Impact of Pro-Inflammatory Mediators on Agonist-Induced Platelet Activation

by

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2022

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A thesis submitted in partial fulfillment of the requirements for the

Degree of Master of Philosophy

in

Biochemistry/Molecular Biology

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2022

CERTIFICATE

This thesis submitted by **Ms. Samra Fatima** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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DECLARATION

I **Samra Fatima** hereby declare that any part of this dissertation is not plagiarized, and all work was carried out in accordance with regulation set by the Quaid-i-Azam University. No part of this thesis has been previously presented for any other degree. If found anything contrary, I shall be held responsible.

Samra Fatima

Dedicated

to

My Parents and Siblings

who are light and love of my life

ACKNOWLEDGEMENTS

First and foremost, I express my deep gratitude to ALLAH (S.W.T), the Most Gracious, the Most Merciful for His blessings and courage to complete my study and this thesis. May Allah's blessing goes to His final Prophet Muhammad (peace be up on him), his family and his companions.

I am grateful to the Chairperson, Department of Biochemistry **Dr Irum Murtaza** for providing us with all research facilities and healthy research environment.

I present my huge respect and gratitude to my supervisor **Dr. M. Rizwan Alam** whose constant support, patience and guidance made me to complete my research work. His motivation, scholarly advice deeply inspired me. Not just as supervisor he is also a best mentor and teacher. I shall always owe the debt of his support and guidance.

I have no words to express my thanks to **Durre Shehwar** who helped me inch by inch throughout my research and thesis writing. Her kind behavior and support made me to work without any tension and stress.

I extend my gratitude to my other laboratory fellows; without those it would be difficult to achieve my goal. I am thankful to my Ph.D. seniors **Saima Barki, Rabia Shireen, Sehrish Gohar Ali, Amjad Ali** for their sincere guidance and kindness. I genuinely represent my huge thanks to my M.phil seniors **Syed Shahab-Ud-Din Shah** and **Maryam Ashraf** for their kind support. My warm thanks to my batch/Lab fellows **Mehreen Tariq** and **Farmanullah** who always provided me a supporting environment and always helped me whenever I needed. I present my million thanks to my lovely juniors **Shafia Khan, Madeeha Rasool,** and **Fazeela Zuhoor** for their love and help.

Importantly, I would like to thanks to my parents and siblings who are my all-time support, love and light of my life and without them I am surely nothing. I also thanks to my friends **Tauheed, Urooj, Hammad, Zarafshan, Shafia, Sabahat, Aqsa, Shumaila, Maryam, Sumbal, Nosheen,** and **Fareeha** who always supported and loved me.

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List of Abbreviations

ATP	Adenosine Triphosphate
ADP	Adenosine 5'-Diphosphate
AGE	Advance Glycation End Product
PAMPs	Pathogens associated molecular patterns
DAMPs	Damage associated molecular patterns
HMGB1	High mobility group box 1
Ca^{2+}	Calcium
IL	interleukin
$CaCl_2$	Calcium Chloride
cAMP	Cyclic AMP
TNF	Tumor necrosis factor
COX-1	Cyclooxygenase-1
DAG	Diacylglycerol
DMSO	Dimethyl Sulphoxide
DTS	Dense Tubular System
EC	Endothelial Cells
ECM	Extracellular Matric
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
FA	Fatty Acids
GPCR	G-Protein Coupled Receptors
GLUT	Glucose Transporter Protein
CCL	Chemoattractant protein

IBC	Institutional Bioethical Committee
IL-6	Interleukin-6
IP3	Inositol trisphosphate
IP3R	Inositol trisphosphate Receptor
KCl	Potassium Chloride
MAPK	Mitogen Activated Protein Kinase
MCU	Mitochondrial Calcium Uniporter
FPR	Formyl peptide receptors
FMLP	N-formyl-methionyl-leucyl-phenylalanine
CLEC	C-type lectin receptor
MM	Mitochondrial Matrix
mM	Millimolar
ILRs	ITAM-linked receptors
mtDNA	Mitochondrial DNA
MTB	Modified Tyrode's Buffer
BSA	Bovine serum albumin
NF-kB	Nuclear Factor Kappa-B
mt protein	Mitochondrial proteins
NSAIDs	Non-Steroidal Anti-inflammatory Drugs
OCS	Open Canalicular System
OMM	Outer Mitochondrial Membrane
OXPHOS	Oxidative Phosphorylation
P2Y1	Class P2Y1 Receptor
PAI-1	Plasminogen

