernatant was removed
- After supernatant removal, amount of resuspension medium equal to the amount of first supernatant removed, was added and again mixed by inverting the bag 3 times
- After washing, serial sampling was done on Day 0 (the day of washing), 1, 2, 4, 7, 10, 14 for CBC, VBG (pH, Potassium), and supernatant preserved for free Hb.
- The experiment yielded that SAGM & SAGM wash caused least amount of potassium leak and hemolyis in comparison to the other two combinations
- Therefore, washing of the bags recruited for thesis was done using SAGM & SAGM combination.
- For number of washes, we collected SOPs from different blood centres and standardised a two round wash for our study.

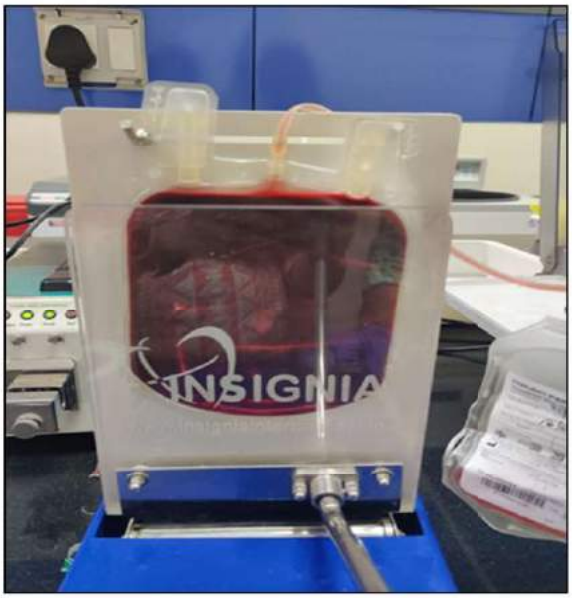


Figure 24: Supernatant removal after washing using manual extractor.

QUALITY CONTROL TESTS:

1. Complete Blood Count:

Using Sysmex XP100, which is a 3-part differential automated hematology analyzer in the Department of Transfusion Medicine, a complete blood count was run to evaluate hemoglobin, hematocrit, cell counts, MCV, MCHC and other related hematological parameters.

2. Blood Gas Analysis:

In the Department of Anesthesiology, blood gas analysis was performed using Cobas Roche b221 to test the pH, potassium, glucose and lactate levels of the samples. In order to calculate electrolytes, the electrolyte module of Cobas has potentiometric electrodes and a reference electrode composed of chloride. In potassium and calcium membranes, neutral carriers are present. Special glasses are used as sensors. Glucose and lactate were assessed by the COOX (co-oxygenator) module. Sufficient dilution of the samples was carried out, measurements were taken and the final value was determined according to the dilution if the values of any of the parameters were not linear.

3. Liver Function Test, LDH, Iron Profile:

Using Beckman Coulter AU-480, the LDH, Iron Profile and LFT parameters were assessed.

4. ATP:

250 uL of PRBC sample was mixed with 250 uL of Trichloroacetic acid (TCA), incubated at 4°C for 5 minutes. The mixture was then centrifuged at 1000g for 5 minutes. The lysate of the samples so obtained, was kept at -80°C. ATP in the lysate was measured using DiaSys ATP Hexokinase FS kit by an AU-480 Beckmann Coulter machine, photometrically, in batch mode.

5. Osmotic fragility:

- Osmotic fragility was determined using Pink test [74](Vettore 1984)\
- The testing solution was prepared by mixing TrisBris powder, Glycerol and Sodium chloride powder in deionized distilled water (DDW) to obtain an osmolarity of 290+/-5 mOsmol/L
- pH was adjusted between 6.6 and 6.7 using hydrochloric acid
- 10 ul of sample was added to 3ml of the testing solution and incubated for 45 minutes at room temperature (20-15°C)
- After incubation, the contents of the test tube were spun in Eppendorf tube centrifuge at 1000 rotations per minute (rpm) for 5 minutes, after which 400 ul of supernatant was separated in Eppendorf tubes and stored at -80°C for future run in batch mode.
- The EON microplate reader in the Department of Biochemistry, AIIMS Jodhpur was used to take photometric readings and the percentage hemolysis was calculated.

6. SLT (Sucrose lysis test):

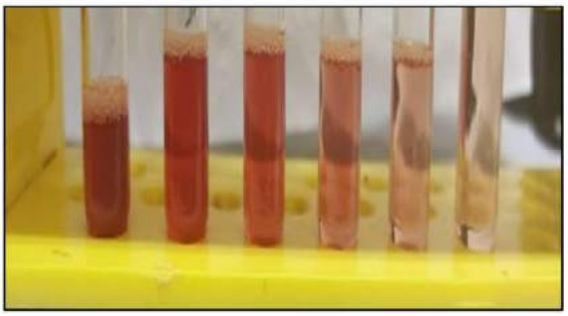
- 50ul of fresh serum of the AB blood group was mixed in a test tube with 900 ul of sucrose solution (92.4g/l)
- 50 ul of the 80% red cell suspension to be tested was mixed in the test tube. At 37°C for 30 minutes, the tube was then incubated.
- After incubation, the contents of the test tube were spun in Eppendorf tube centrifuge at 1000 rotations per minute (rpm) for 5 minutes, after which 400 ul of supernatant was separated in Eppendorf tubes and stored at -80°C for future run in batch mode.
- In the EON microplate reader in the Department of Biochemistry, AIIMS Jodhpur, the photometric readings were taken and percentage hemolysis was calculated.

7. Morphology:

Using Leishman stain, blood smears were prepared to study the morphology of stored red blood cells.

8. Free Hb:

The supernatants separated from samples were read with the EON microplate reader in the Department of Biochemistry, AIIMS Jodhpur and the quantitative hemoglobin calculation was performed by micro-plate spectrophotometric method [75](Harboe Method).



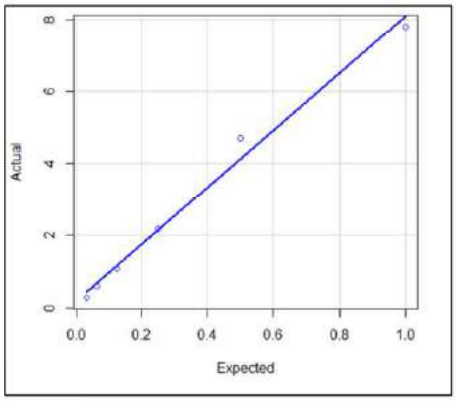


Figure 25: Serial dilutions for Hb estimation & Calibration verification for hemoglobin (g/dL)

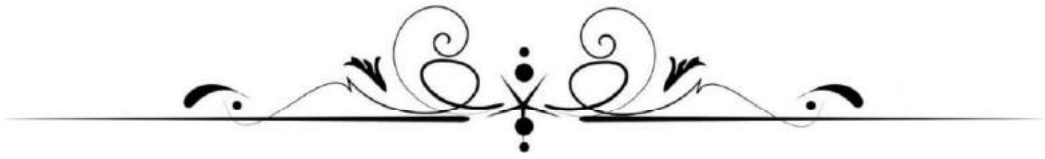
9. NTBI (Non-Transferrin Bound Iron):

NTBI was measured using a method adapted from Ikuta et al. The original method was performed on Hitachi 7700 biochemistry autoanalyzer, so we modified the method to match the technical specifications of Beckman Coulter AU 480 present in our Biochemistry Laboratory. Reagents were prepared in our laboratory.

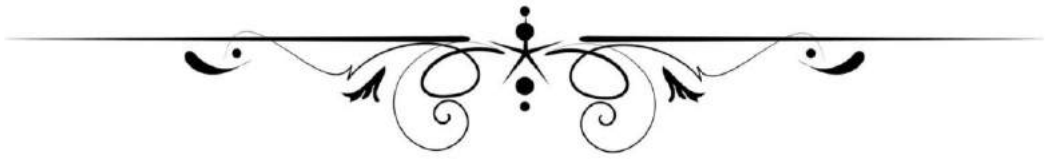
R1 was prepared with Tris buffer, Triton X and Nitrilotriacetic acid (NTA). NTA is a chelator that extracts iron from weak ligands such as citrate, phosphate, albumin etc and transfers it to the signal producing moiety.

R2 was prepared using Tris buffer, triton X, ascorbic acid and Nitroso PSAP which is a chromogen and gives an absorption peak of 750nm with Iron. Ascorbic acid helps to keep iron in ferrous state but the reagent is stable only for a narrow range of 4-5 days as ascorbic acid gets oxidised and hinders with the signal.

Figure 26: Beckman Coulter AU-480, AU-680, AU-5800 Biochemistry Auto Analyzer



RESULTS



RESULTS

DONOR DEMOGRAPHIC SECTION

IEC approved informed consents for this project was collected from 56 male donors whose details are summarised below.

Table 4: Descriptive Statistics of donor demographics (Overall)

	N	Range	Minimum	Maximum	Mean	Std. Deviation
Donor age(yrs.)	56	32	18	50	27.32	7.107
Donor body Wt.(kg)	56	55	52	107	74.64	10.793

Table 5: Independent Sample t-Test between LR and NLR groups for donor demographic parameters

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Donor age (yrs.)	Equal variances assumed	3.925	.053	.186	54	.853	.357	1.916	-3.485	4.199
	Equal variances not assumed			.186	46.507	.853	.357	1.916	-3.499	4.213
Donor body Wt.(kg)	Equal variances assumed	1.012	.319	.098	54	.922	.286	2.911	-5.550	6.122
	Equal variances not assumed			.098	53.590	.922	.286	2.911	-5.551	6.123

The above table shows that the LR and NLR groups have no significant allocation bias regarding age and weight between the groups.

Table 6: Descriptive Statistics of donor demographics (Group)

Group		N	Mean	Std. Deviation	Std. Error Mean
Donor age(yrs.)	LR	28	27.50	5.548	1.048
	NLR	28	27.14	8.488	1.604
Donor body Wt.(kg)	LR	28	74.79	10.404	1.966
	NLR	28	74.50	11.358	2.146

The above table shows the distribution of donor demographics for the two main groups.

Table 7: Distribution of samples in subgroups across the study period (4 weeks)

		Time Series (conc)				
		I_W	N	W	W_W	Total
Day	Day 00	7	7	7	7	28
	Day 07	7	7	7	7	28
	Day 14	7	7	7	7	28
	Day 14.5	7	0	7	7	21
	Day 21	7	7	7	7	28
	Day 28	7	7	7	7	28
	Day 28.5	7	0	0	7	14
Total		49	35	42	49	175

The above table shows the distribution of numbers for all the timed samples in the NLR group.

Table 8: Distribution of samples in subgroups across the study period (4 weeks)

		Time Series (conc)				
		I_W	N	W	W_W	Total
Day	Day 00	7	7	7	7	28
	Day 00.5	7	7	7	7	28
	Day 07	7	7	7	7	28
	Day 14	7	7	7	7	28
	Day 14.5	7	0	7	7	21
	Day 21	7	7	7	7	28
	Day 28	7	7	7	7	28
	Day 28.5	7	0	0	7	14
Total		56	42	49	56	203

The above table shows the distribution of numbers for all the timed samples in the LR group. In this group there is an extra time of denoted day 00.5 which is the post leukoreduction sample.

As described in the Methods section, a number of storage lesion parameters and iron related parameters were measured in these samples whose t-test results are given in the below table.

Table 9: Independent Sample t-Test between LR and NLR groups for the measured parameters

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Diff.	Std. Error Diff.	95% Confidence Interval of the Difference	
									Lower	Upper
WBC (10^3 / µl)	Equal variances assumed	5.409	.021	8.798	403	.000	1.686	0.192	1.310	2.063
	Equal variances not assumed			8.738	375.566	.000	1.686	0.193	1.307	2.066
HGB (g/dl)	Equal variances assumed	7.269	.007	-6.821	403	.000	-1.088	0.160	-1.402	-0.774
	Equal variances not assumed			-6.510	285.845	.000	-1.088	0.167	-1.417	-0.759
RBC (10^6 /µl)	Equal variances assumed	.726	.395	-1.994	403	.047	-0.119	0.060	-0.237	-0.002
	Equal variances not assumed			-1.964	358.983	.050	-0.119	0.061	-0.239	0.000
HCT(%)	Equal variances assumed	4.709	.031	-4.214	403	.000	-1.449	0.344	-2.125	-0.773
	Equal variances not assumed			-4.111	337.637	.000	-1.449	0.352	-2.142	-0.756
MCV (fL)	Equal variances assumed	2.555	.111	.932	403	.352	3.809	4.086	-4.223	11.842
	Equal variances not assumed			.843	183.189	.400	3.809	4.517	-5.103	12.722
MCH (pg)	Equal variances assumed	2.291	.131	-4.437	403	.000	-0.981	0.221	-1.416	-0.547
	Equal variances not assumed			-4.391	368.436	.000	-0.981	0.224	-1.421	-0.542
MCHC (g/dl)	Equal variances assumed	2.664	.103	-8.824	403	.000	-0.928	0.105	-1.134	-0.721

	Equal variances not assumed			-8.683	357.018	.000	-0.928	0.107	-1.138	-0.718
PLT (10^3 /µl)	Equal variances assumed	2.319	.129	11.434	401	.000	39.316	3.439	32.556	46.076
	Equal variances not assumed			11.615	400.044	.000	39.316	3.385	32.662	45.971
RDW -SD (fL)	Equal variances assumed	2.591	.108	.253	402	.801	0.094	0.370	-0.634	0.822
	Equal variances not assumed			.248	345.786	.805	0.094	0.378	-0.650	0.837
RDW -CV (fL)	Equal variances assumed	8.267	.004	2.459	402	.014	0.274	0.112	0.055	0.494
	Equal variances not assumed			2.374	310.553	.018	0.274	0.116	0.047	0.502
pH	Equal variances assumed	.792	.374	-.344	404	.731	-0.007	0.021	-0.049	0.034
	Equal variances not assumed			-.341	371.400	.733	-0.007	0.021	-0.049	0.035
K+	Equal variances assumed	.222	.638	1.167	404	.244	2.333	2.000	-1.598	6.263
	Equal variances not assumed			1.171	392.232	.242	2.333	1.992	-1.584	6.249
Glucose	Equal variances assumed	10.446	.001	8.457	404	.000	95.042	11.238	72.949	117.134
	Equal variances not assumed			8.681	403.026	.000	95.042	10.948	73.520	116.564
final Lac	Equal variances assumed	.312	.577	1.576	404	.116	1.600	1.015	-0.396	3.596
	Equal variances not assumed			1.580	390.886	.115	1.600	1.013	-0.391	3.591
LDH(U/L)	Equal variances assumed	36.192	.000	4.665	404	.000	200.385	42.958	115.935	284.834

	Equal variances not assumed			4.322	226.081	.000	200.385	46.363	109.026	291.744
TOTAL BILIRUBIN (mg/dL)	Equal variances assumed	2.533	.112	-6.409	404	.000	-0.052	0.008	-0.068	-0.036
	Equal variances not assumed			-6.420	389.703	.000	-0.052	0.008	-0.068	-0.036
TOTAL PROTEIN (g/L)	Equal variances assumed	6.598	.011	-2.606	404	.009	-0.098	0.037	-0.171	-0.024
	Equal variances not assumed			-2.551	346.395	.011	-0.098	0.038	-0.173	-0.022
AST (U/L)	Equal variances assumed	59.341	.000	5.994	404	.000	6.270	1.046	4.214	8.327
	Equal variances not assumed			5.625	250.944	.000	6.270	1.115	4.075	8.466
TIBC (ug/L)	Equal variances assumed	.688	.407	2.955	404	.003	8.987	3.041	3.008	14.965
	Equal variances not assumed			2.949	383.887	.003	8.987	3.048	2.995	14.979
UIBC	Equal variances assumed	11.414	.001	5.679	404	.000	13.909	2.449	9.094	18.724
	Equal variances not assumed			5.770	402.834	.000	13.909	2.411	9.170	18.648
IRON (ug/dL)	Equal variances assumed	15.154	.000	-3.223	404	.001	-4.922	1.527	-7.924	-1.920
	Equal variances not assumed			-3.311	402.679	.001	-4.922	1.486	-7.844	-2.000
Transferrin Saturation	Equal variances assumed	24.285	.000	-2.355	404	.019	-31.509	13.379	-57.810	-5.208
	Equal variances not assumed			-2.528	317.949	.012	-31.509	12.465	-56.034	-6.985
ATP(umol/g Hb)	Equal variances assumed	3.506	.062	1.563	403	.119	1.970	1.260	-0.507	4.447

	Equal variances not assumed			1.412	181.257	.160	1.970	1.395	-0.783	4.722
Free Hb	Equal variances assumed	17.781	.000	-4.007	404	.000	-0.377	0.094	-0.562	-0.192
	Equal variances not assumed			-4.133	400.294	.000	-0.377	0.091	-0.557	-0.198
Pink %	Equal variances assumed	20.782	.000	2.634	403	.009	1.581	0.600	0.401	2.761
	Equal variances not assumed			2.566	333.984	.011	1.581	0.616	0.369	2.793
SLTx	Equal variances assumed	59.648	.000	3.308	403	.001	1.252	0.378	0.508	1.996
	Equal variances not assumed			3.141	273.620	.002	1.252	0.399	0.467	2.037
NTBI	Equal variances assumed	6.156	.014	-1.328	230	.185	-0.515	0.388	-1.280	0.249
	Equal variances not assumed			-1.369	228.296	.172	-0.515	0.377	-1.258	0.227

The parameters above are analysed in further detail along with time profile graphs below. Kindly refer to the discussion section for interpretation of all of the graphs below.

IRON RELATED PARAMETERS

NTBI

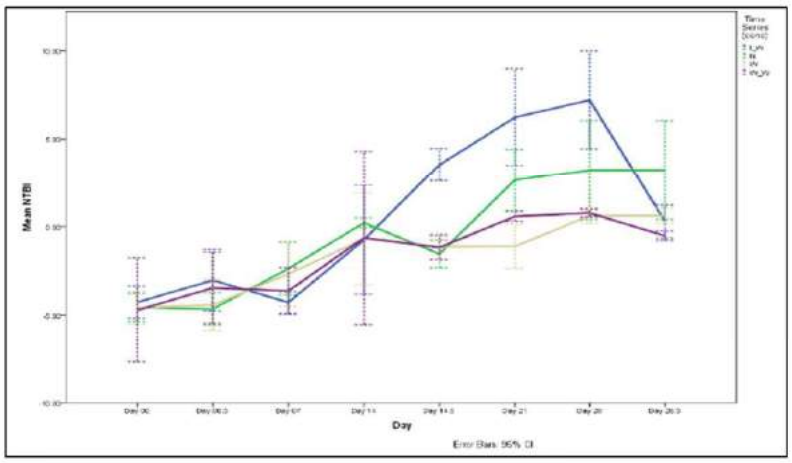


Figure 27: Storage time trend of NTBI in Leukoreduced bags.

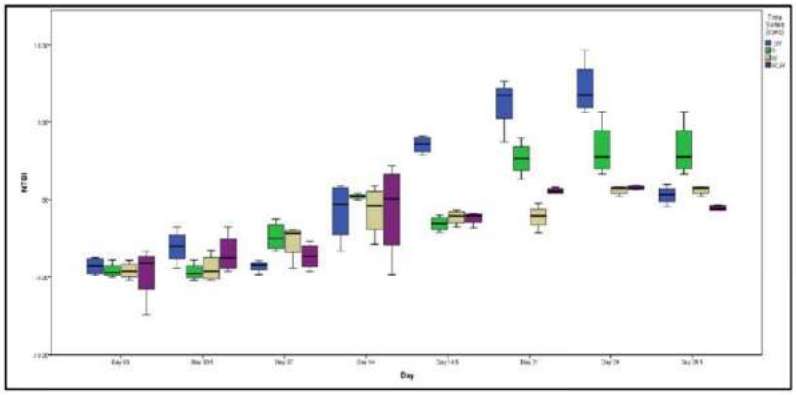


Figure 28: Storage time trend of NTBI in Leukoreduced bags (BOX PLOT).

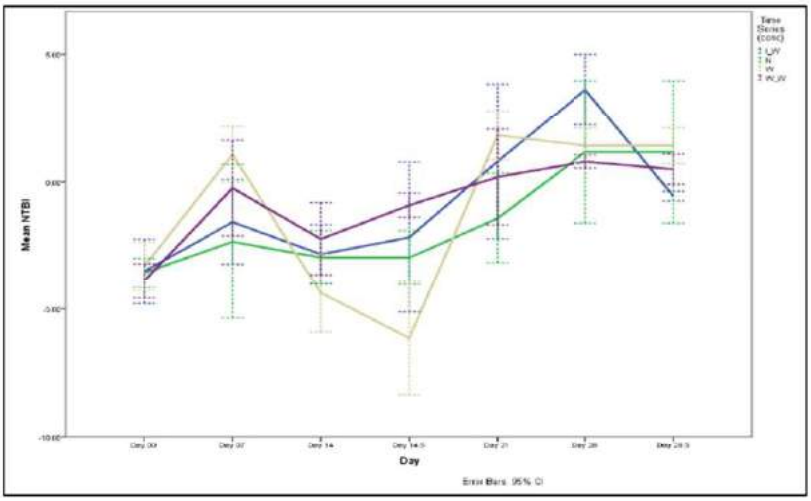


Figure 29: Storage time trend of NTBI in Non-Leukoreduced bag

UIBC

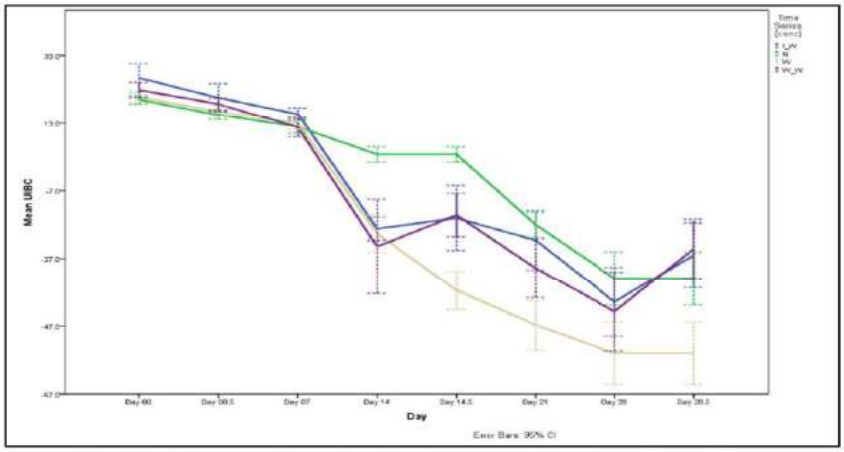


Fig.30: Storage time trend of UIBC in Leukoreduced bags.

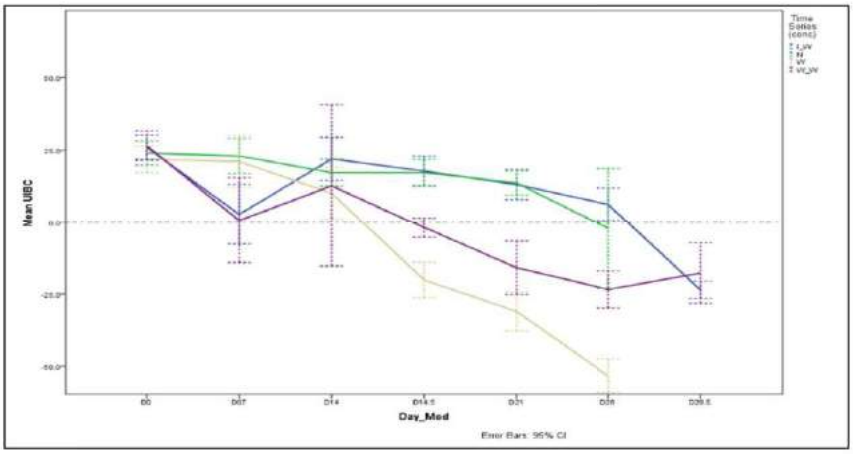


Fig.31: Storage time trend of UIBC in Non-Leukoreduced bags.

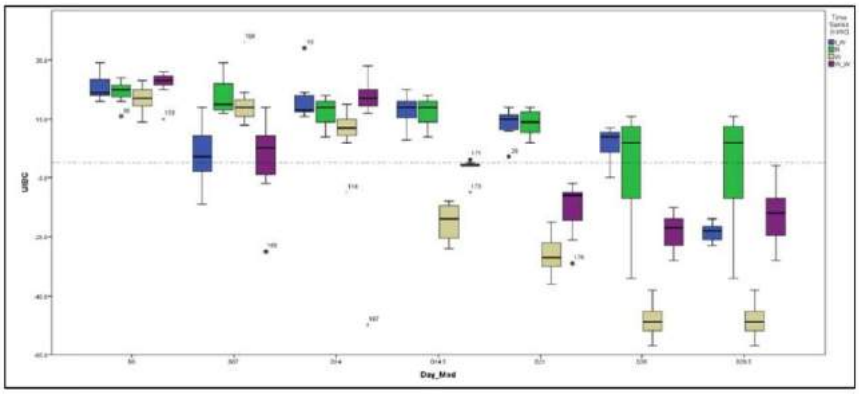


Figure.32: Storage time trend of UIBC in Non-Leukoreduced bags.(Box plot)

TIBC

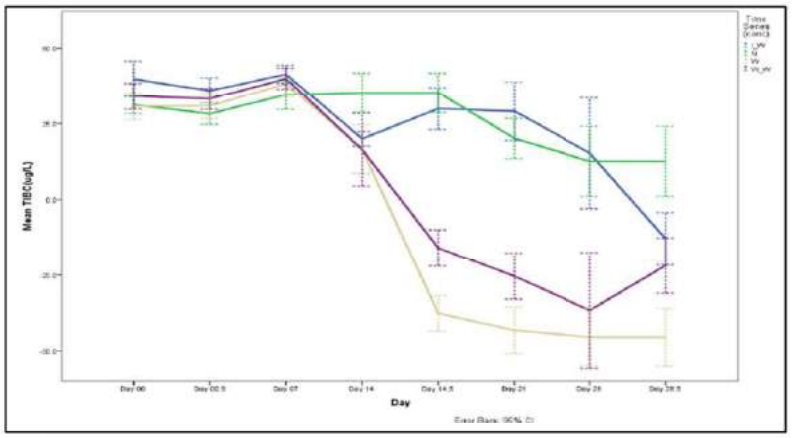


Fig.33: Storage time trend of TIBC in Leukoreduced bags.

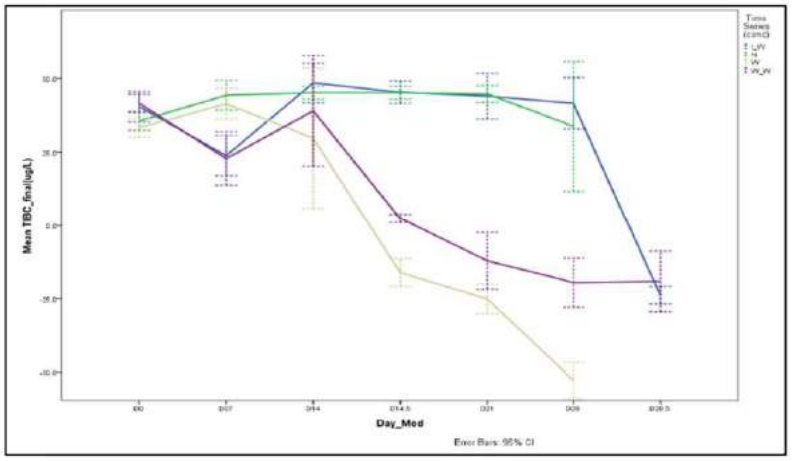


Fig.34: Storage time trend of TIBC in Non-Leukoreduced bags.

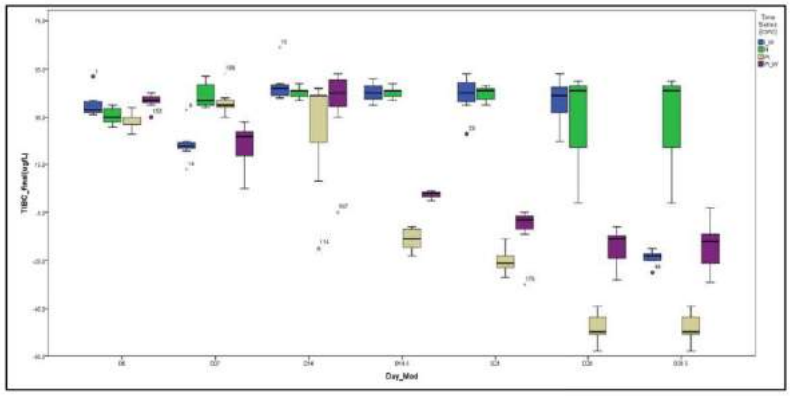


Figure.35: Storage time trend of TIBC in non Leukoreduced bags.(Box Plot)

TRANSFERRIN SATURATION

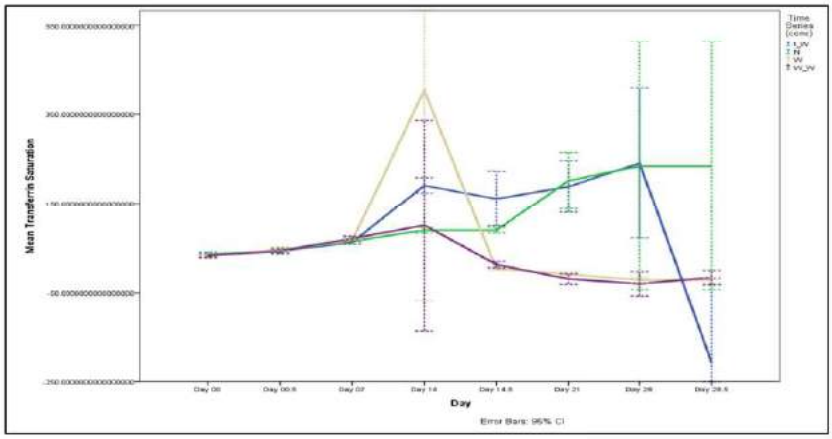


Fig.36 : Storage time trend of transferrin Saturation in Leukoreduced bags.

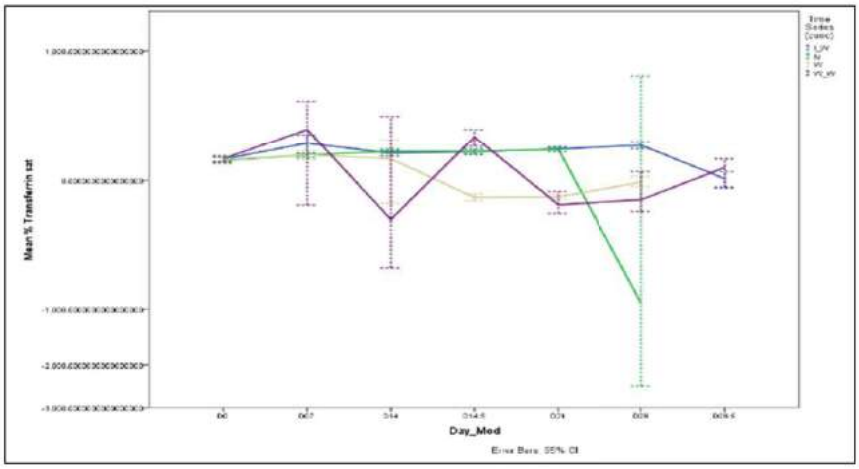


Fig.37: Storage time trend of transferrin Saturation in non Leukoreduced bags.

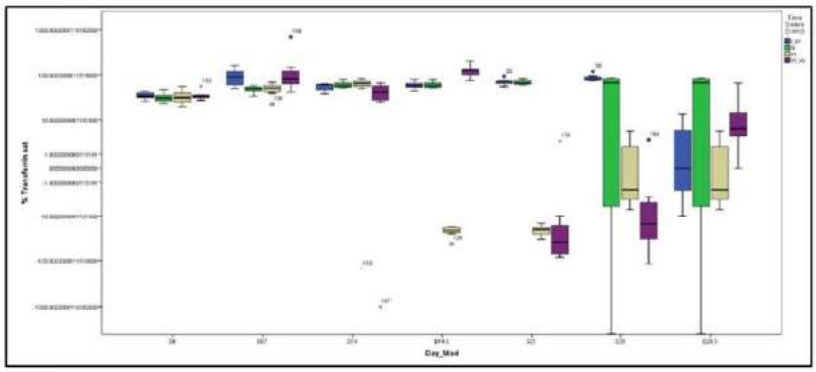


Figure 38: Storage time trend of transferrin saturation in Non-Leukoreduced bags (BOX PLOT).

AUTOMATED BLOOD COUNT RELATED PARAMETERS

MCV

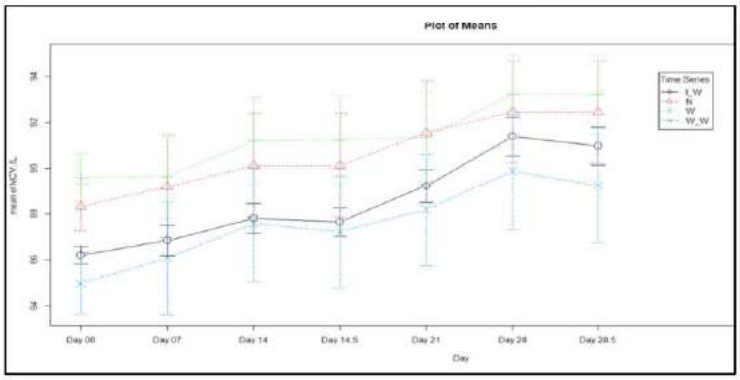


Figure 39: Storage time trend of MCV in Leukoreduced bags

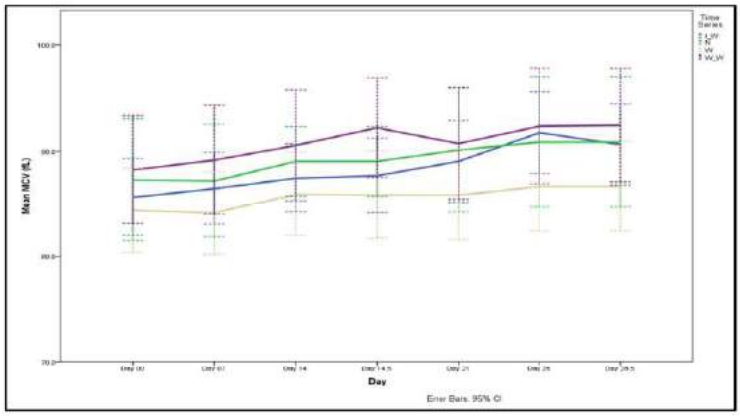


Figure 40: Storage time trend of MCV in Non-Leukoreduced bag

MCH

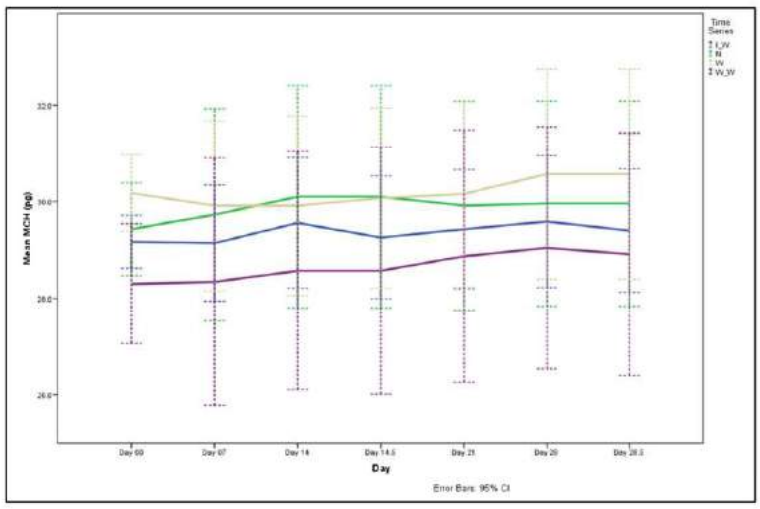


Figure.41: Storage time trend for MCH in Leukoreduced bags.

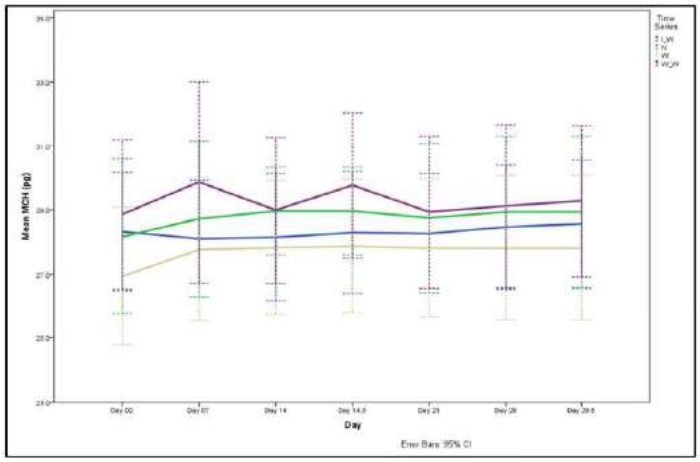


Figure.42: Storage time trend of MCH in Non-Leukoreduced bags.

MCHC

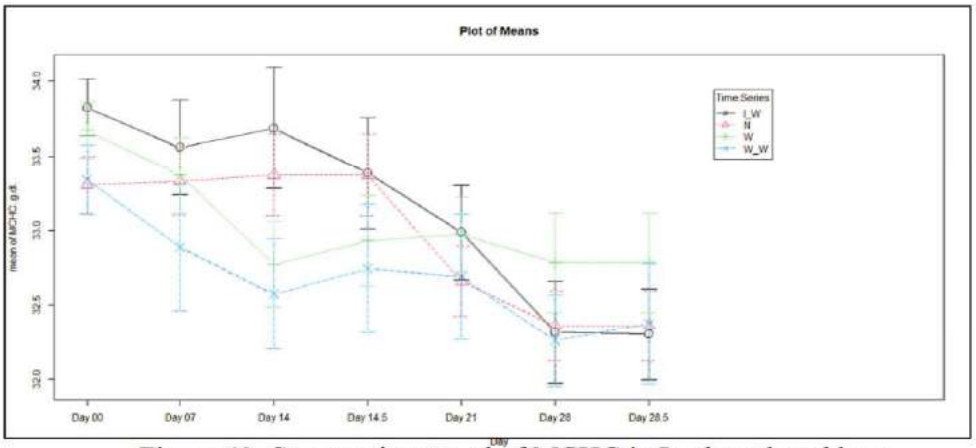


Figure.43: Storage time trend of MCHC in Leukoreduced bags.

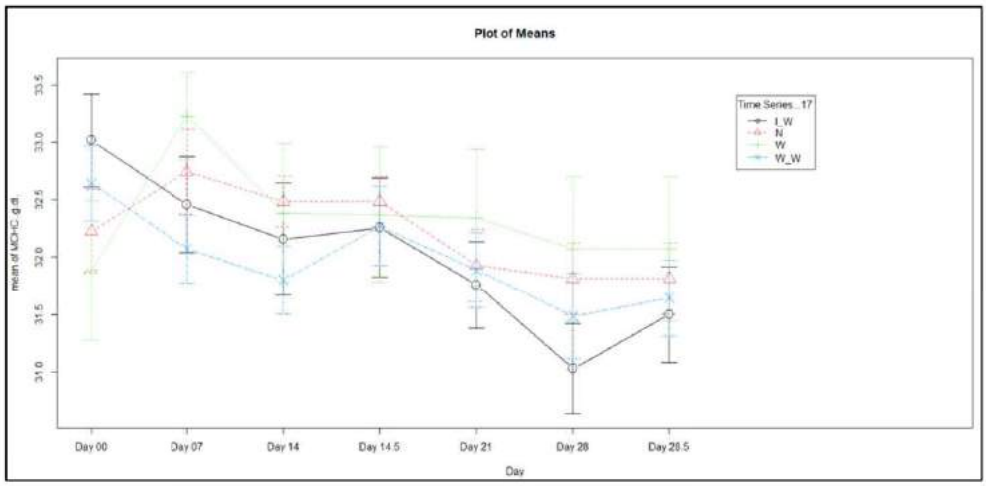


Figure 44: Storage time trend of MCHC in NON Leukoreduced bags

HEMATOCRIT

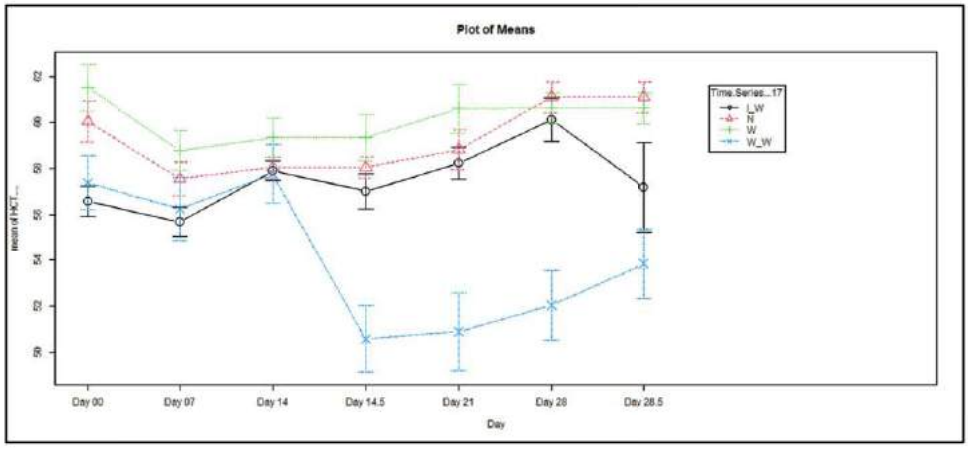


Figure 45: Storage time trend of haematocrit in Leukoreduced bags.

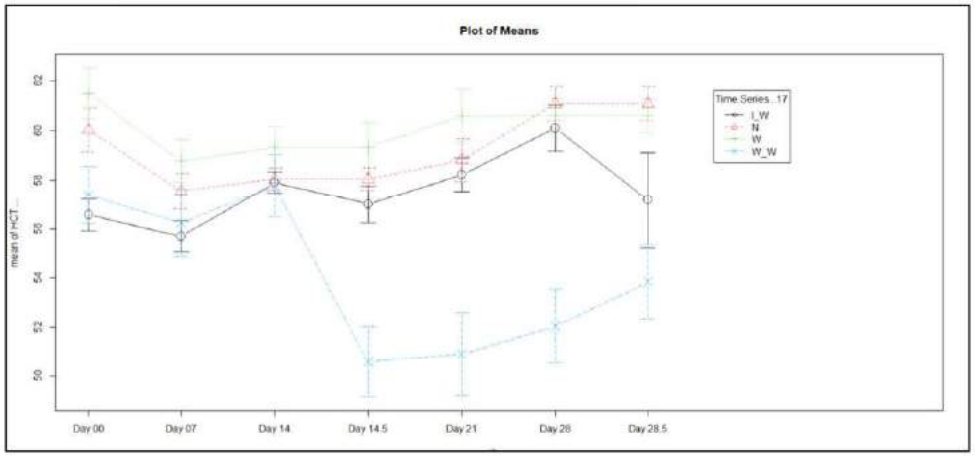


Figure.46: Storage time trend of haematocrit in Non-Leukoreduced bags.

HEMOGLOBIN

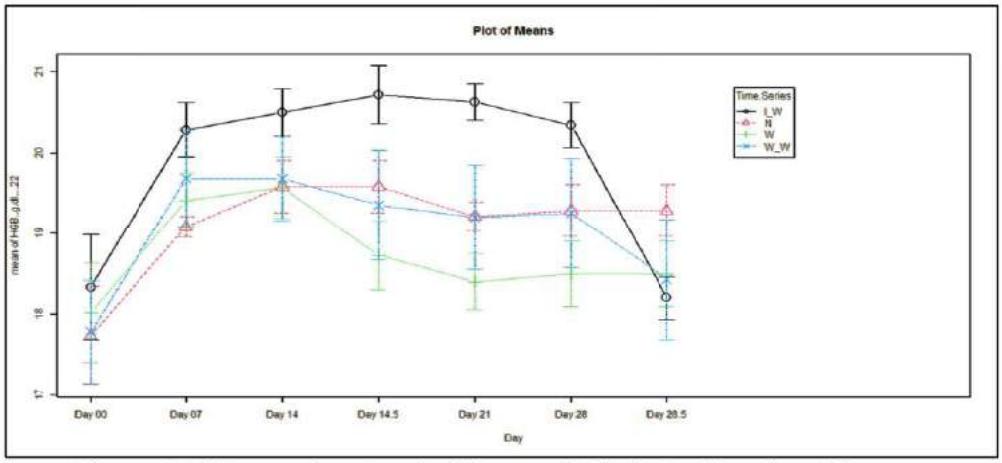


Figure 47: Storage time trend of haemoglobin in Leukoreduced bags.