PAR1	Protease Activating Receptor 1
PAR4	Protease Activating Receptor 4
PGI_2	Prostaglandin I2
NMDA	N-methyl-D-aspartate
PIP2	Phosphatidylinositol 4, 5-Bisphosphate
PKA	Protein Kinase C
PLC	Phospholipase C
PM	Plasma Membrane
TRAP 6	Thrombin receptor activator peptide 6
PRP	Platelets Rich Plasma
PS	Phosphatidylserine
PTP	Protein Tyrosine Phosphorylation
NO	Nitric oxide
TLRs	Toll like receptors
ROS	Reactive oxygen species
RT	Room Temperature
SERCA	Sarco/Endoplasmic Reticulum Calcium ATPase
SOCE	Store Operated Calcium Entry
SOCs	Store Operated Calcium Channels
STIM1	Stromal Interaction Molecule 1
TF	Transcription Factor
TGF-β	Tumor Growth Factor Beta
TNF-α	Tumor Necrosis Factor Alpha
TSC	Trisodium Citrate

TxA_2	Thromboxane A2
VDAC	Voltage-Dependent Anion Channel
vWF	Von Willebrand Factor
WBCs	White Blood Cells
WHO	World Health Organization

Abstract

Platelets are multi-functional cells which besides playing their role in hemostasis also contribute to immunity when activated via their immune receptors causing granule secretion, chemotaxis, and phagocytosis. All these immunological functions are pre-hallmarks of platelets activation which can be enhanced by various pro-inflammatory mediators. In this study we investigated the role of different pro-inflammatory molecules on the priming of agonist-induced platelet aggregation responses. For this purpose, we employed several Pathogen/Damage associated molecular patterns (PAMP/DAMPS) like FMLP, glucose, FMLP treated blood cell's supernatant, mitochondrial DNA (mtDNA) and mitochondrial proteins (mt proteins). We isolated platelets, manually counted them, and after treatment with a priming agent and stimulated with adenosine-diphosphate (ADP) and adrenaline (AD). Extent of aggregation was noted by using a time-lapse spectrophotometric assay. A notable aggregation of platelets in response to ADP and AD was found, and also a significant difference of aggregation between mtDNA primed group followed by adrenaline stimulation and only adrenaline treated group was observed. We surprisingly found no significant alteration in platelet aggregation profiles after pre-treatment with other priming agents (FMLP, Glucose, FMLP treated Blood cell's lysate, Mitochondrial protein). In short, except mtDNA we unexpectedly did not find any remarkable effect of pro-inflammatory mediators on platelets aggregation, but this finding still requires more exploring regarding its incubation time and proinflammatory mediators' concentrations.

Key words: Hemostasis, Proinflammatory mediators, PAMPs, DAMPs, Immunity

INTRODUCTION

1.1. Immune system

Human body have a specialized system to keep itself safe from external pathogens, infection, and other diseases. This system is known as immune system (Yazdanpanah *et al*., 2020). Immune system in turn has a vast system of proteins, cells and even organs to protect our body from many pathological abuses by following several mechanisms. These mechanisms remove the harmful pathogens and toxic compounds, heal the injured tissue or a cell and restitute the normal functional status of the cell or tissue (Medzhitov, 2010). One of the well-known mechanisms that an immune system uses to evade external invaders is known as inflammation.

1.2. Inflammation

Inflammation is a protective response to defend the body against damage caused by infection and tissue injury. Inflammatory response is a key feature of tissue's response that involves the activation of various cell types like leukocytes, lymphocytes, neutrophils and macrophages to fight the insults caused by inflammatory agents and to maintain tissue homeostasis. (Soto-Heredero *et al.,* 2020*)*. This inflammatory response is evoked upon detection of PAMPs (Pathogens associated molecular patterns) or DAMPs (damage associated molecular pattern).

1.2.1. PAMPs and DAMPs

When any kind of pathogen invades inside our body, innate immune cells recognize the harmful signals present on pathogens, these molecules are known as pathogens associated molecular patterns (PAMPs). PAMPs are molecules generated by pathogens and microbes such as lipopolysaccharides, β-glycans, and formyl peptides etc. (Zindel *et al*.,2020). PAMPs are considered as major culprit in generation of inflammatory responses and that inflammatory response is known as non-sterile inflammation. PAMPs are recognized by innate immune cells through their cell surface receptors and by a receptor confined to intracellular compartments collectively known as pattern recognition receptors (PRRs) (Walsh *et al*.,2013).