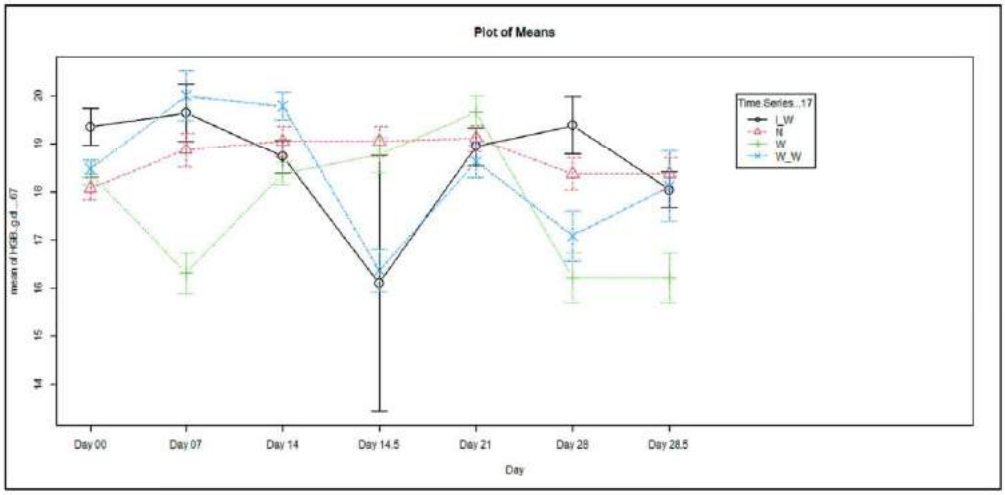


Figure 48: Storage time trend of Haemoglobin in Non Leukoreduced bags.

RDW CV

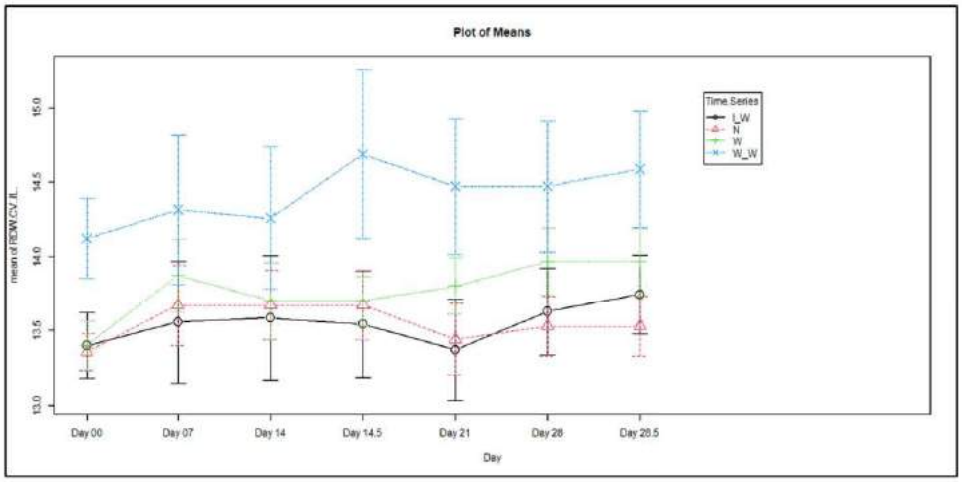


Figure 49: Storage time trend of RDW-CV in Leukoreduced bags.

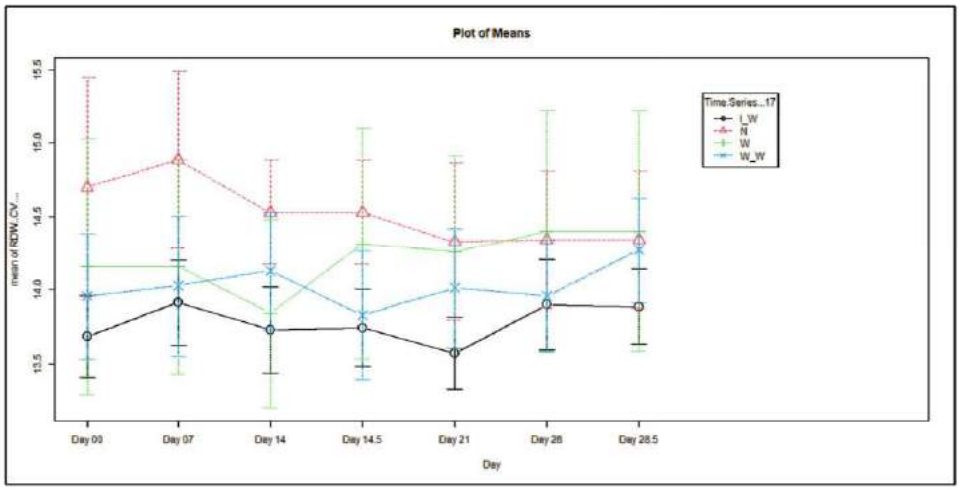


Figure 50: Storage time trend of RDW-CV in Non-Leukoreduced bags

RDW SD

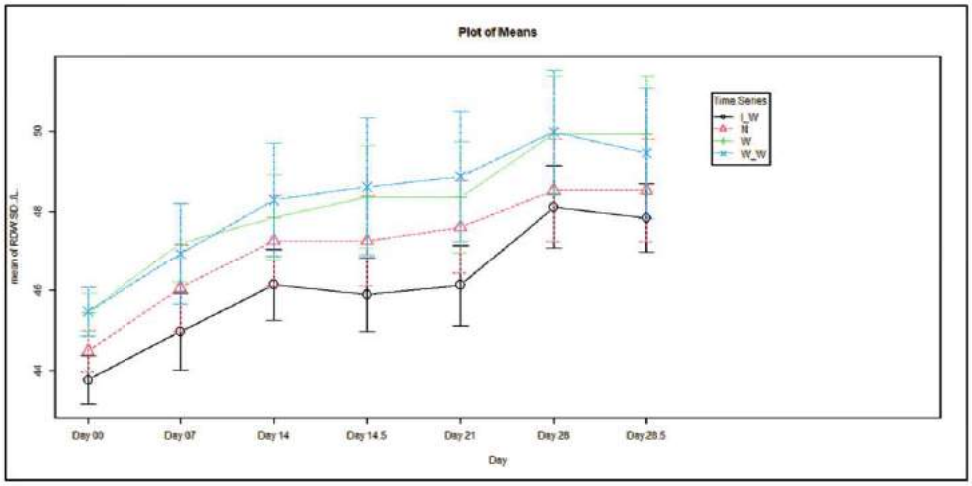


Figure 51: Storage time trend of RDW-SD in Leukoreduced bags.

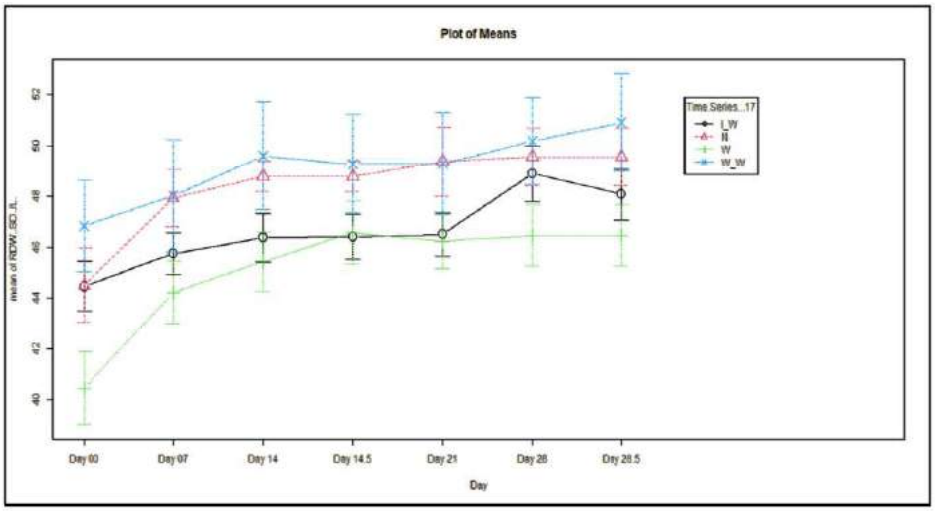


Figure 52: Storage time trend of RDW-SD in Non-Leukoreduced bags

AUTOMATED VBG RELATED PARAMETERS

For VBG parameters pH and glucose was done in neat samples while potassium and lactate were done at higher dilutions.

pH

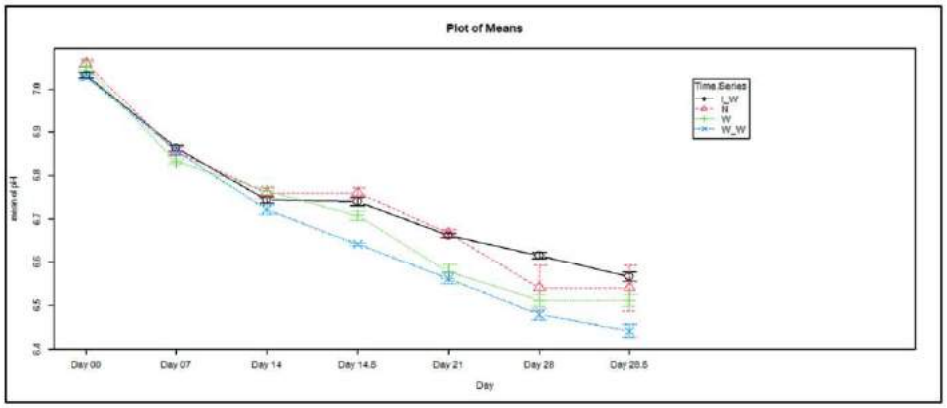


Figure 53: Storage time trend of pH in Leukoreduced bags.

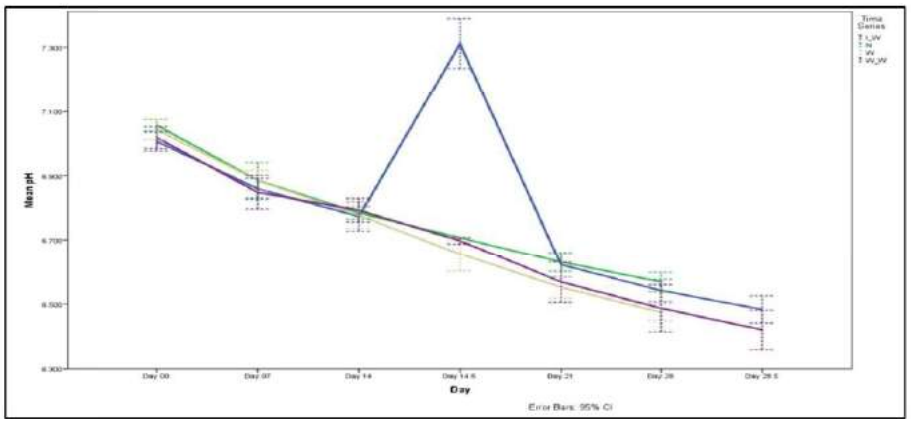


Figure 54: Storage time trend of pH in Non-Leukoreduced bags.

LACTATE

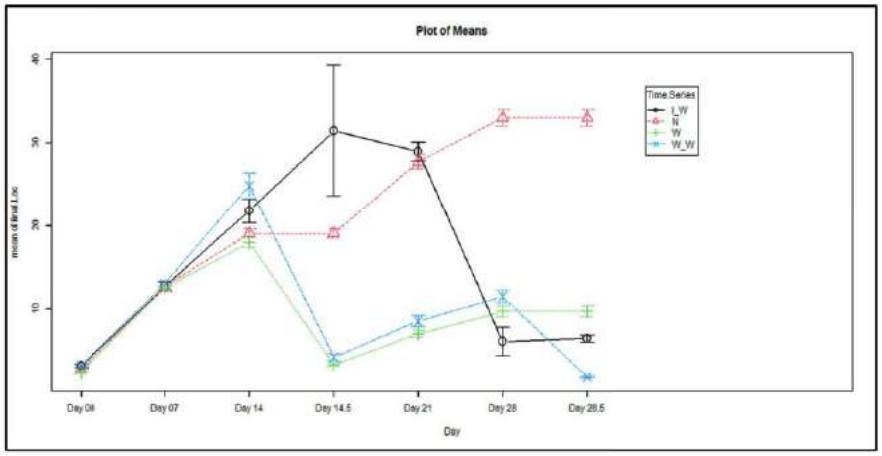


Figure 55: Storage time trend of lactate in Leukoreduced bags.

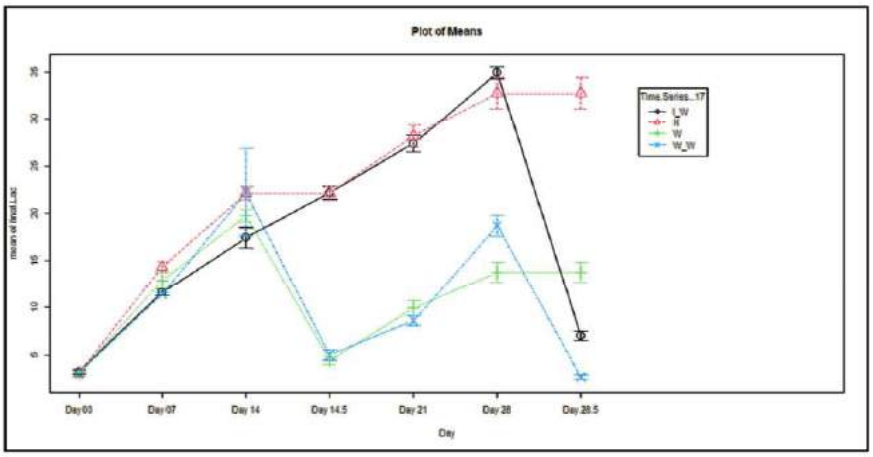


Figure 56: Storage time trend of Lactate in Non-Leukoreduced bags.

POTASSIUM

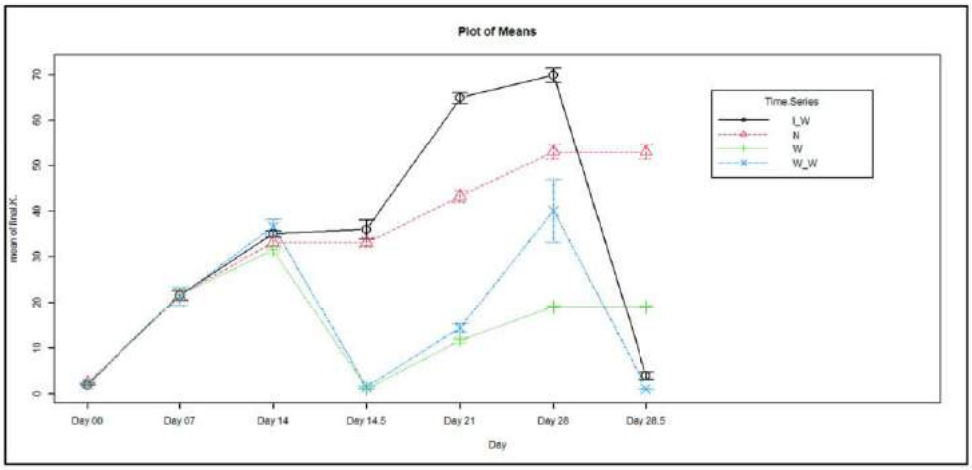


Figure 57: Storage time trend of potassium in Leukoreduced bags.

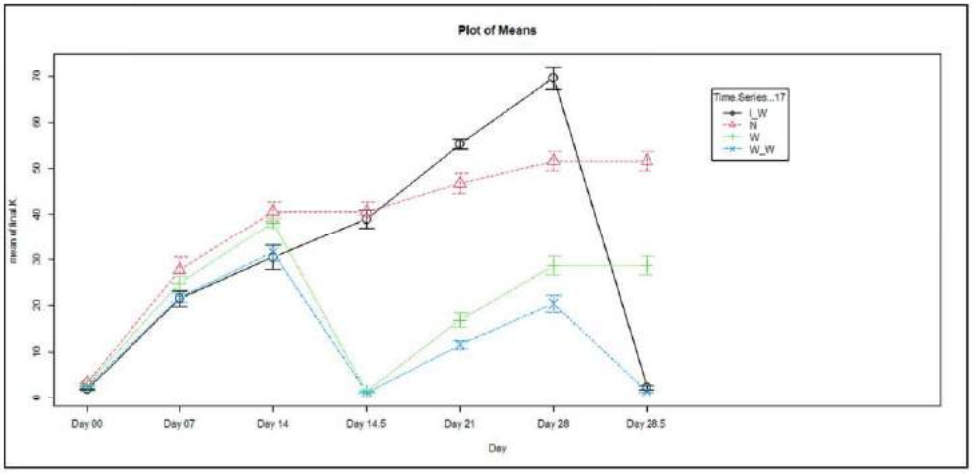


Figure 58: Storage time trend of potassium in Non-Leukoreduced bags.

GLUCOSE

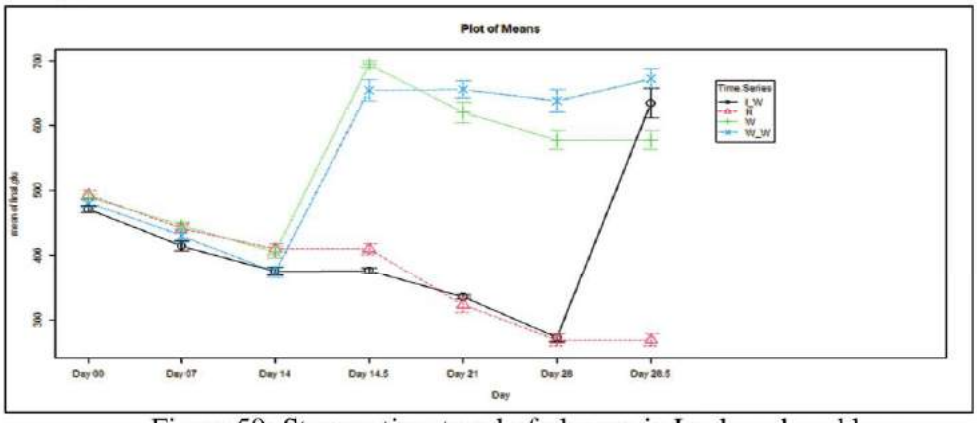


Figure 59: Storage time trend of glucose in Leukoreduced bags

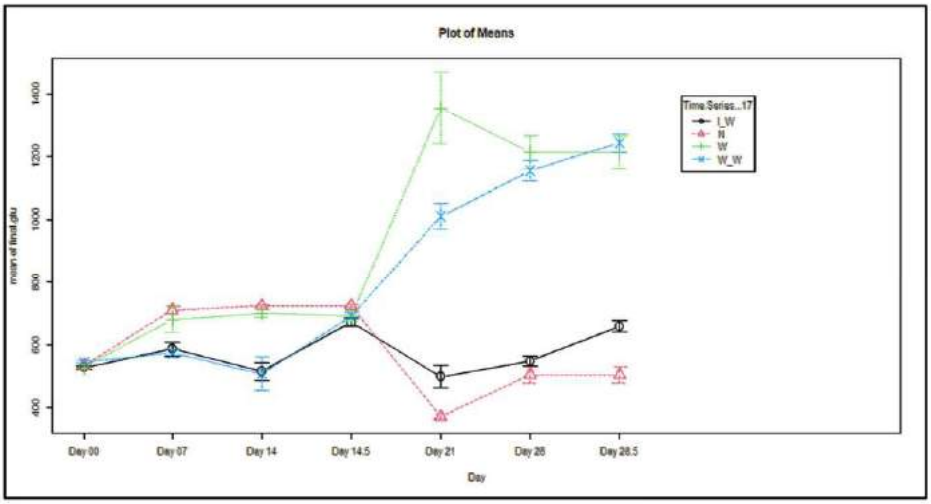


Figure 60: Storage time trend of Glucose in Non-Leukoreduced bags.