Besides the attack of pathogens, our body's tissues or cells go through a stress and releases many endogenous molecules which are signaled as a "Danger molecule ", which can also activate innate immune system (Vénéreau et al., 2015) and an innate immune system deals with them by the generation of different inflammatory responses (MG *et al*., 2017). These danger molecules are known as DAMPs (Danger/Damage associated molecular patterns)**.** DAMPs are non-infectious normal physiological molecules inside the cells, but when releases outside the cells they trigger a sterile inflammatory response. Examples of DAMP include ATP, uric acid, histones, DNA, mitochondrial DNA, IL-33, HMGB1, mitochondrial formylated proteins and others (Andersson *et al.,*2018). DAMPs may be released due to physical factor like burns, trauma, radiation, or any chemical factors like sugars, alcohol, unwanted heavy metals, toxins, fatty acids etc. (Rho & Sohn, 2018).

1.2.2. Pro-inflammatory mediators

The first step in inflammation is the recognition of the PAMPs or DAMPs by PRRs of an innate immune cell**.** Inflammatory process constitutes a series of events which not only recruit immune cells but also brings a temporary change in the vasculature (Grete *et al*.,2019). At the site of injury or any pathological attack site, the tissue resident macrophages start their initial defense mechanism after recognizing the alarmins and then an inflammatory activation pathway runs inside a cell. Commonly known inflammatory pathways are NF-κB, MAP kinase pathway and JAK-STAT pathway (Chen *et al*.,2018, Chatterjee *et al*.,2020). These inflammatory pathways lead a cell to come in action by phagocytosis and by the release of soluble vasoactive molecules such as histamine, prostaglandins, and several leukotrienes which increase permeability of vessels helping other immune cells to recruit at the site of inflammation in order to fight the infection (Chen *et al*.,2018). The whole process of vasodilation and the recruitment of other active immune cells occurs within minutes and the maintenance of all this system until the infection is fully eradicated is highly important and these processes are managed by many soluble bioactive chemical mediators released by our circulatory system, immune cells, or from a site of an injury. These soluble chemical mediators are known as "pro-inflammatory mediators" (Demine *et al*.,2020).

There comes a huge list of these pro inflammatory mediators, including cytokines/chemokines, but according to their chemical nature they can be classified into following categories: (a) Interleukins (like IL-1, -2, -4, -5, -6, TNF- α , and GM-CSF), (b) Arachidonic acid derivatives (like prostaglandins), (c) Vasoactive peptides (like histamine and serotonin, and (d) Phospholipids activating factors (like Platelet activation factor) (Abdul Khaleq *et al.,* 2018).

As discussed earlier these proinflammatory mediators cause an infiltration of other leukocytes to resolve the infection by capturing, phagocytizing, processing of the antigens and by releasing many antimicrobial proteins as shown in *figure-1.1* (Jordan *et al*., 2020). In this mechanism Antigen Presenting Cells (APCs like macrophages, monocytes and neutrophils which internalize an external moiety and process it. They, by the help of their major histocompatibility protein complexes (MHCs) present the processed antigens to adaptive immune cells. This causes in generating an effective and long-term immune response against that pathogen which includes antibodies production and memorizing that antigen. Thus resolving an inflammation as shown in *fig-1.2* (Ashley *et al*., 2012).

All these features of inflammation are due to this immune system and this immune system is comprised of two big units.

1.2.2.1. Innate immune System

Immune system is broadly divided into an innate and adaptive immune system. Innate immune system provides the first line of defense, which comprises of physical and chemical barriers. Innate immune system play its role in body by the activation of different cells including granulocytes, monocytes, macrophages, natural killer cells (Lenz & Nelson, 2018) and platelets (Ali *et al*., 2015). This innate immune system has preliminary approach to deal with an invading pathogen and this process in a continuity is handed over to an adaptive immune system.

1.2.2.2. Adaptive immune System

Adaptive immune system's response solely depends upon innate immune system, and it is activated as a specific response based on the nature antigen presented to it by the innate

immune system. Adaptive immune system includes antigen specific T cells, antigen presenting cells and B cells (Ley, 2020). The most enormous quality of adaptive immune system is that they "memorize" a specific antigen in case, if again an infection occurs because of that antigen, it is quickly recognized and neutralized (Bonilla & Oettgen.,2010)