HEMOLYSIS AND HEMOLYTIC POTENTIAL RELATED PARAMETERS

% HEMOLYSIS

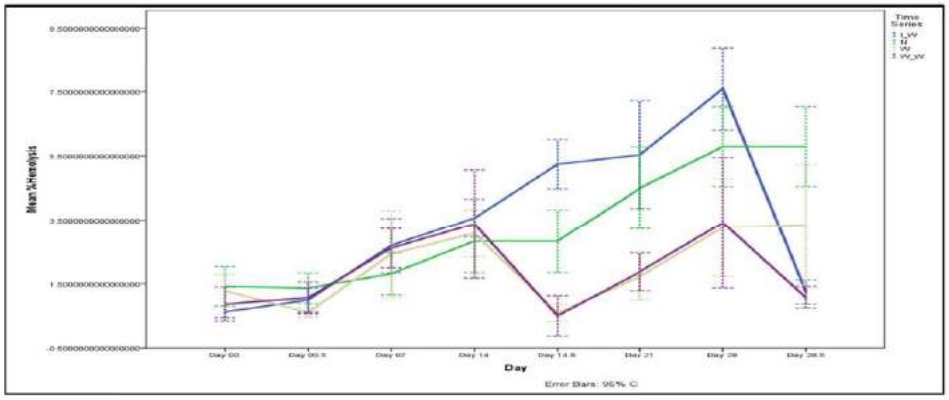


Figure 61: storage time trend of percentage hemolysis in Leukoreduced bags

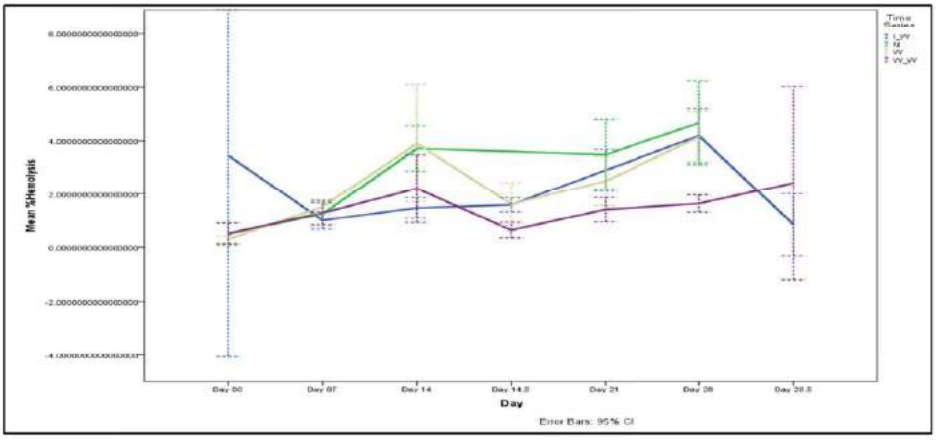


Figure 62: storage time trend of percentage hemolysis in Non-Leukoreduced bags

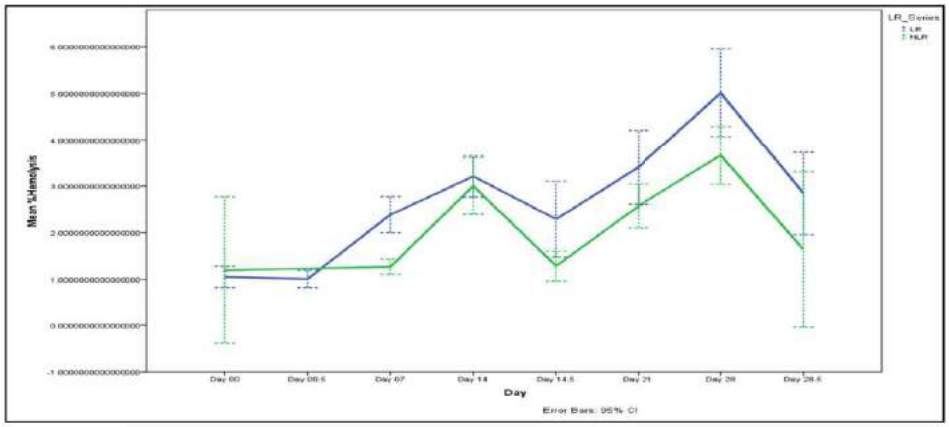


Figure 63: Storage time trend of % hemolysis in LR vs NLR bags

LDH

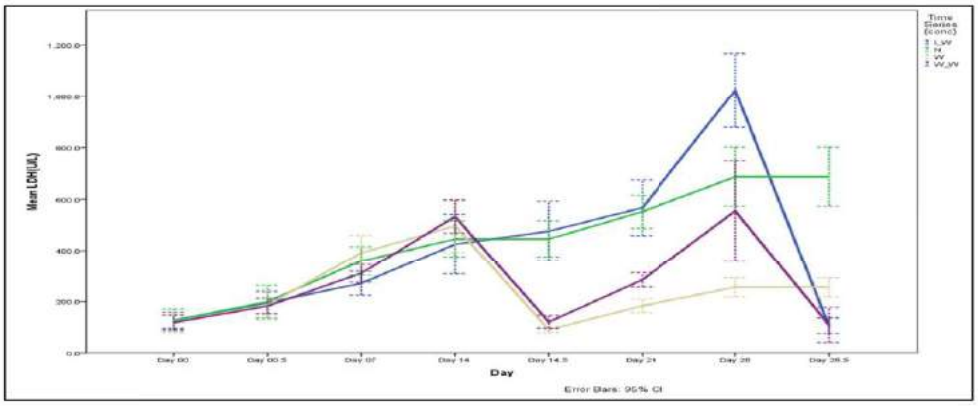


Figure 64: storage time trend of LDH in Leukoreduced bags

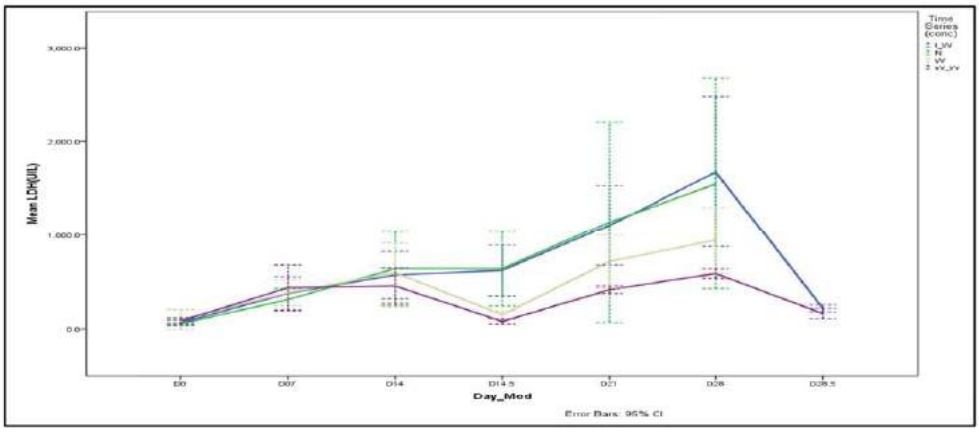


Fig.65: Storage time trend for lactate dehydrogenase in Non-Leukoreduced bags.

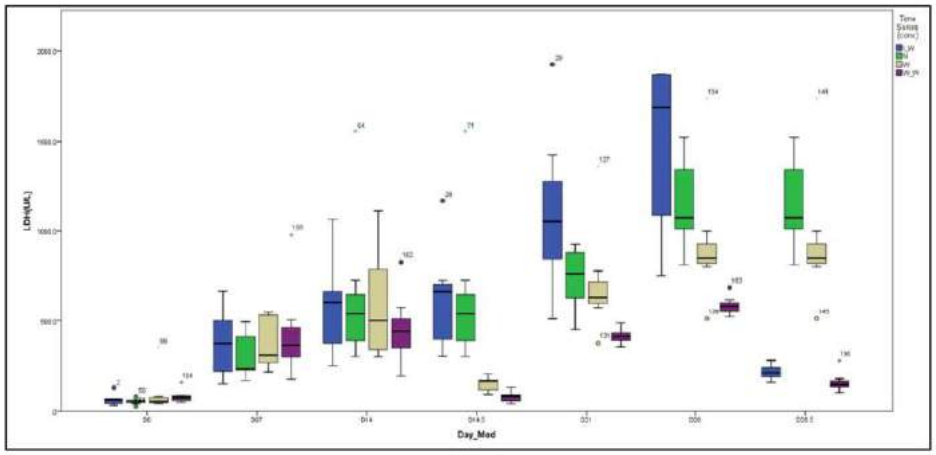


Figure 66: Storage time trend of LDH in Non-Leukoreduced bags.

TOTAL BILLIRUBIN

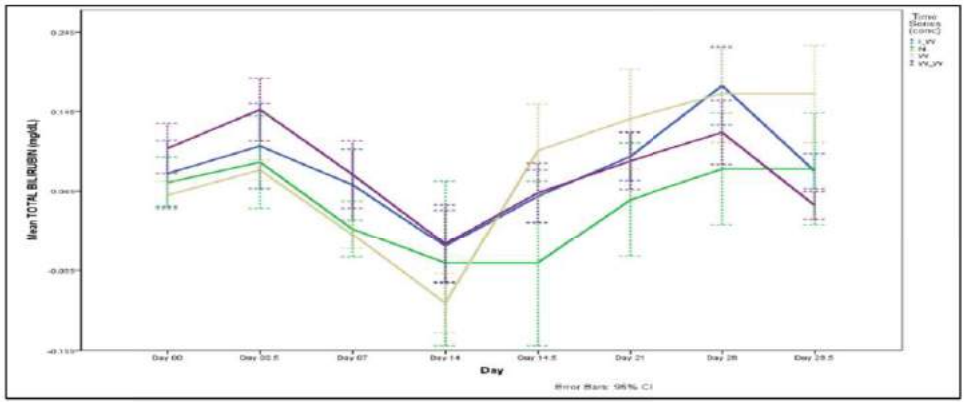


Figure 67: Storage time trend of Total Bilirubin in Leukoreduced bags

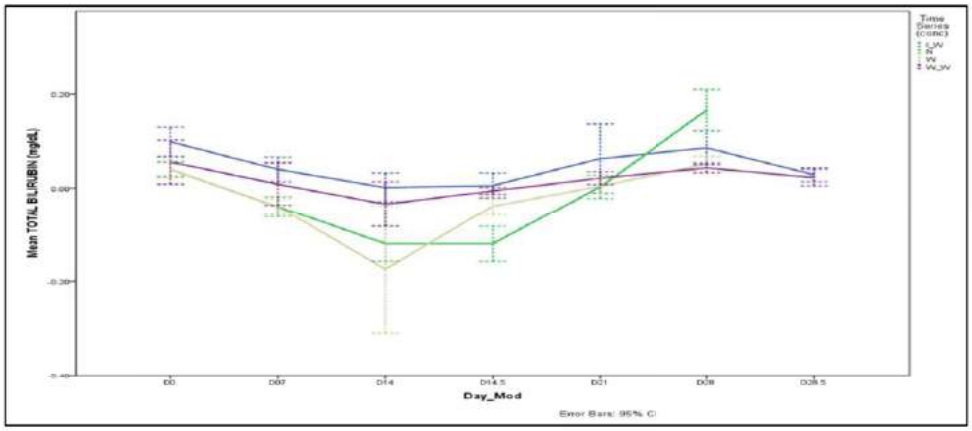


Fig 68: Storage time trend of Total bilirubin in Non-Leukoreduced bag

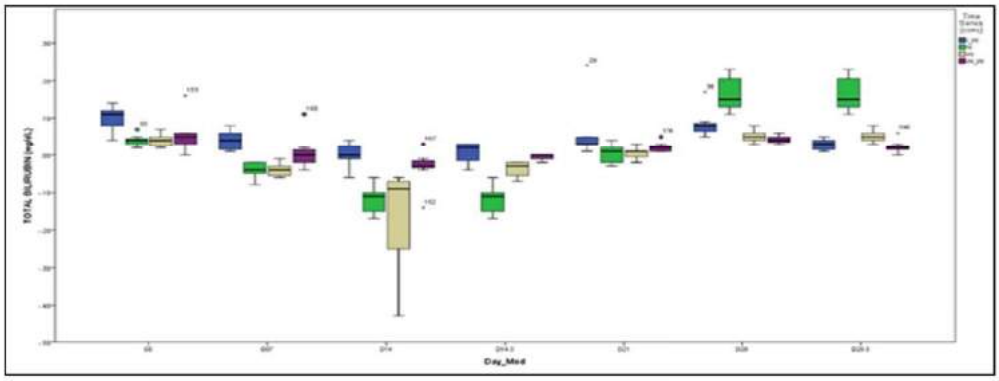


Fig 69: Storage time trend of Total bilirubin in Non-Leukoreduced bag

AST

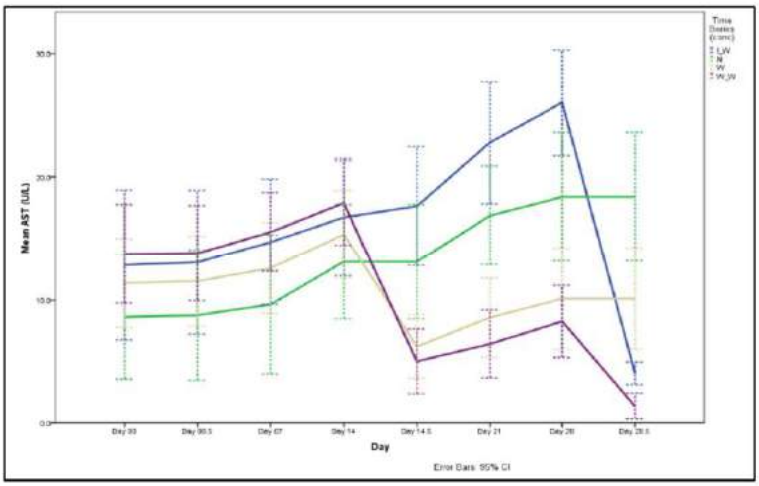


Figure 70: Storage time trend of AST in Leukoreduced bags

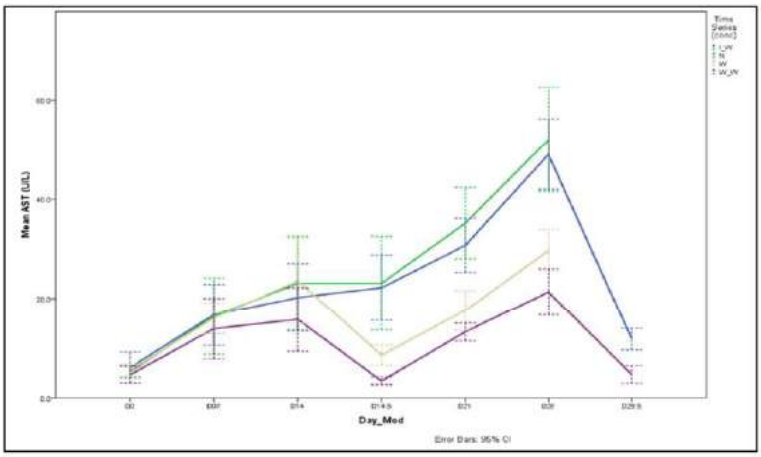


Figure 71: Storage time trend of AST in Non-Leukoreduced bags

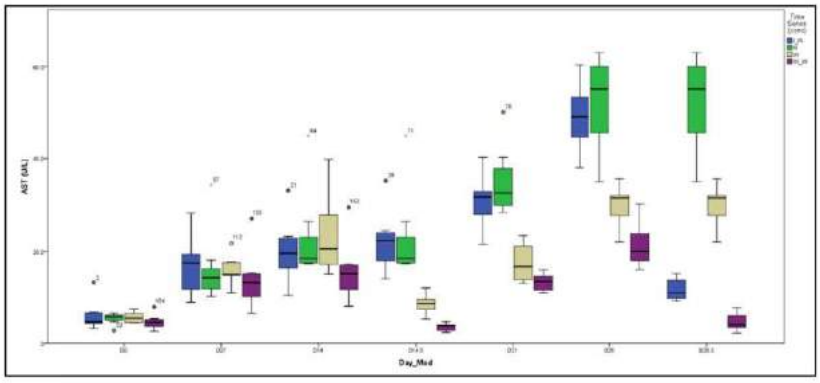


Figure 72: Storage time trend of AST in Non-Leukoreduced bags

TOTAL PROTEIN

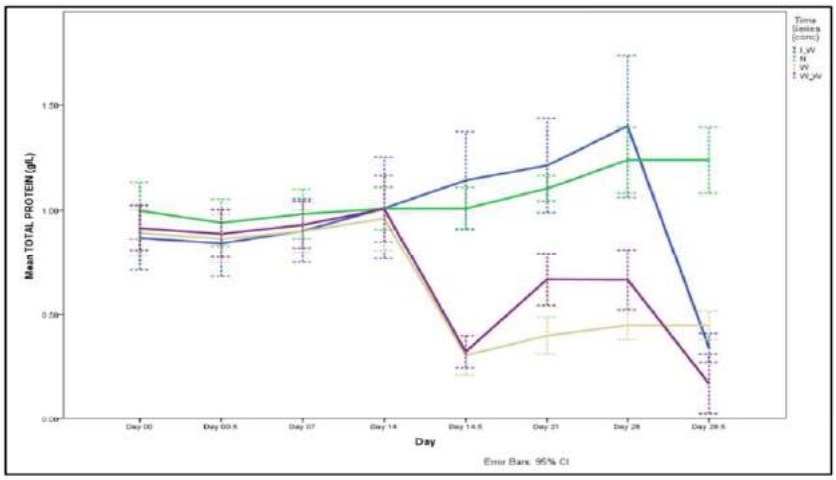


Figure 73: storage time trend of total protein in Leukoreduced bags.

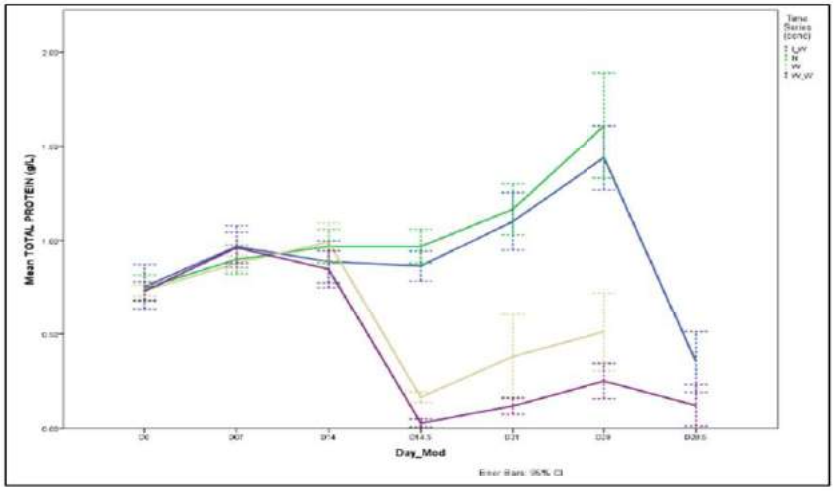


Figure 74: Storage time trend of Total protein in Non-Leukoreduced bags.

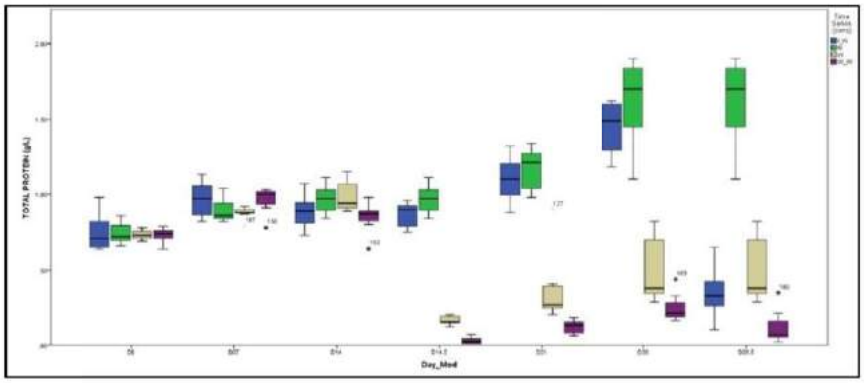


Figure 75: Storage time trend of total protein in Non-Leukoreduced bags.

Free Hb

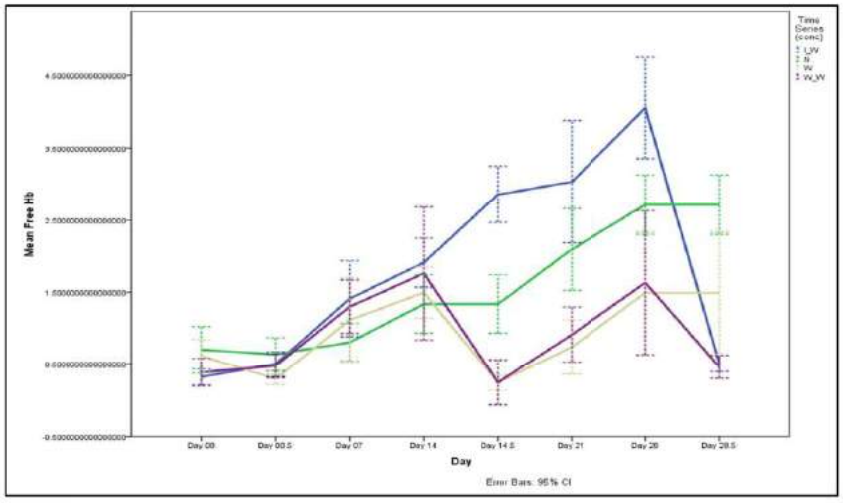


Figure 76: Storage time trend of free hemoglobin in Leukoreduced bags.

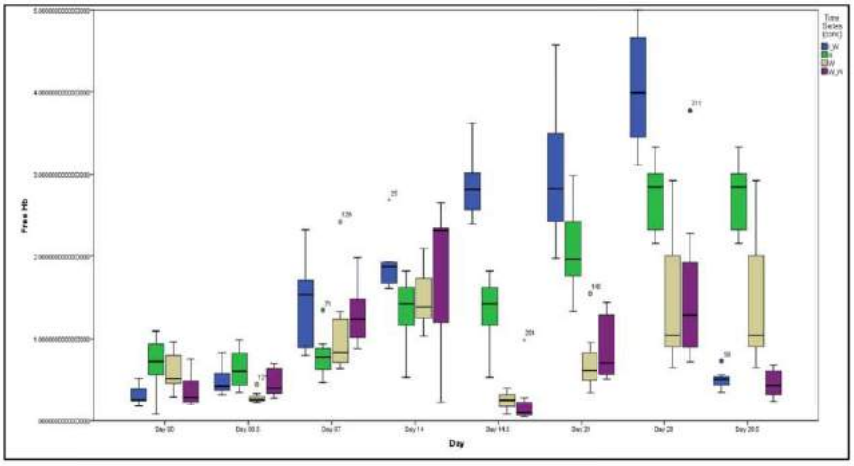


Figure 77: Storage time trend of free hemoglobin in Leukoreduced bags

Free Hb

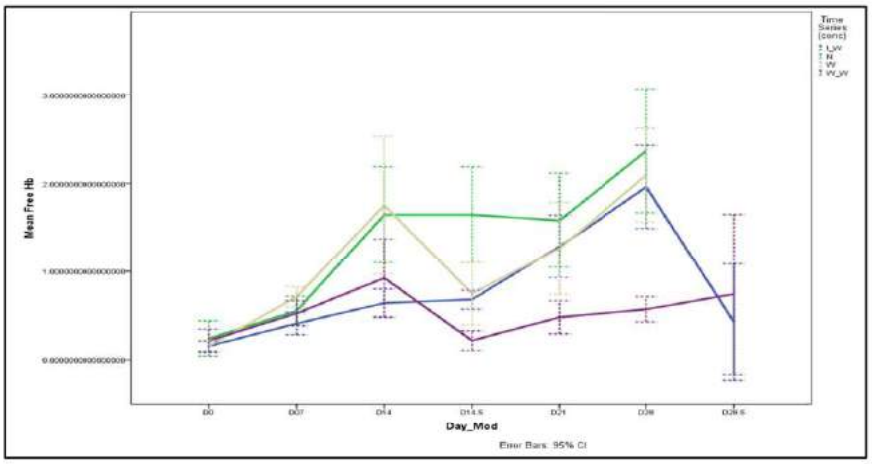


Figure 78: Storage time graph of free hemoglobin in Non-Leukoreduced bags.

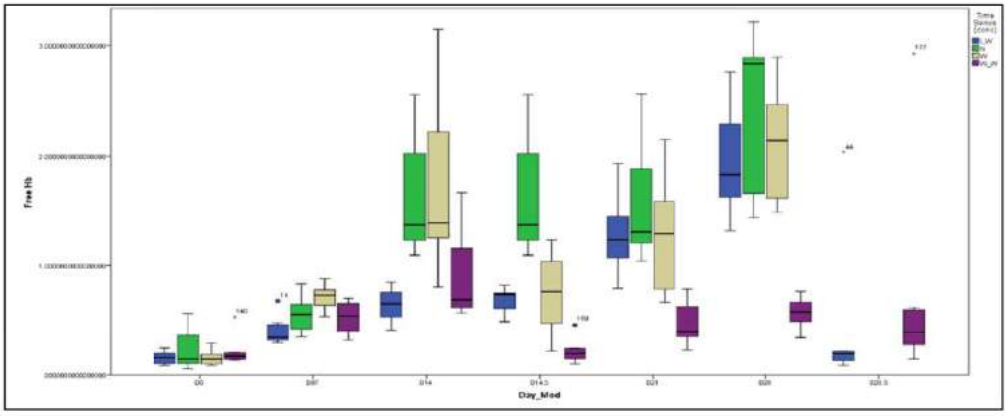


Figure 79: Storage time trend of Free Hemoglobin in Non-Leukoreduced bags.

Pink test

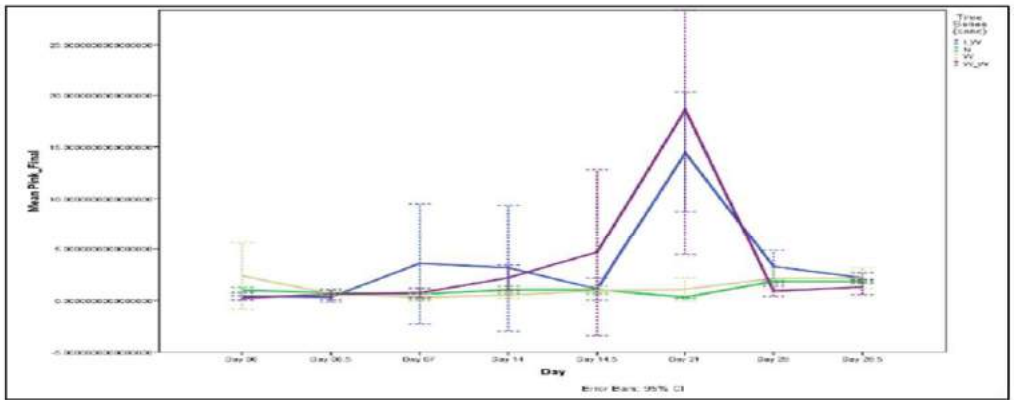


Figure 80: Storage time trend of Pink test in Leukoreduced bags.

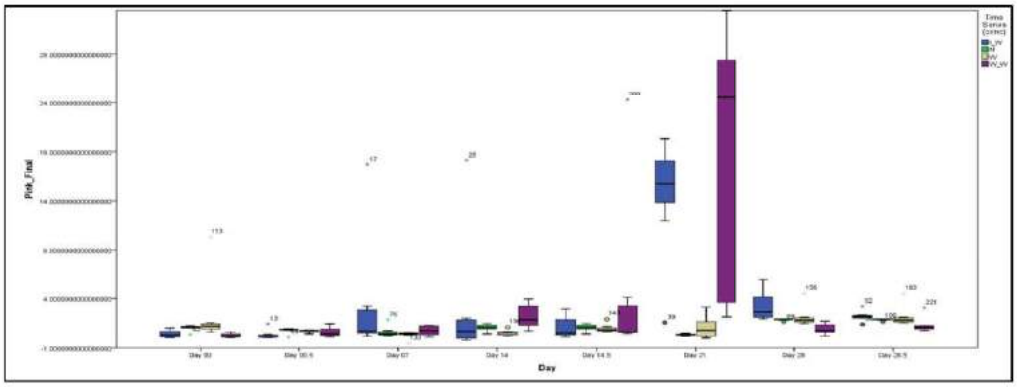


Figure 81: Storage time trend of Pink test in Leukoreduced bags.

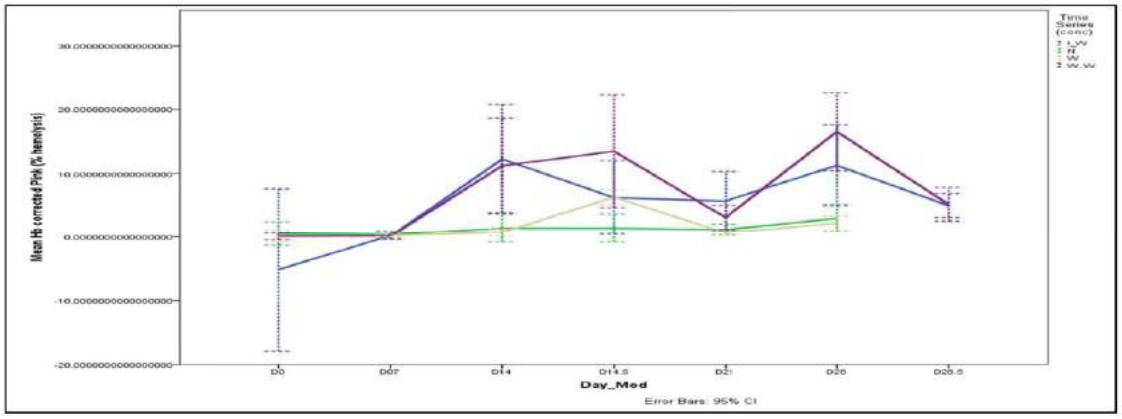


Figure 82: Storage time trend of Pink test in Non-Leukoreduced bags.

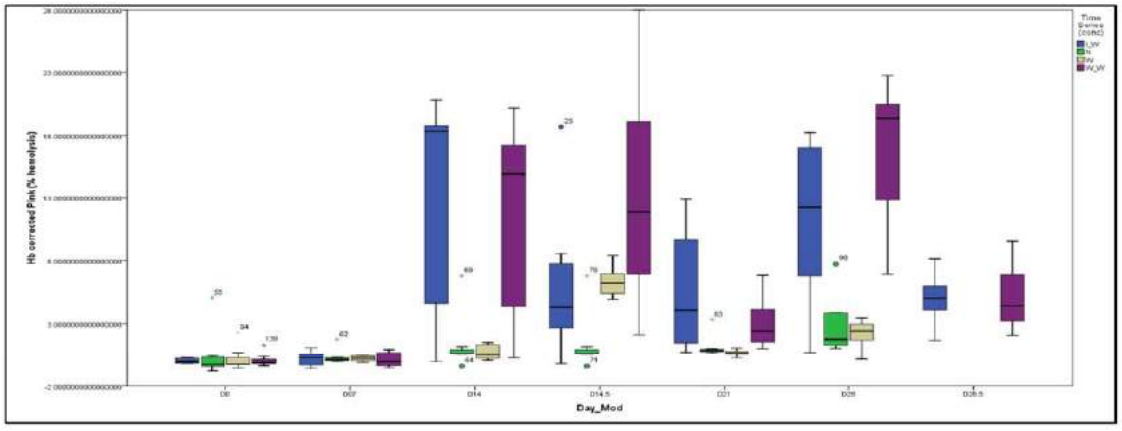


Figure 83: Storage time trend of Pink test in Non-Leukoreduced bags

SLT

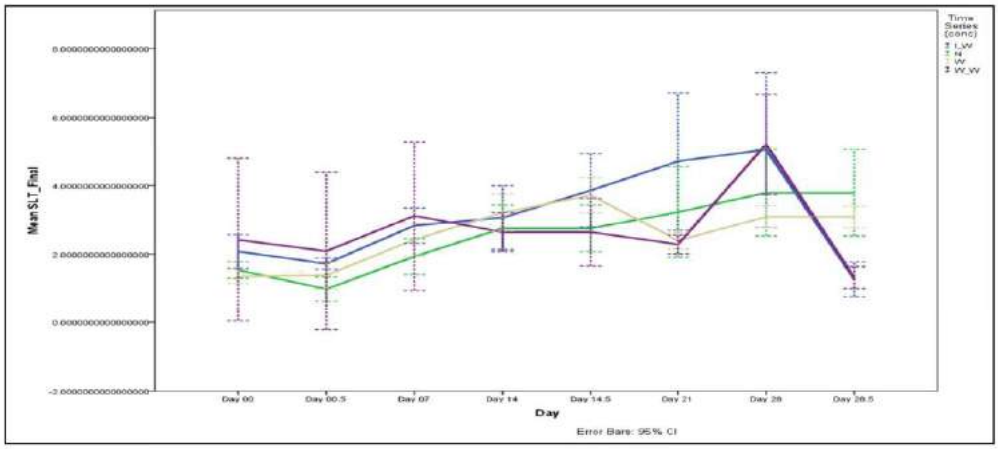


Figure 84: Storage time trend of SLT in Leukoreduced bags.

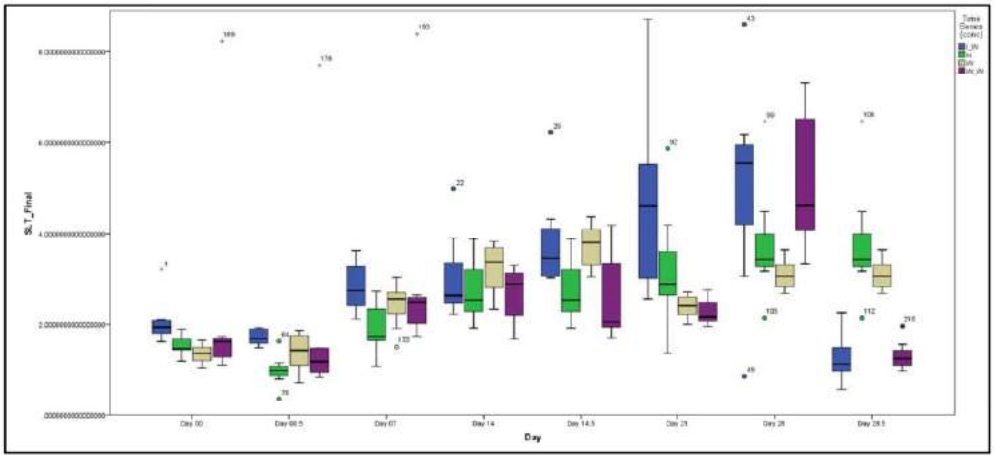


Figure 85: Storage time trend of SLT in Leukoreduced bags.

SLT

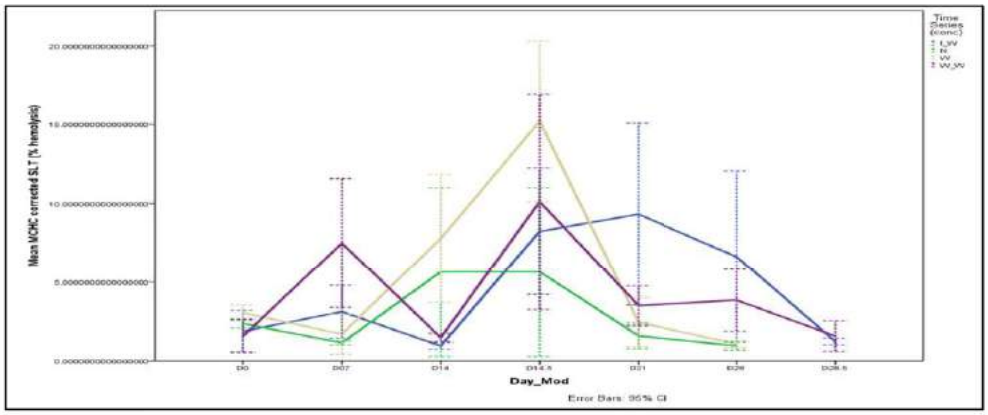


Figure 86: Storage time trend of SLT in non Leukoreduced bags.

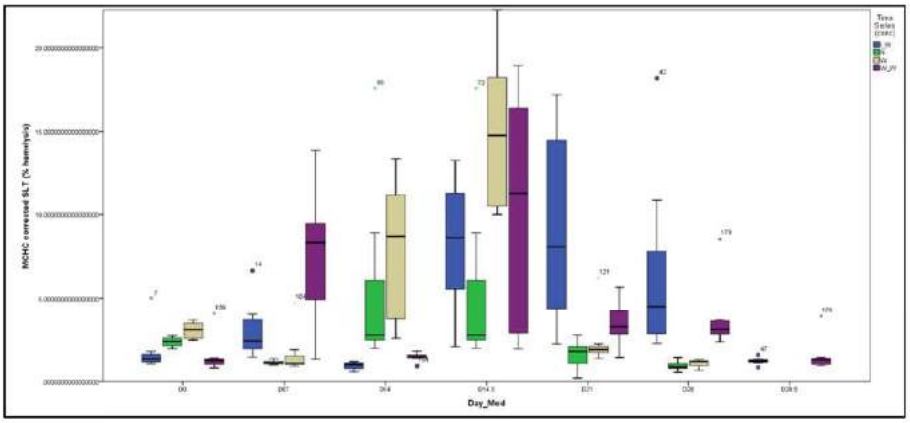


Figure 87: Storage time trend of SLT in Non-Leukoreduced bags

Correlations

		NTBI	Day_n	K+	TOTAL BILIRUBIN (mg/dL)	UIBC	ATP(umol/g Hb)	Free Hb	pH	Glucose	final Lac	LDH(U/L)	TOTAL PROTEIN (g/L)	AST (U/L)	TIBC(ug/L)	IRON (ug/dL)	Transferrin Saturation	% HEMOLYSIS X	Plrk%	MCV(fL)	MCHC (g/dl)	RDW-SD (fL)
Pearson Correlation	NTBI	1.000	0.894	0.594	0.278	-0.586	-0.435	0.550	-0.595	-0.210	0.534	0.348	0.188	0.260	-0.292	0.399	0.370	0.505	0.209	0.206	-0.188	0.218
	Day_n	0.894	1.000	0.465	0.155	-0.746	-0.496	0.445	-0.893	0.151	0.459	0.386	-0.167	0.250	-0.581	0.091	0.070	0.465	0.292	0.507	-0.546	0.418
	K+	0.594	0.465	1.000	-0.045	-0.143	-0.098	0.733	-0.277	-0.551	0.943	0.620	0.655	0.676	0.265	0.767	0.487	0.887	0.167	0.158	-0.213	0.201
	TOTAL BILIRUBIN (mg/dL)	0.278	0.155	0.045	1.000	-0.297	-0.282	0.030	-0.119	-0.034	-0.081	-0.062	0.008	-0.120	-0.217	0.064	-0.014	-0.016	0.014	0.131	0.105	0.018
	UIBC	-0.586	-0.746	-0.143	-0.297	1.000	0.644	-0.359	0.738	-0.147	-0.113	-0.097	0.269	0.079	0.865	0.049	-0.036	-0.345	-0.079	-0.317	0.048	-0.326
	ATP(umol/g Hb)	-0.435	-0.496	-0.098	-0.282	0.644	1.000	-0.277	0.499	0.040	-0.059	-0.039	0.075	0.079	0.503	-0.075	-0.076	-0.253	0.029	-0.248	-0.156	-0.265
	Free Hb	0.550	0.445	0.733	0.030	-0.359	-0.277	1.000	-0.304	0.494	0.671	0.517	0.531	0.518	-0.020	0.561	0.455	0.971	0.042	0.189	-0.087	0.246
	pH	-0.595	-0.893	-0.277	-0.119	0.738	0.499	-0.304	1.000	-0.209	-0.266	-0.278	0.285	-0.128	0.658	0.075	0.019	-0.360	-0.253	-0.230	0.333	-0.388
	Glucose	-0.210	0.151	0.551	-0.034	-0.147	0.040	0.494	-0.209	1.000	0.543	0.287	-0.792	-0.216	-0.519	-0.767	-0.515	0.433	0.167	0.009	-0.122	0.127
	final Lac	0.534	0.459	0.943	-0.081	-0.113	-0.059	0.671	-0.266	-0.543	1.000	0.596	0.618	0.670	0.292	0.769	0.566	0.629	0.205	0.159	-0.216	0.204
	LDH(U/L)	0.348	0.386	0.620	-0.062	-0.097	-0.039	0.517	-0.278	-0.287	0.596	1.000	0.471	0.765	0.101	0.365	0.319	0.543	0.090	-0.097	-0.390	0.163
	TOTAL PROTEIN (g/L)	0.188	0.167	0.655	0.008	0.269	0.075	0.531	0.285	-0.795	0.618	0.471	1.000	0.585	0.598	0.740	0.482	0.470	-0.083	-0.097	0.024	-0.062
	AST (U/L)	0.260	0.250	0.676	-0.120	0.079	0.079	0.518	-0.128	-0.316	0.670	0.765	0.585	1.000	0.279	0.422	0.286	0.519	0.108	0.026	-0.233	0.182
	TIBC(ug/L)	-0.292	-0.581	0.265	-0.217	0.865	0.503	-0.020	0.658	-0.519	0.292	0.101	0.598	0.279	1.000	0.544	0.279	-0.045	-0.091	-0.216	0.066	-0.281
	IRON (ug/dL)	0.399	0.091	0.767	0.064	0.049	-0.075	0.561	0.075	-0.767	0.769	0.365	0.740	0.422	0.544	1.000	0.615	0.488	-0.049	0.101	0.049	-0.013
	Transferrin Saturation	0.370	0.070	0.487	-0.014	-0.036	-0.076	0.439	0.019	-0.515	0.566	0.319	0.482	0.286	0.279	0.615	1.000	0.396	-0.029	0.030	0.009	-0.028
	% HEMOLYSIS X	0.505	0.465	0.887	-0.016	-0.345	-0.253	0.971	-0.360	-0.433	0.629	0.543	0.470	0.519	-0.045	0.488	0.396	1.000	0.056	0.164	-0.161	0.245
	Plrk%	0.209	0.292	0.167	0.014	-0.079	0.029	0.042	-0.253	0.167	0.205	0.090	-0.083	0.108	-0.091	-0.049	-0.029	0.056	1.000	0.005	-0.234	0.021
	MCV(fL)	0.206	0.507	0.158	0.131	-0.317	-0.248	0.189	-0.230	0.009	0.159	-0.097	-0.097	0.026	-0.216	0.101	0.030	0.164	0.005	1.000	0.364	0.657
	MCHC (g/dl)	-0.188	-0.546	-0.213	0.105	0.048	-0.156	-0.087	0.333	-0.122	-0.216	-0.390	0.024	-0.233	0.065	0.049	0.009	-0.161	-0.234	0.364	1.000	-0.001
	RDW-SD (fL)	0.218	0.418	0.201	0.018	-0.326	-0.265	0.246	-0.388	0.127	0.204	0.163	-0.062	0.182	-0.281	-0.013	-0.028	0.245	0.021	0.657	-0.001	1.000
Sig. (1-tailed)	NTBI		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.000
	Day_n	0.000		0.000	0.009	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.005	0.000	0.000	0.083	0.145	0.000	0.000	0.000	0.000	0.000
	K+	0.000	0.000		0.248	0.015	0.069	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.008	0.001	0.001
	TOTAL BILIRUBIN (mg/dL)	0.000	0.009	0.248		0.000	0.000	0.326	0.035	0.304	0.110	0.173	0.280	0.034	0.000	0.166	0.419	0.401	0.418	0.023	0.055	0.391
	UIBC	0.000	0.000	0.015	0.000		0.000	0.000	0.000	0.013	0.043	0.070	0.000	0.115	0.000	0.228	0.293	0.000	0.114	0.000	0.234	0.000
	ATP(umol/g Hb)	0.000	0.000	0.069	0.000	0.000		0.000	0.000	0.273	0.186	0.279	0.129	0.117	0.000	0.129	0.123	0.000	0.332	0.000	0.009	0.000
	Free Hb	0.000	0.000	0.000	0.326	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.383	0.000	0.000	0.000	0.261	0.002	0.094	0.000
	pH	0.000	0.000	0.000	0.095	0.000	0.000	0.000		0.001	0.000	0.000	0.000	0.026	0.000	0.126	0.385	0.000	0.000	0.000	0.000	0.000
	Glucose	0.001	0.011	0.000	0.364	0.015	0.273	0.000	0.001		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.443	0.032	0.027
	final Lac	0.000	0.000	0.000	0.110	0.043	0.186	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.008	0.000	0.001
	LDH(U/L)	0.000	0.000	0.000	0.173	0.070	0.279	0.000	0.000	0.000	0.000		0.000	0.000	0.062	0.000	0.000	0.000	0.085	0.079	0.000	0.006
	TOTAL PROTEIN (g/L)	0.002	0.005	0.000	0.283	0.000	0.129	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.104	0.071	0.358	0.107
	AST (U/L)	0.000	0.000	0.000	0.034	0.115	0.117	0.000	0.026	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.051	0.345	0.000	0.003
	TIBC(ug/L)	0.000	0.000	0.000	0.000	0.000	0.000	0.383	0.000	0.000	0.000	0.062	0.000	0.000		0.000	0.000	0.246	0.080	0.000	0.164	0.000
	IRON (ug/dL)	0.000	0.083	0.000	0.166	0.228	0.129	0.000	0.126	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.229	0.063	0.231	0.422
	Transferrin Saturation	0.000	0.145	0.000	0.418	0.293	0.123	0.000	0.065	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.445	0.006
	% HEMOLYSIS X	0.000	0.000	0.000	0.401	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.246	0.000	0.000		0.188	0.006	0.007	0.000
	Plrk%	0.001	0.000	0.005	0.418	0.114	0.332	0.261	0.000	0.002	0.001	0.085	0.104	0.051	0.083	0.229	0.330	0.188		0.471	0.000	0.372
	MCV(fL)	0.001	0.000	0.008	0.023	0.000	0.000	0.002	0.000	0.443	0.008	0.070	0.071	0.345	0.000	0.063	0.322	0.008	0.471		0.000	0.000
	MCHC (g/dl)	0.002	0.000	0.001	0.055	0.234	0.009	0.094	0.000	0.032	0.000	0.000	0.358	0.000	0.164	0.231	0.445	0.007	0.000	0.000		0.491
	RDW-SD (fL)	0.000	0.000	0.001	0.391	0.000	0.000	0.000	0.000	0.027	0.001	0.006	0.107	0.003	0.000	0.422	0.336	0.000	0.372	0.000	0.491	

Figure 88: Correlation of NTBI with storage lesion parameters that gave significant values.

232 list-wise complete.

DISCUSSION

DISCUSSION

Prof. Paul M. Ness, from John Hopkins University who has been Chief Editor of the Journal "Transfusion" has often said "***Whether 'old' RBCs are less safe than fresh RBCs is the most critical issue facing transfusion medicine today***."[76] The technical name for the subject of changes seen in old RBCs is called red cell storage lesions. The occurrence of storage lesions though a well-established phenomenon, remains partly understood till date. In the present era, apart from elaborating on the mechanisms of already known storage lesions, there has been increasing demand for figuring out the measurable storage lesions which might be better indicators of potential harm from transfusing old units, as well as methods for mitigating or reversing the effects of storage lesions. Washing is one such modification which has been employed to remove unwanted accumulated substances in blood due to storage or irradiation. Washing/volume reduction of red cell units is performed for various indications such as to remove or reduce harmful effects of storage lesions e.g., post-irradiation accumulation of potassium, free Hb, NTBI etc. and/or removing or reducing substances to which a patient might be specifically intolerant to e.g., Ig A/ haptoglobin in cases of anaphylaxis, cytokines in cases of severe recurrent FNHTR, Anti HLA antibody in cases of NAIT, mannitol/adenine additives in cases of massive transfusion in neonates etc.

The effects of shear force and mechanical hemolysis on manipulated RBCs have been well described and washing being a harsh modification invites attention on post wash quality of red cells. Plasma potassium and free Hb are reduced immediately post wash but their subsequent rise varies with the washing solution and resuspension medium used, which in turn is climacteric in determining the shelf life of washed PRBC. NS doesn't contain Glucose or adenine as nutrients that can sustain ATP concentration in solution. Whereas some of the automated washers of newer generation use solutions eg NS 0.9% plus Dextrose 0.2% during washing and resuspension in Additive Solutions.

Internationally, three rounds of washing the norm. However, in our study due to small initial volume and too many parameters to measure, multiple rounds of washing was logistically unfeasible. In India, multiple rounds of manual washing is not routinely practiced. However we standardized 2 rounds of washing to remove effects of hemolysis during the first round of wash.

Studies have been published to compare manual vs automated washing [42](Proffitt, 2018) and to compare various wash fluids suitable for washing. Young et al compared different cell washers and different wash fluids for extracellular potassium and hemolysis index. For our study we compared NS and SAGM in different combination in manual method and also standardised single vs double wash. Post wash rise in Potassium and free hemoglobin was observed in all 3 combinations but did not reach the pre wash levels in 14 days in any case. The Pearson Coefficient between potassium concentration and hemolysis in NS & NS, NS & SAGM and SAGM & SAGM was 5.0671, 10.396 & 7.6595 and p values were highly significant (as 0.002295, 0.00004639 & 0.0002688).

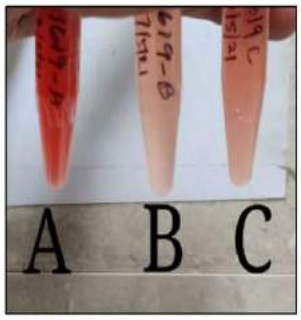


Figure 89: Visible hemolysis in: A. NS+SAGM, B:SAGM+SAGM, C:NS+NS on Day 7 post Wash

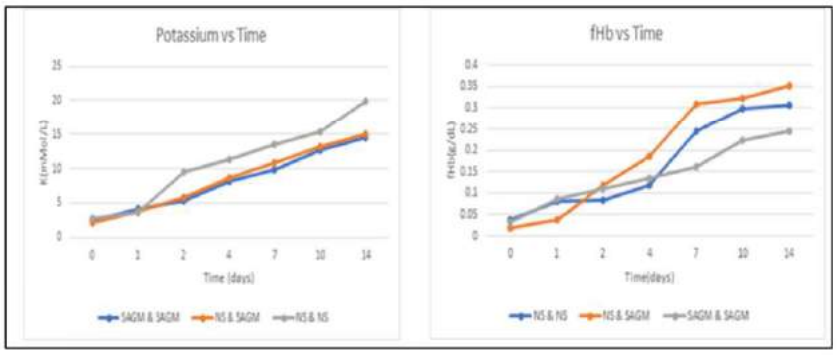


Figure 90: Time trend plot of Potassium and Lactate over the storage period

This pilot study showed maximum post wash increase in Potassium concentration in NS & NS group and least in SAGM & SAGM group while hemolysis was seen maximum in NS & SAGM group and least in SAGM & SAGM group. The osmolarities of NS and SAGM are 308 and 376 mOsm/L. Red cells equilibrate with SAGM when stored for extended periods of time, thus washing with NS and resuspension in SAGM manifests hypo-osmotic shock

followed by hyperosmotic shock to the cells which deranges cell membrane integrity and results in hemolysis. Higher rise in Potassium in NS & NS wash is likely due to potassium leak along with hemolysis. The red cells tend to be less osmotically fragile if the storage, washing and resuspension media is same as is supported by our data too. Therefore, SAGM-wash & SAGM-resuspension combination was found to be most suitable and safest for washing in comparison to the other two groups

NTBI

To the best of our knowledge, we developed the second NTBI assay made in India, and among the fully automated NTBI assays fourth in the world and first in India. Our assay was adapted and standardized in the Beckman Coulter AU series instruments which are capable of a throughput of 2000 tests an hour for photometric tests in contrast to the tedious methods presently available even internationally.

The field of NTBI though 50 years old (Hershko, 1978), the analytical methods of NTBI measurements have progressed very slowly. The first interlaboratory comparison appeared in 2005 (Jacob, 2005) and the 2nd round happened only in 2016 (Swart, 2016). Even in the 2nd round there were only five labs all over the world and only ten methods. Majority of these methods are either HPLC based or spectrofluorometric (including the previous one made in India) and involve hours of tedious sample processing.

There is no consensus regarding exact reference range for physiologic NTBI levels. Though, majority of methods correlate fairly well within each other but the results exactly don't match. Nearly half of the methods start from negative value at the lower end. So in between these where the true value lies is still an open question.

Millions of Iron overload patients exist all over the world. A large fraction of them die due to cardiac failure, endocrine failure and suffer from a lot of morbidity. Not all of them received iron chelators and even some those who receive iron chelators either don't receive optimal dose or don't respond or don't tolerate these drugs. Hence, iron chelators don't always extend their longevity. All these patients could benefit a lot if a true NTBI assay would be easily available for clinical purposes which would be a way better marker than ferritin which is presently being used and less invasive than liver biopsy. The T2* weighted MRI for non-invasive iron assessment if not locally validated against Prussian blue stained liver biopsies

may sometimes give misleading results leading to over or under treatment or even potential fatalities.

All the NTBI methods presently are research use only (RUO) and are mostly not available in under developed countries. There have been very few publications in India which use NTBI assay. One publication that we could trace (Tripathi et al, 2019) used an assay from Afferrix Tel Aviv Israel and presently cost approximately 1400 USD (approx. Rs 1 lac) and can test only 44 samples including control per kit. Thus, there is a huge unmet requirement for a fully automated easy clinical grade NTBI assay.

After a thorough study of the literature, we could find only two fully automated NTBI methods and one fully automated LPI method. Of the two fully automated NTBI methods, one[61] (Prezelj et al, 2010) had only one publication and the other[68] (Ito et al, 2014) had two posters and two publications. They use fairly similar principles of homogenous colorimetric assay. We chose the method by Ito et al because it has a claim for a larger dynamic analyte measurement range and a slightly greater number of publication (two rather than one) and reproduced it in our setup. It was originally done in a different fully automated analyser and used a different compound as a calibrator. We used several alternative calibrators, troubleshooted there turbidity issues, and after a lot of pilot experiments narrowed down two materials that could give satisfactory results. After twelve different programs in the automated analyser tried out with various permutations and combinations of filter settings and reading time point settings and other parameters, we narrowed down the various constraints and managed to get a fairly precise results for our NTBI assay which finally used in this study on the frozen supernatant of the timed samples of our storage lesions study.

Selection of calibrator:

We evaluated several iron compounds for developing our in-house calibrator for the NTBI assay. Ferric chloride, Ferrous Ascorbate and Ferrous Ammonium Sulphate were compared because these compounds have been used as calibrators in various published studies.

1) **Ferrous Ascorbate**: The compound was dissolved in distilled water to prepare a 20 micromolar solution. The iron content of the solution was confirmed using the Beckman Coulter Serum Iron assay. The consistency between the measured value and the spiked-in expected value in the serum iron assay established that it did not cause analytical interference in the serum iron assay. The solution was kept at $4^{O}C$ wrapped in aluminium

foil to protect it from potential oxidation and development of turbidity. However, on measuring the same solution for iron on 3 subsequent days, the values obtained had a decreasing trend, possibly because of either oxidation or hydrolysis. Thus, it was deciphered that the compound was not stable enough to qualify as a calibrator.

2) **Ferric chloride**: When we scanned the freshly prepared Ferric chloride solutions of pH 5 and 8 in a spectrophotometer capable of plotting the absorption spectra across the wavelengths we noticed that instead of showing an absorption peak there was a pattern of exponentially increasing absorbance as we go down the wavelengths which is characteristic of a turbid solution even though the solution looked visibly clear to the naked eye.

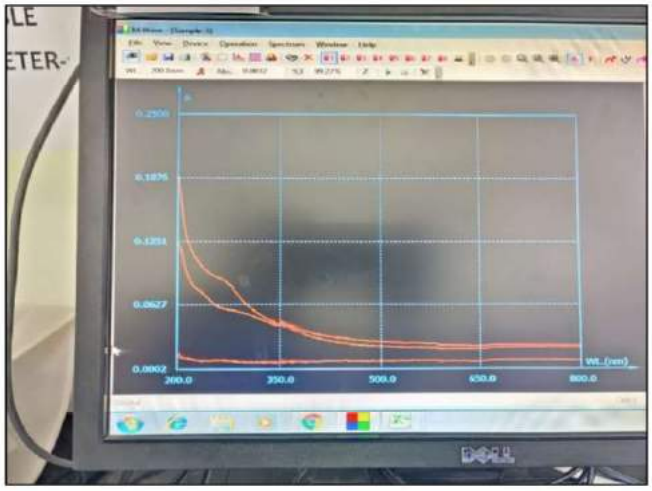


Figure 91: Absorption scan across wavelengths for $FeCl_3$ solutions at different pH.

The topmost curve is the $FeCl_3$ at pH 8 and the next lower curve is the $FeCl_3$ at pH 5 both of which show micro (invisible to naked eye) turbidity pattern and the pH 8 solution is more turbid implying $FeCl_3$ is not truly soluble at either pH and more so at pH 8. We had a premonition that this compound is unlikely to become a choice for our inhouse calibrator because our reactions were to happen at pH 8, albeit at a dilution and in presence of mobilization agent. However, when we attempted to dissolve the $FeCl_3$ with more acidic concentrated HCl or long incubation with Iron mobilization agent present in our reagent it still failed to give values even close to the expected range by the serum Iron assay despite this assay working at a lower pH than our NTBI assay. Our tentative explanation for this discrepancy is that $FeCl_3$ undergoes instantaneous hydrolysis at such pH ranges and a large part of the Iron gets trapped into insoluble polymeric forms

inaccessible to our colour development reagent which requires the iron to become completely monomeric.

3) **Ferrous ammonium sulphate**: Ferrous ammonium sulphate was dissolved in 0.2 N HCl to make two solutions: 5 uM and 10 uM. Both were measured with Beckman Coulter Serum Iron assay. The value obtained were 4.8 uM and 18.9 uM. Since we wanted our inhouse to be sensitive at very low range we selected the 4.8 uM solution as our in-house calibrator and it was used fresh to avoid hydrolysis on standing.

Before we had arrived at the conclusion that Ferrous ammonium sulphate will be our preferred calibrator we have been verifying the absorption peak of various combinations of our iron solution, blank solution, and reagent solutions by scanning through 12 different wavelengths between 300-800 nm.

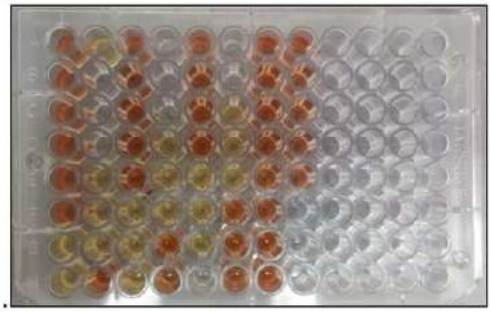


Figure 92: Microwell plate for spectroscopy

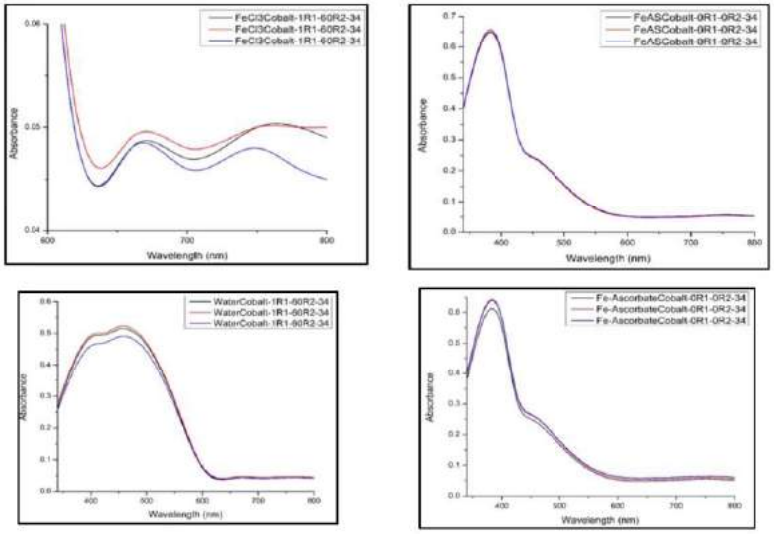


Figure 93: absorption scan of different combination of potential calibrators and various reagent components.

We tried to explore Cobalt as a potential blocker for unsaturated transferrin sites which worked in our hands. However, we did not incorporate blocking in our final experiments because all the newer assays incorporate in the final round robin (de Swarts et al, 2016) didn't use any blocking and mentioned that such blocking might overestimate NTBI rather than underestimation of NTBI without blocking feared by the older literature.

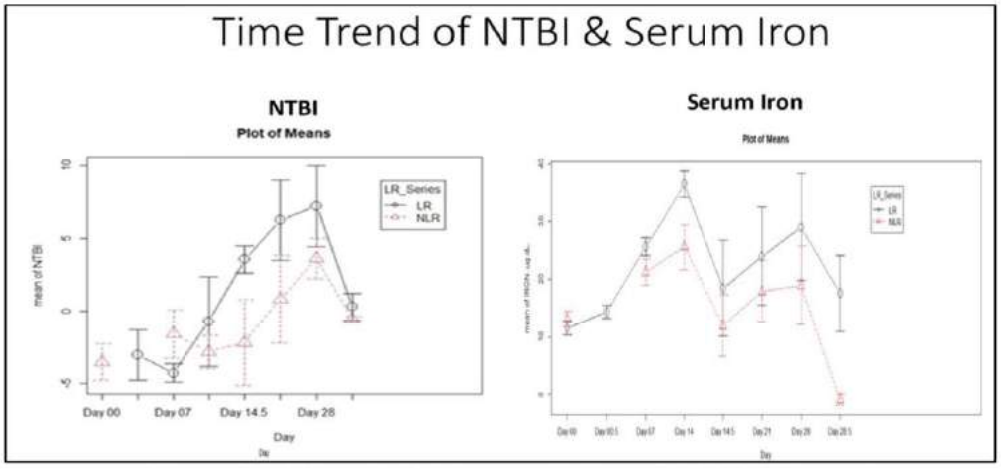


Figure 94: LR vs NLR Time trend plots of NTBI & Serum Iron

The NTBI value are starting from around -4 reaches close to zero on day 7 for NLR group and day 14 for LR group. 4 of the 10 assays in Second Round Robin gave negative values.[62] And the NLR group shows a temporary but significant drop around day 14 followed by rise parallel to the NLR group with a time lag.

NTBI change due to washing is a phenomenon which shows high donor variability. On day 14 the groups W and WW have undergone same handling; however, one is showing rise and other is showing drop in NTBI which is likely due to donor variability.

Serum Iron is showing very clear parallelism to free Hb rather than LDH and is higher in the LR group, whereas LDH is higher in NLR group. It is tempting to speculate that during leukoreduction process there is a burst of pinching off of Hb containing membrane micro vesicles some of which got released in form of free iron, which contributed to the serum iron assay. The free Hb and serum iron are both easily washable whereas NTBI washing seems to be variable.

A novel finding of our study is that in LR units NTBI levels remained "below zero" up to 2 weeks which coincide with the freshness cut off or Leukoreduced units traditional transfused

to thalassaemics. However, in NLR units NTBI levels may counter-intuitively remain "below zero" up to 3 weeks. This might suggest that NLR units which are commonly used in a country like India may be usable in thalassaemics for longer than previously thought and make this precious resource available for a longer utilization window that may benefit thousands.

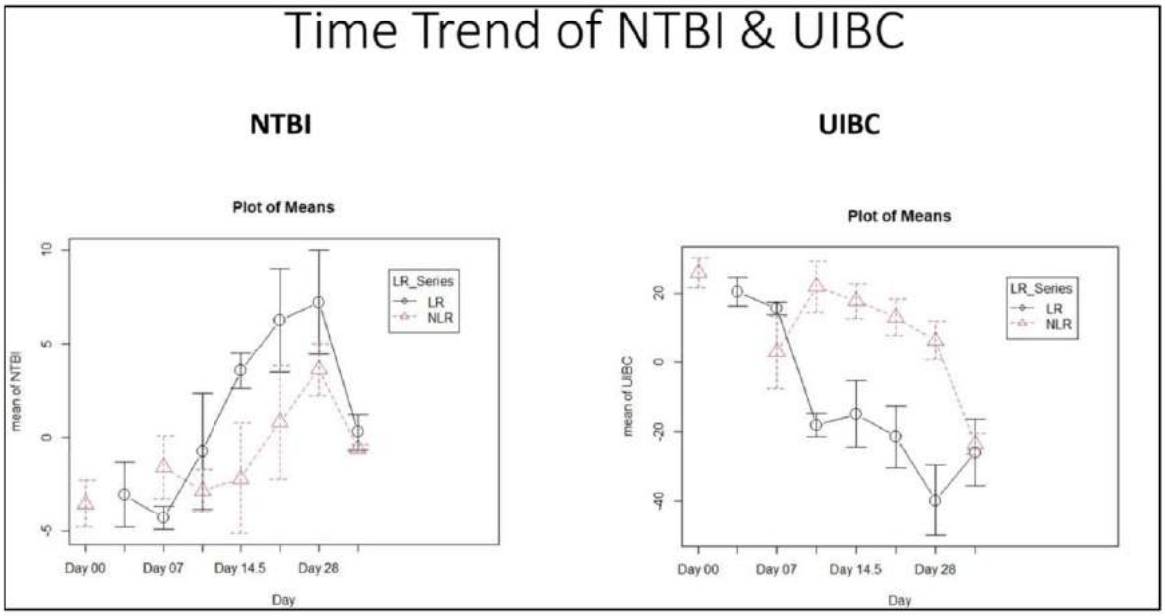


Figure 95: LR vs NLR time trend plots of NTBI and UIBC

LR group seems to have significantly higher NTBI which is somewhat consistent with the hypothesis that a fraction of free Hb converts to NTBI, however, our study also has a confounding factor of skewed MCHC distribution between the two groups.

In our study accidentally it so happens that NLR group has lower MCHC likely because of subclinical iron deficiency. Thus, in case, higher MCHC also contribute to higher NTBI, we would not be able to decipher it.

Table 10: Correlations of NTBI with different hemolysis related parameters										
		NTBI	Free Hb	% Hemo LYSIS	Pink %	SLTx	IRON (ug/dL)	TOTAL BILIRUBIN (mg/dL)	AST (U/L)	LDH (U/L)
NTBI	Pearson Correlation	1	.550**	.505**	.209*	-0.076	.399**	.278**	.260**	.348**
	Sig. (2-tailed)		0.000	0.000	0.001	0.249	0.000	0.000	0.000	0.000
	N	232	232	232	232	232	232	232	232	232

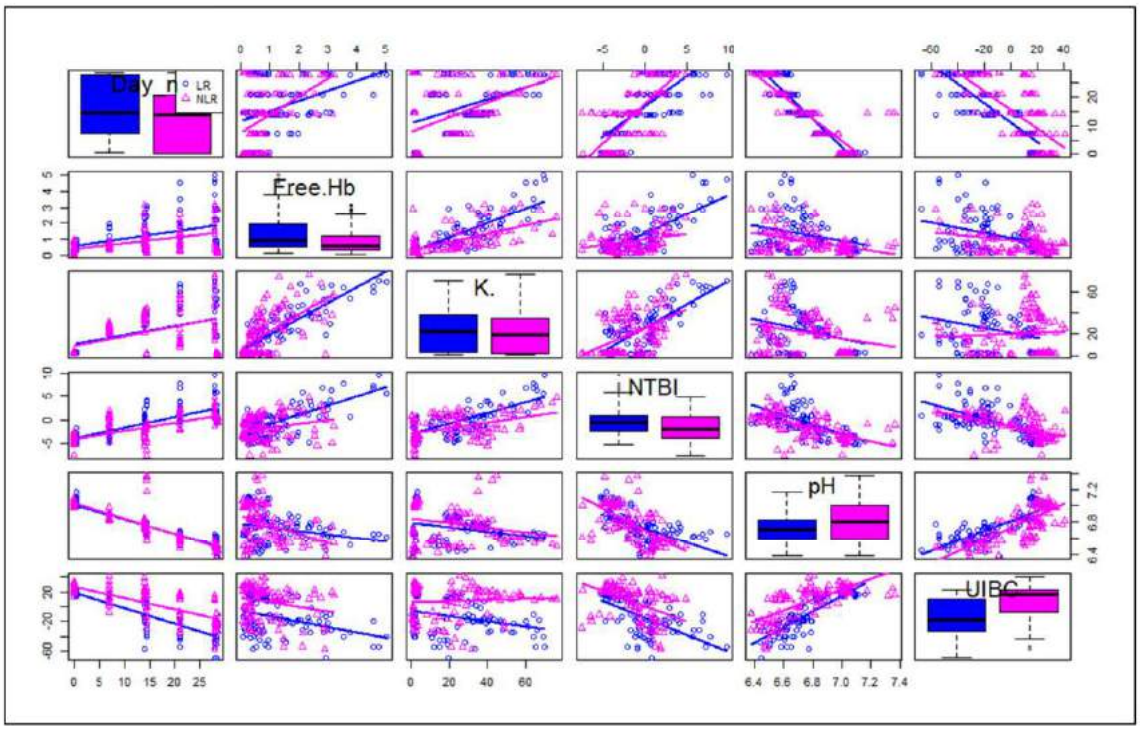


Figure 96: Matrix Plot Showing Correlation and Group-wise regression lines of NTBI versus some of the best correlating parameters (Red is Non-Leukoreduced, and Blue is Leukoreduced). Diagonals show the Box plot of LR vs LNR and the other cells show pairwise Linear-regressions.

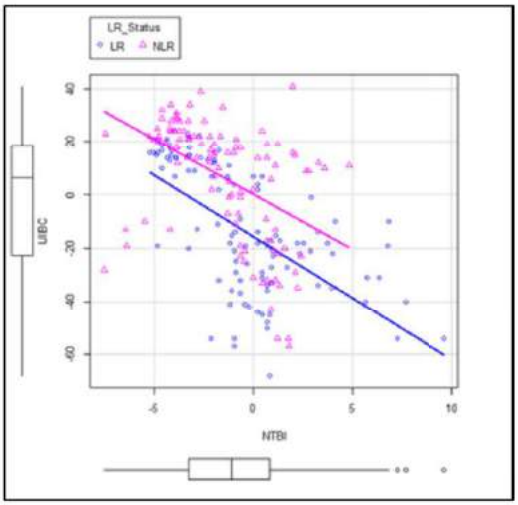


Figure 97: Correlation of NTBI with UIBC

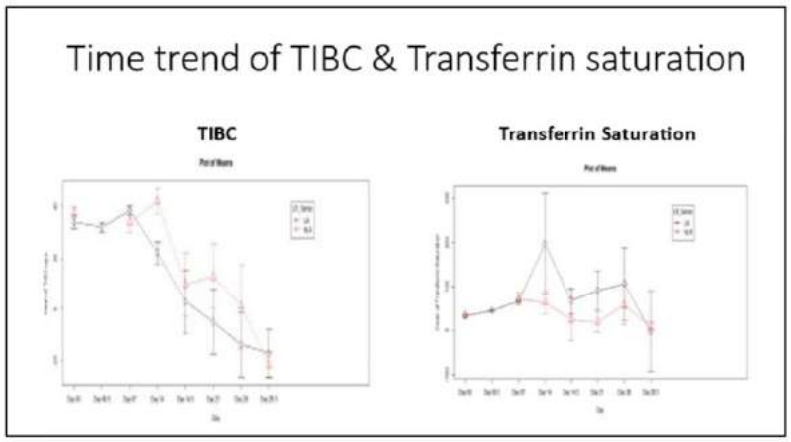


Figure 98: LR VS NLR time trend plots of TIBC & Transferrin saturation

The rise in NTBI has caused a negative value for UIBC. And since TIBC is equal to serum Iron plus UIBC the drop in UIBC seems to be more dominant than the rise in serum iron. The LR group which has higher free Hb and higher free NTBI naturally also has higer trend of transferrin saturation and it is at a time point where transferrin saturation reaches 100 and NTBI reaches 0.

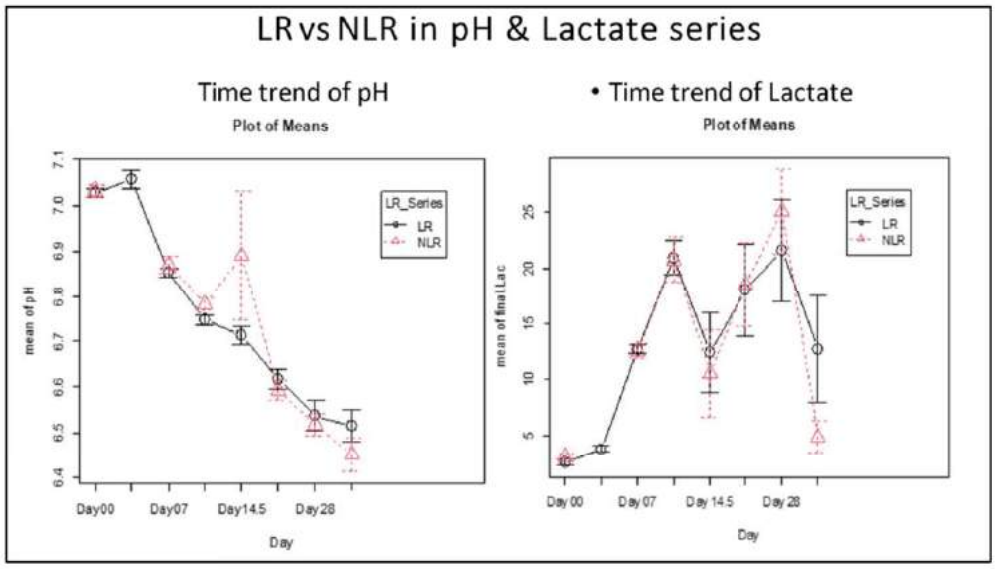


Figure 99: LR vs NLR time trend plots for pH and Lactate

There is a transient rise of pH four hours post wash in the NLR group but not in the LR group. This corroborates with corresponding ATP rise in the NLR group.

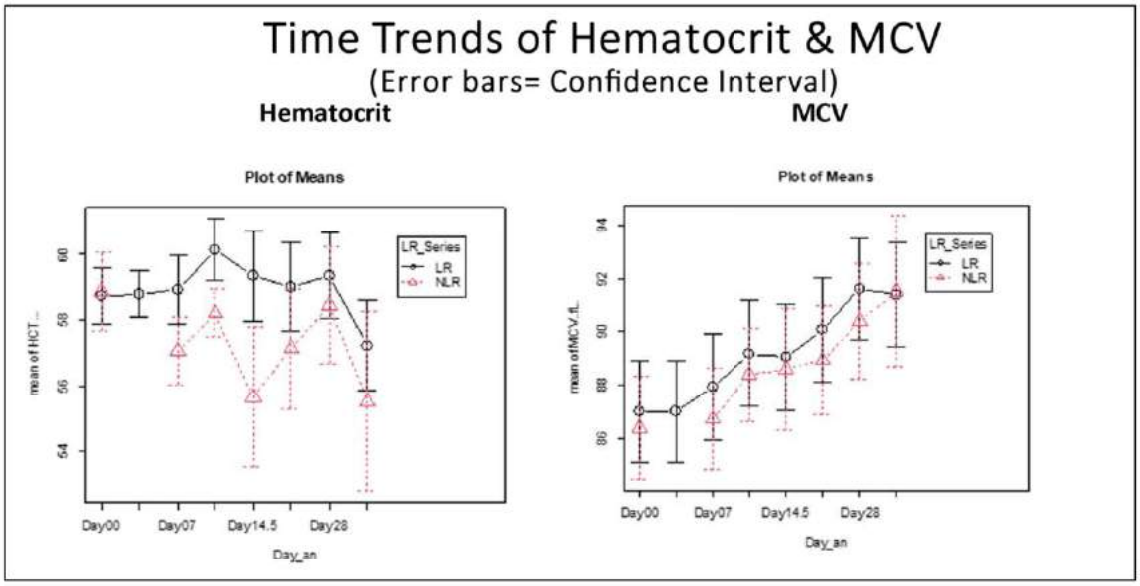


Figure 100: LR vs NLR time trend plots for Hematocrit and MCV

Trend of lower MCV in the NLR group, though statistically not significant, is consistent with subclinical iron deficiency.

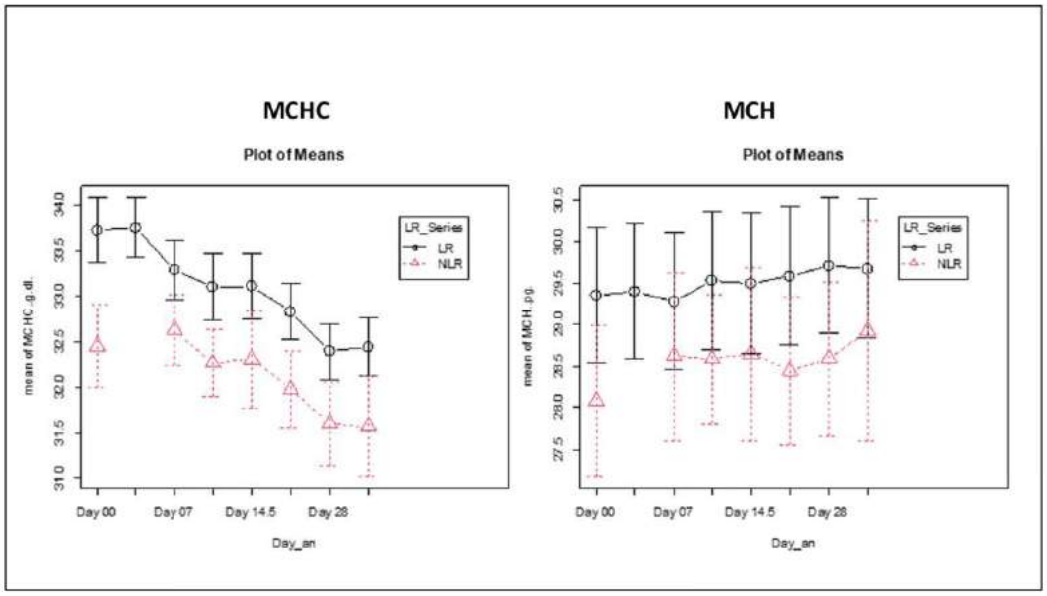


Figure 101: LR vs NLR time trend plots for MCHC and MCH

Due to logistic reasons people who were recruited in the LR series accidently happen to be first time voluntary donors in a camp outside the blood bank, whereas people in the NLR series included 12 replacement donors and 16 repeat voluntary donors. This is consistent with possible subclinical iron deficiency among majority of NLR group donors as evidenced by lower MCHC and MCH which explains why the LR group also has a better trend of Hb and hematocrit as well across the storage period.

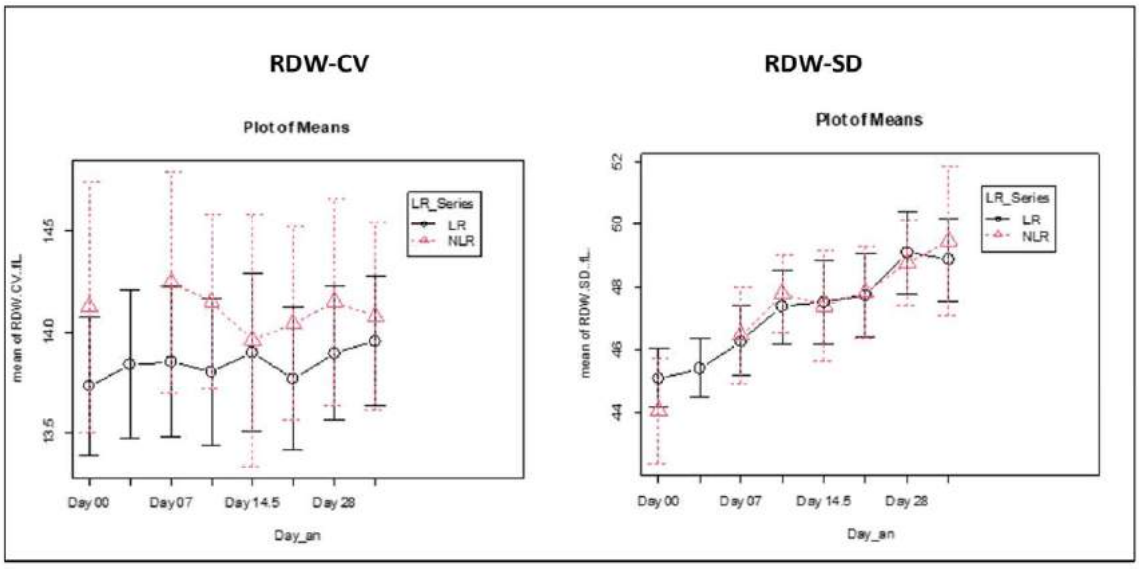


Figure 102: LR vs NLR time trend plots for MCHC and MCH

The higher RDW trend though stastically not significant is consistent with subclinical iron deficiency in the NLR group. Since RDW uses MCV in the denominator it is a more sensitive indicator than RDW-SD.

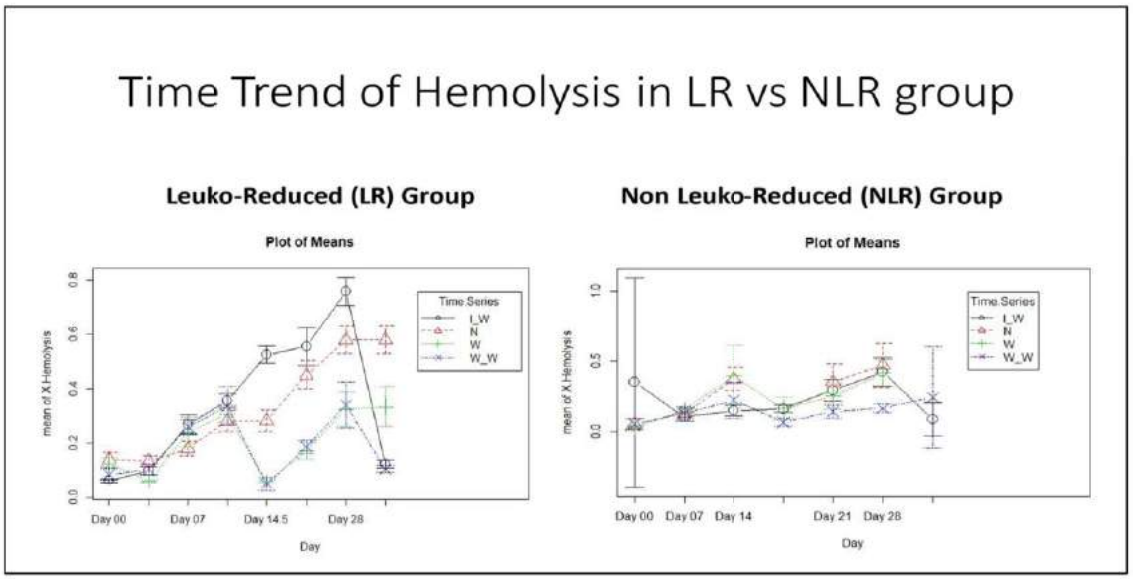


Figure 103: LR vs NLR time trend plots of hemolysis.

Subclinical iron deficiency cannot explain the difference between LR and NLR group because physiologically free Hb is barely detectable in both groups.

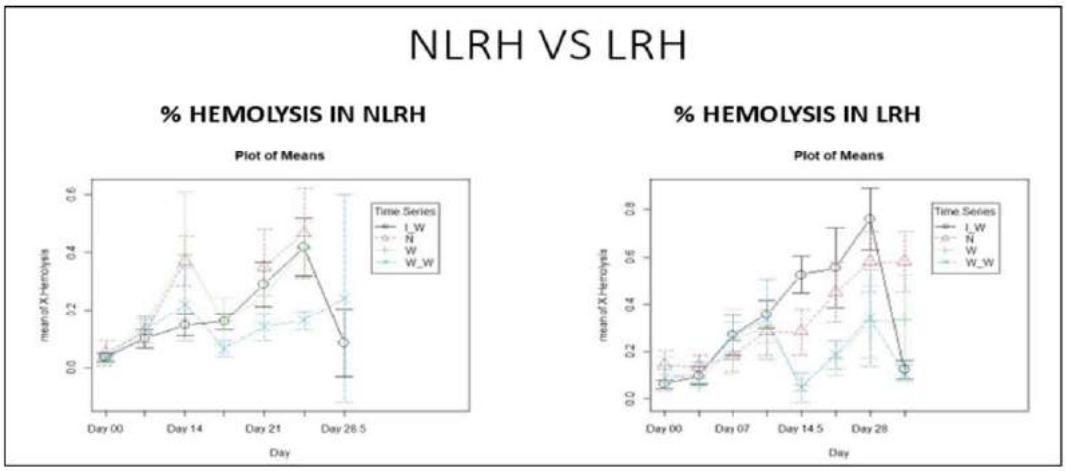


Figure 104: LR vs NLR time trend plots of hemolysis .

Subclinical iron deficiency cannot explain the difference between LR and NLR group because physiologically free Hb is barely detectable in both groups.

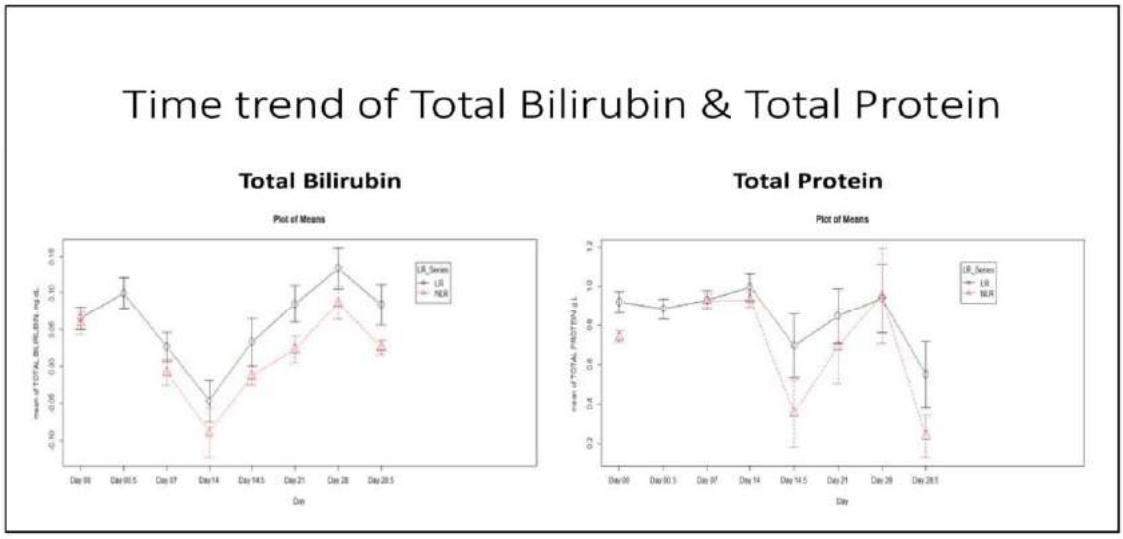


Figure 105: LR vs NLR time trend plots of Total Bilirubin & Total Protein

The total bilirubin is showing trends parallel to free Hb (LR > NLR) which means that even though we assume that leukocytes are degenerated and barely metabolically active at cold temperature, bilirubin synthesis must have happened even in the cold, and in the same pattern as free Hb (LR> NLR) after bilirubin has been washed away.

Total protein shows drop during washing as expected. The Leukoreduced group shows a mild rise in total Hb during the first 20 days and lesser drop than NLR group during washing. The NLR group shows a much steeper drop than LR group in the total protein during both wash 1 and wash 2.

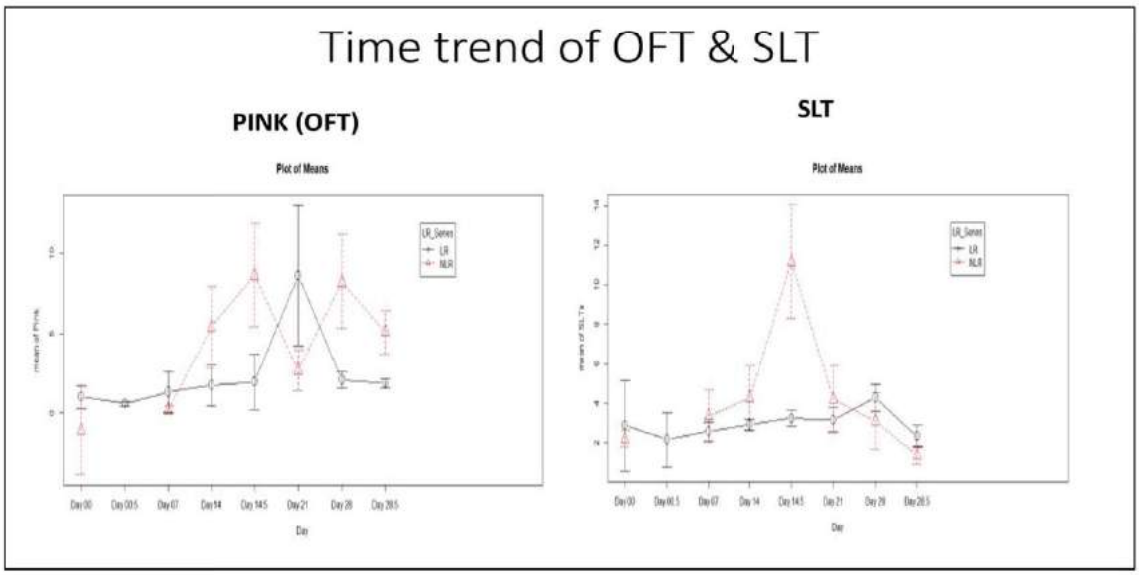


Figure 106: LR vs NLR time trend plot of OFT & SLT

The NLR is showing a trough of osmotic fragility on day 21, which is somewhat similar to the literature (Blasi et al, 2013 and stone et al, 2018) however, the LR group is showing a transient rise in day 21.

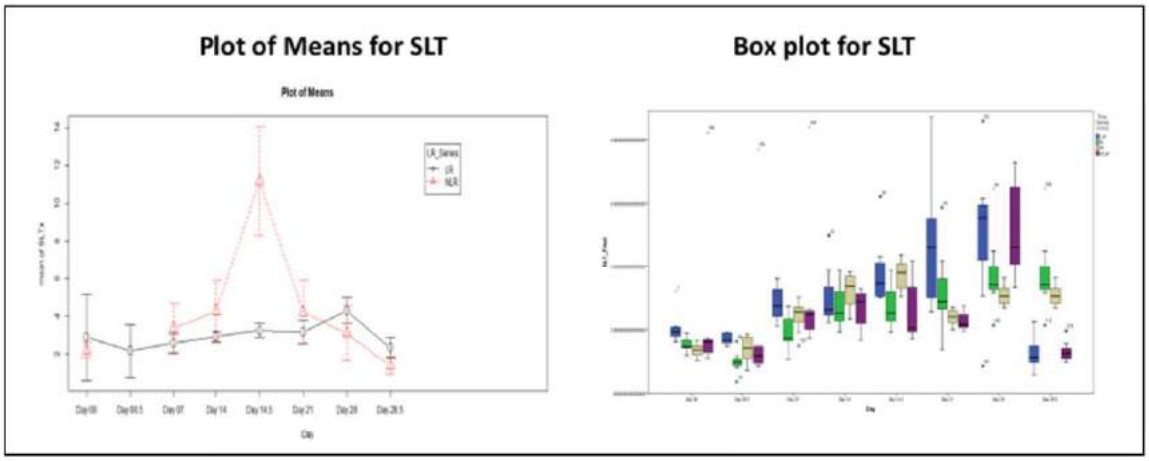


Figure 107: Plot of means and Box plot for SLT

The NLR group is showing a transient but highly significant rise in complement fixing potential (SLT) at day 14.5 that is at 4 hours after washing which is not seen at all in the LR group.The LR group, from day 0.5 should have no leukocytes or platelets. Whereas, the NLR group will have degenerating WBCs and platelets which may have contributed to the transient complement fixing phenomenon during washing in the NLR group.

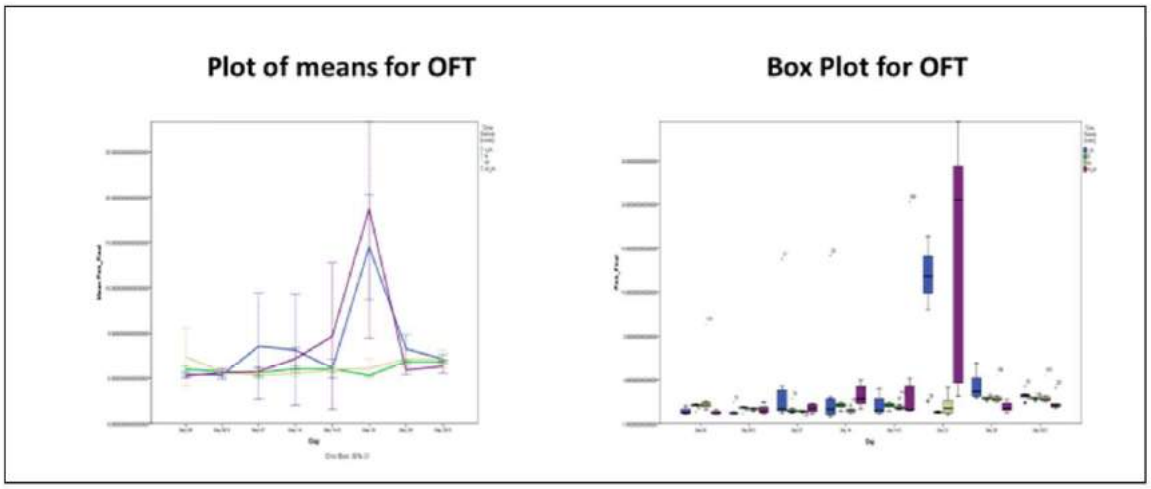


Figure 108: Plot of means & Box plot for OFT

Irradiation and washing are both harsh modifications which cause persistent rise in osmotic fragility in these two subgroups till day 21.

The leukoreduction happened on Day 0 whereas washing and irradiation happened 4 hours before sampling time 14.5 and the osmotic fragility peak happened only in irradiated and washed subgroup but not in non-irradiated non-washed group.

There are very few literatures about trend of osmotic fragility during storage Blasi et al 2013 used classical osmotic fragility test and showed that there is a reduction in osmotic fragility till mid storage followed by further rise. Stone et al 2018, used pink test and showed similar findings however, neither Blasi nor stone show any data regarding effect of irradiation and washing on osmotic fragility. Our data showed a trough of osmotic fragility on day 28. while irradiation on day 14 shows a transient sharp rise in osmotic fragility, washing shows rise in osmotic fragility only in subset, e.g. More in WW than W but all subside down to the trough by day 28.

STRENGTH:

1. We have developed a single load walk away automation which takes less than a minute to program and and the result is obtained within 20 minutes. A throughput of 2000 tests/ hour can be obtained with this method
2. Our study shows that in case NTBI in supernatant is a critical factor determining the utilisation window of ageing red cells then NLR units may be able to serve longer possibly 3 weeks rather than 2 weeks.
3. Sample size of 56 gave us significant p value in several parameters between NLR and LR group.
4. Such extensive systematic exploration of questions as fundamental as whether leukoreduction and washing in various combinations are beneficial or harmful to quality of units.

LIMITATIONS:

1. We have an unintended confounding factor as MCHC due to recruitment of donors on different days with an imbalance in the number of voluntary repeat donors in the NLR and LR group
2. We have studied the blood bags for 28 days but whether studying them for 42 days would cause any drastic change in the time trend was outside the scope of this study.
3. We have done the study with 50 mL bag volume which is equivalent of pediatric aliquot bags (pedipack) but our present study did not include any full size bag. However our data largely corroborates with similar data from full and half sized and smaller bag sizes studied by our group earlier, except the NTBI part which is so new that it has not been studies in other bag sizes yet.
4. We have used fresh reagents for every run because when we tried to use the reagent after 4 days of preparation, we didn't get satisfactory result. We did not try to pin-point the exact stability of the reagent. However, all the thesis samples were run with the same batch of fresh reagents to avoid batch to batch variation.
5. Our study is completely in vitro, so observing the effects in vivo will be our future goal

CONCLUSION

CONCLUSION

This is the first ever study in the world investigating NTBI ex-vivo in relation to blood unit irradiation and leuko-reduction. We successfully developed from scratch a fully automated homogenous colorimetric NTBI assay including all its reagent and calibrators by adapting from one of the only two published fully automated NTBI assays available in the literature till date, and used it for our study. The biological behaviour of our measured NTBI parameter in the stored blood was consistent with the scant literature available so far and also showed several novel but clinically useful findings.

The closed system washing & assessment of its effectiveness in this study might help extend the utilization window of precious antigen typed units for thalassemia patients beyond 2 weeks from collection. It will also help extend the safety-window of post-irradiation-storage. Despite legal acceptance of viability, irradiated units are rarely used in the later part of the legal time window for many patients because validation data for washing such units are unavailable, especially in our country.

We have a novel finding that surprisingly NLR group has lower NTBI than LR group and maintains a relatively safe NTBI levels till day 21 in contrast to similar level at day 14 for the LR group. The international guideline of less than 2 weeks fresh RBCs to be transfused to Thalassemics coincide with the NTBI crossing the safe levels in the Leukoreduced units. However, it might be possible that Non-Leukoreduced units might be safer in terms of NTBI for iron overload patients up to 3 weeks.

In the western countries transfusion dependent thalassaemic patients are generally given LR units. Though leukoreduction reduces true hemolysis as evidenced by LDH and AST, our study shows that during the early storage (even up to 4 weeks) Potassium leak and free Hb (which includes Hb in micro vesicles shed by RBCs) remain higher in the LR units and it correlates with NTBI more than LDH or AST. Despite benefit of reducing chances of HLA allo-immunisation, leukoreduction seems to have this relatively less known disadvantage of higher early apparent hemolysis (% hemolysis calculated from plasma Hb) which translates to higher NTBI regarding which to the best of our knowledge there is no published data in the searchable domain.

We observed that on Day 14 after washing the NTBI decreases in the NLR group but unexpectedly increased in the LR group though not at statistically significant levels. However, at day 28 washing significantly reduced the NTBI. During early storage since NTBI level is below zero washing does not further reduce NTBI rather might induce mild hemolysis and NTBI rise which is statistically insignificant. However, in late storage when NTBI is detectable at positive levels or aggravated post irradiation, washing is able to reduce the NTBI level to below zero.

Irradiation does seem to increase hemolysis as well as NTBI as speculated. However, Leukoreduction seem to cause higher NTBI. Though apparently counter intuitive it is consistent with higher apparent hemolysis during early storage in the LR group and is likely to be caused by vesiculation of hemoglobin containing micro vesicles during leukofiltration which contribute to free Hb but not so much to LDH.

In a country like India where leukofilters are too costly to be available to poor patients suffering from thalassaemia Non-leukoreduced units are commonly used for most of them.
Our study suggest that NLR units may be safe to transfuse to thalassaemic patients for a safety window (Possibly 3 weeks) longer than previously thought (2 weeks), and thus might make this precious resource available for a longer utilization window that may benefit thousands.

BIBLIOGRAPHY

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ANNEXURES

ANNEXURES – 1

A. ICMR GRANT LETTER

भारतीय आयुर्विज्ञान अनुसंधान परिषद
स्वास्थ्य अनुसंधान विभाग, स्वास्थ्य और परिवार
कल्याण मंत्रालय, भारत सरकार

Indian Council of Medical Research
Department of Health Research, Ministry of Health
and Family Welfare, Government of India

No.3/2/June-2020/PG-Thesis-HRD (29) **Dated: 02.09.2020**

Dr. Richa Mishra
All India Institute of Medical Sciences,
Basni Industrial Area, Mia, 2nd Phase, Basni
Jodhpur-342005, Rajasthan
Registration No. MD20JUN-0169

Subject: - Award of ICMR Financial Support for the MD/MS/DM/MCh/MDS dissertation /thesis for June 2020 batch-reg.

Dear Dr. Richa Mishra,

This is with reference to your application seeking financial assistance from the ICMR for MD/MS/DM/MCh/MDS dissertation/thesis entitled **"Ex-vivo effects of washing and leukoreduction on iron profile, including non-transferrin-bound-iron and storage lesions in non-irradiated and irradiated packed red blood cell (PRBC) units"**.

I am glad to inform you that, based on the recommendation of Expert Committee, Director General, ICMR, has approved your application / thesis for the financial support of **Rs. 50,000/- (Fifty thousand only)** as stated above, which will be disbursed in two /three installments. Initial amount of **Rs. 30,000/-** will be released after receipt of the Undertaking as per the guidelines and remaining amount of **Rs. 20,000/-** on receipt of the electronic copy and summary of work done of your dissertation / thesis duly approved by the University/ Institute along with one publication in an indexed Journal. Mandatory requirement to avail this opportunity is to submit an Undertaking duly forwarded through the Guide, to the undersigned, enabling us to release the first Installment.

Kindly also submit the Guide details as well as the MANDATE FORM (available on ICMR website) along with a photocopy of a Cancelled Cheque (Please ignore, if already submitted) latest by 21st September, 2020 for receiving e-payment for purpose of verification of the concerned bank account where money is to be remitted.

Yours faithfully
(Ishwar Likhar)
Administrative Officer
(For Director General)

Copy to:

1. Guide: - Dr. Saptarshi Mandal, Dept. of Transfusion Medicine and Blood Bank , All India Institute of Medical Sciences, Basni Industrial Area, Mia, 2nd Phase, Basni, Jodhpur-342005, Rajasthan

वी. रामलिंगस्वामी भवन, पोस्ट बॉक्स नं. 4911,
अंसारी नगर, नई दिल्ली - 110 029, भारत
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B. Ethical Clearance Certificate

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2020/ 2091 **Date: 01/01/2020**

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2019-20/1012**

Project title: **"Ex-Vivo Effects of Washing and Leukoreduction on Iron Profile, including "Non-Transferrin-Bound-Iron" (NTBI), and "Storage Lesions" in Non-Irradiated and Irradiated Packed Red Blood Cell (PRBC) Units"**

Nature of Project:	**Research Project**
Submitted as:	**M.D. Dissertation**
Guide Name:	**Dr. Saptarshi Mandal**
Student Name:	**Dr. Richa Mishra**
Co-Guide:	**Dr. Mahendra Kumar Garg, Dr. Pradeep Kumar Bhatia, Dr. Poonam Elhence, Dr. Anuradha Sharma, Dr. Mithu Banerjee & Dr. Pradeep Dwivedi**

This is to inform that members of Institutional Ethics Committee (Annexure attached) met on **23-12-2019** and after through consideration accorded its approval on above project. Further, should any other methodology be used, would require separate authorization.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Pravesh Sharma
Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Enclose:
1. Annexure 1

Page 1 of 2

Basni Phase-2, Jodhpur, Rajasthan-342005, Website: www.aiimsjodhpur.edu.in, Phone: 0291-2740741 Extn. 3109
Email: ethicscommittee@aiimsjodhpur.edu.in

Annexure I

Institutional Ethics Committee
All India Institution of Medical Sciences, Jodhpur

Meeting of Institutional Ethics committee held on **23-12-2019 at 10:00 AM** at Committee Room, Admin Block AIIMS Jodhpur.

Following members were participated in the meeting:-

S/No.	Name of Member	Qualification	Role/Designation in Ethics Committee
1.	Dr. F.S.K Barar	MBBS, MD (Pharmacology)	**Chairman**
2.	Justice N.N Mathur	LLB	Legal Expert
3.	Dr. Varsha Sharma	M.A (Sociology)	Social Scientist
4.	Mr. B.S.Yadav	B.Sc., M.Sc. (Physics), B.Ed.	Lay Person
5.	Dr. K.R.Haldiya	MD (General Medicine)	Clinician
6.	Dr. Arvind Mathur	MBBS, MS (General Medicine)	Clinician
7.	Dr. Surajit Ghatak	MBBS, MS (Anatomy)	Basic Medical Scientist
8.	Dr. Vijaya Lakshmi Nag	MBBS, MD (Microbiology)	Basic Medical Scientist
9.	Dr. Sneha Ambwani	MBBS, MD (Pharmacology)	Basic Medical Scientist
10.	Dr. Kuldeep Singh	MBBS, MD (Paediatric), DM (General Medicine)	Clinician
11.	Dr. Abhinav Dixit	MBBS, MD (Physiology), DNB (Physiology)	Basic Medical Scientist
12.	Dr. Pradeep Kumar Bhatia	MBBS, MD (Anaesthesiology)	Clinician
13.	Dr. Tanuj Kanchan	MBBS, MD (Forensic Medicine)	Basic Medical Scientist
14.	Dr. Pankaj Bhardwaj	MBBS, MD (CM&FM)	Clinician
15.	Dr. Praveen Sharma	M.Sc., Ph.D. (Biochemistry)	Member Secretary

Dr. Praveen Sharma

ANNEXURES - 2

All India Institute of Medical Sciences, Jodhpur

Informed Consent Form

Title of the project: - Ex-vivo effects of washing and leukoreduction on iron profile, including non-transferrin-bound-iron and storage lesions in non-irradiated and irradiated packed red blood cell (PRBC) units

Name of the PG Student : Dr. Richa Mishra

Tel. No. (Mobile) : +91-9460458328

Registration/unit No: _____

I, _____S/o or D/o_____

R/o_____

give my full, free, voluntary consent to be a part of the study -" Ex-vivo effects of washing and leukoreduction on iron profile, including non-transferrin-bound-iron and storage lesions in non-irradiated and irradiated packed red blood cell (PRBC) units", the procedure and nature of which has been explained to me in my own language to my full satisfaction. I confirm that I have had the opportunity to ask questions.

I have been explained that in India there are 2 types of licensed blood bags(350&450ml) which are routinely used. I have been informed that my blood donated in a 450 ml bag will be converted to its 350 ml equivalent and the extra volume so obtained will be used in the study and the rest will be available to be transfused to patients.

I understand that my participation is voluntary and I have been made aware of the fact that my donated blood will be given to patient if it meets the quality and safety standards.

I am aware of my right to opt out of the study at any time without giving any reason.

I understand that the information collected about me and any of my medical records/test reports may be looked at by responsible individual from AIIMS Jodhpur or from regulatory authorities. I give permission for these individuals to have access to my records.

Date: _____ _____

Place: _____ Signature/Left thumb impression

This to certify that the above consent has been obtained in my presence.

Date: _____ _____

Place: _____ Signature of PG Student

1. Witness 1

Signature

Name: _____

Address: _____

2.Witness2

Signature

Name:_____

Address: _____

ANNEXURES - 3

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर

सूचित सहमति प्रपत्र

अध्ययन का शीर्षक: पैक्ड रेड ब्लड सेल (पी आर बी सी) में नॉन ट्रांसफरिन बाऊंड आयरन समेत आयरन प्रोफाइल एवं भंडारण क्षति पर ल्यूकोरीडक्शन एवं वाशिंग के एक्स विवो प्रभाव का अध्ययन

अन्वेषक का नाम: डॉ.ऋचा मिश्र

दूरभाष नंबर (मोबाइल): + 91-9460458328

पंजीकरण/ यूनिट संख्या : _____

मैं, _____ **पिता/ पति** _____

पता _____ **अध्ययन का एक हिस्सा बनने के लिए मेरी पूर्ण, स्वतंत्र, स्वैच्छिक सहमति दें :"पैक्ड रेड ब्लड सेल (पी आर बी सी) में नॉन ट्रांसफरिन बाऊंड आयरन समेत आयरन प्रोफाइल एवं भंडारण क्षति पर ल्यूकोरीडक्शन एवं वाशिंग के एक्स विवो प्रभाव का अध्ययन ", जिसकी प्रक्रिया और प्रकृति को मेरी अपनी भाषा में मेरी पूर्ण संतुष्टि के लिए समझाया गया है मैं पुष्टि करता हूं कि मुझे प्रश्न पूछने का अवसर मिला है।**

मुझे समझाया गया है कि भारत में 2 प्रकार के लाइसेंस प्राप्त रक्त बैग (350 और 450 मिली) हैं जो नियमित रूप से उपयोग किए जाते हैं। मुझे सूचित किया गया है कि 450 मिलीलीटर की थैली में दान किया गया मेरा रक्त इसके 350 मिलीलीटर के बराबर में परिवर्तित हो जाएगा और प्राप्त अतिरिक्त मात्रा का उपयोग अध्ययन में किया जाएगा और शेष रोगियों को रक्त आधान के लिए उपलब्ध होगा।

मैं समझता हूं कि मेरी भागीदारी स्वैच्छिक है और मुझे इस तथ्य से अवगत करा दिया गया है कि अगर मेरा रक्त सुरक्षा एवं गुणवत्ता मानकों को पूरा करता है तो मेरा दान रक्त रोगी को दिया जाएगा।

मुझे इस तथ्य से अवगत करा दिया गया है कि मुझे बिना किसी भी कारण के किसी भी समय अध्ययन से बाहर निकलने का मेरा अधिकार है।

मैं समझता हूं कि मेरे और मेरे किसी भी मेडिकल रिकॉर्ड/ जाँच रिपोर्ट के बारे में एकत्रित की गई जानकारी को एम्स, जोधपुर या विनियामक प्राधिकरणों से जिम्मेदार व्यक्ति द्वारा देखा जा सकता है। मैं इन लोगों के लिए मेरे रिकॉर्डों तक पहुंच की अनुमति देता हूं

तारीख: _____ _____

जगह: _____ **हस्ताक्षर / बाएं अंगूठे का छाप**

यह प्रमाणित करने के लिए कि मेरी उपस्थिति में उपरोक्त सहमति प्राप्त की गई है

तारीख: _____ _____

जगह: _____ **स्नातकोत्तर छात्र का हस्ताक्षर**

गवाह 1 **गवाह 2**

हस्ताक् _____ **हस्ताक्षर** _____

नाम: _____ **नाम:** _____

पता: _____ **पता:** _____

ANNEXURES - 4

All India Institute of Medical Sciences, Jodhpur

Information sheet

1. Title of the study: Ex-vivo effects of washing and leukoreduction on iron profile, including non-transferrin-bound-iron and storage lesions in non-irradiated and irradiated packed red blood cell (PRBC) units
2. Purpose of Study: Blood stored in the Blood Bank uses only licensed material and licensed methods and perform quality tests routinely. However in order to further improve the quality of the stored blood research is ongoing in the whole world to find out more sophisticated quality assessment parameters. We will be testing additional investigational storage quality parameters discovered by latest research.
3. Study procedures to be followed: Participants attending Blood Bank at AIIMS, Jodhpur are routinely evaluated for:

a) Parameters: Weight, Pulse, Blood Pressure, Temperature, Hemoglobin etc.

b) Blood Grouping

c) Transfusion Transmitted Infection testing

If all the above tests fit in the inclusion criteria for our study and if selected by "random allocation" (like a lottery) for special tests then your donated blood may undergo a series of additional advanced research quality parameters.

1. Benefits from the study: not directly but contribution to the society and patients to whom the red cells will be transfused
2. Risks of the study: None
3. Complications of the study: None
4. Confidentiality: Data collected from the participant shall not be shared with anyone except the study investigators.
5. Rights of participants: Participants would have the freedom to share their data and to continue or leave the study if they desire so.

ANNEXURES - 5

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर

सूचना पत्रक

1. **अध्ययन का शीर्षक: : पैक्ड रेड ब्लड सेल (पी आर बी सी) में नॉन ट्रांसफरिन बाऊंड आयरन समेत आयरन प्रोफाइल एवं भंडारण क्षति पर ल्यूकोरीडक्शन एवं वाशिंग के एक्स विवो प्रभाव का अध्ययन**
2. अध्ययन का उद्देश्य: पैक्ड रेड ब्लड सेल (पी आर बी सी) में नॉन ट्रांसफरिन बाऊंड आयरन समेत आयरन प्रोफाइल एवं भंडारण क्षति पर ल्यूकोरीडक्शन एवं वाशिंग के एक्स विवो प्रभाव का अध्ययन
3. अध्ययन प्रक्रिया: एम्स जोधपुर में प्रत्येक प्रतिभागी का निम्नलिखित मूल्यांकन किया जाता है।

   क) वजन - पल्स, ब्लड प्रेशर, तापमान, हिमोग्लोबिन

   ख) रक्त ग्रुपिंग

   ग) खून से फैलने वाला संक्रमण का परीक्षण

   यदि उपरोक्त सभी जाँचे हमारे अध्ययन में शामिल किए जाने वाले मानकों में सफल होती हैं, और यदि विशेष परीक्षणों के लिए "रेण्डम" (लॉटरी की तरह) आवंटन द्वारा चुना जाता है तो आपके द्वारा दान किए गए भंडारित रक्त की हम कुछ उन्नत जाँचे करेंगे ।
4. अध्ययन से लाभ: सीधे नहीं, लेकिन समाज और मरीजों में योगदान, जिन्हें लाल रक्त कोशिकाओं को ट्रांसफ्यूज़ किया जाएगा
5. अध्ययन के जोखिम: अध्ययन से संबंधित नहीं
6. अध्ययन की जटिलताओं: कोई नहीं
7. गोपनीयता: प्रतिभागी से एकत्रित आंकड़े अध्ययन जांचकर्ताओं को छोड़कर किसी के साथ साझा नहीं किया जाएगा।
8. प्रतिभागियों के अधिकार: प्रतिभागियों को अपने डेटा साझा करने और यदि वे चाहें तो अध्ययन जारी रखने या छोड़ने की स्वतंत्रता होगी।

ANNEXURES - 6

CASE RECORD FORM

DEMOGRAPHIC DATA

Name……Age……..Yrs Sex-M/F

Registration/unit No…….. Bag Segment no……

Contact No……..

Marital status: …… Occupation:……Monthly income: ……

Anthropometric & other baseline parameters:

Variable	
Height (meters)	
Weight (kg)	
Waist circumference (cm)	
Blood Pressure (mm Hg)	
Body Mass index (kg/meter2)	

Other relevant demographic data / history cropped from routine donation form:

ANNEXURES – 7

Parameters Chart

Parameters	Sampling/subsampling date	Value
CBC		
IRON PROFILE		
LIVER FUNCTION TEST (Total protein, Bilirubin, AST)		
LDH		
FREE Hb		
NTBI /LPI		
VBG (Electrolytes, Glucose, Lactate)		
MORPHOLOGY RELATED PARAMETERS		
COMPLEMENT SENSITIVITY TEST (Microplate Sucrose lysis)		
RBC OSMOTIC FRAGILITY TEST (AGLT)		
ATP (intracellular)		

ANNEXURES – 8

Master Chart