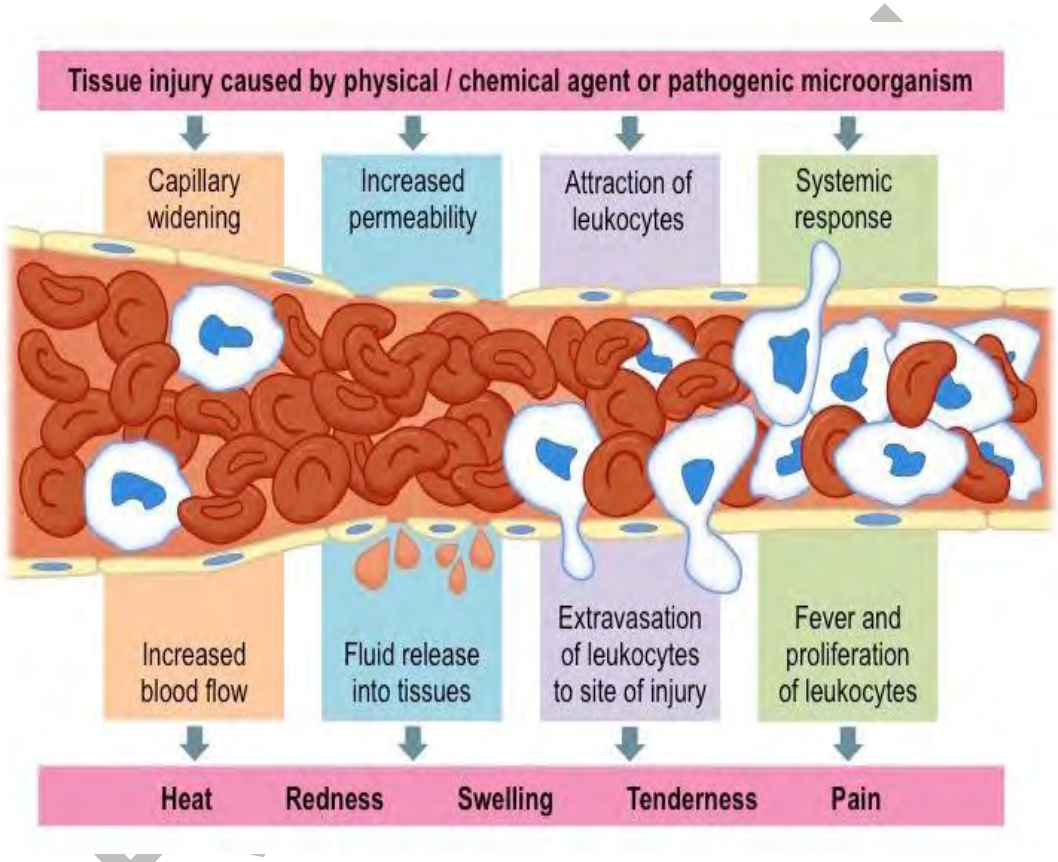

Fig:1.1. *Inflammatory response upon exposure to external invasion. Heat, redness, swelling, and pain are the cardinal signs of inflammation. Vasodilation (capillary widening), vascular permeability and leukocytes infiltration are primary inflammatory responses. Adapted from Bioninja.com*

1.3-Role of platelets in inflammation

The long-established role of platelets is well known that they are involved in hemostasis, but now platelets have also been reported for their role in inflammation (Margraf & Zarbock, 2019). When any injury happens, platelets go to that site where they play their role of attaching to the endothelium, facilitate clot formation and later help in wound healing (Etulain,2018). When platelets are activated, the transmembrane protein P-selectin is translocated from intracellular granule to plasma membrane and makes an interaction with leukocyte's cell surface ligand known as PSGL-1 which helps in hemostasis and inflammation known as immunothrombosis (Margraf & Zarbock, 2019b). Platelets-leukocyte interaction helps in extravasation of leukocytes to the site of inflammation (Assinger *et al*., 2019). They also sense PAMPs or DAMPs by a plethora of immune receptors (Aslam *et al*., 2006) and when activated they release chemokines/cytokines to activate leukocytes especially neutrophils to come and play their role at infectious site (Schrottmaier *et al*., 2015). Platelets have also been reported to phagocyte the pathogens (Jansen *et al.,* 2016). Platelet's immunity roles are depicted in *figure-1.2*.

Platelets have many storage granules, and in these granules, they have many anti-microbial protein, bio active chemicals, and inflammatory molecules playing a crucial role in innate immunity. For example, histamine and serotonin present in dense granules have a proinflammatory response to monocytes and T cells. Moreover, in α-granules many cytokines and chemokines are present such as CXCL1,CXCL4,CXCL5,CXCL7,CXCL 8 and CCL2, CCL3, CCL5 which recruits leukocytes to a site of infection and antimicrobial proteins such as kinocidins, clathecidins, thrombocidins and defensins (Yeaman.,2014)(Portier & Campbell, 2021). Platelets play their role of immunity by having and by releasing these biomolecules which plays a vital role in resolving inflammation.

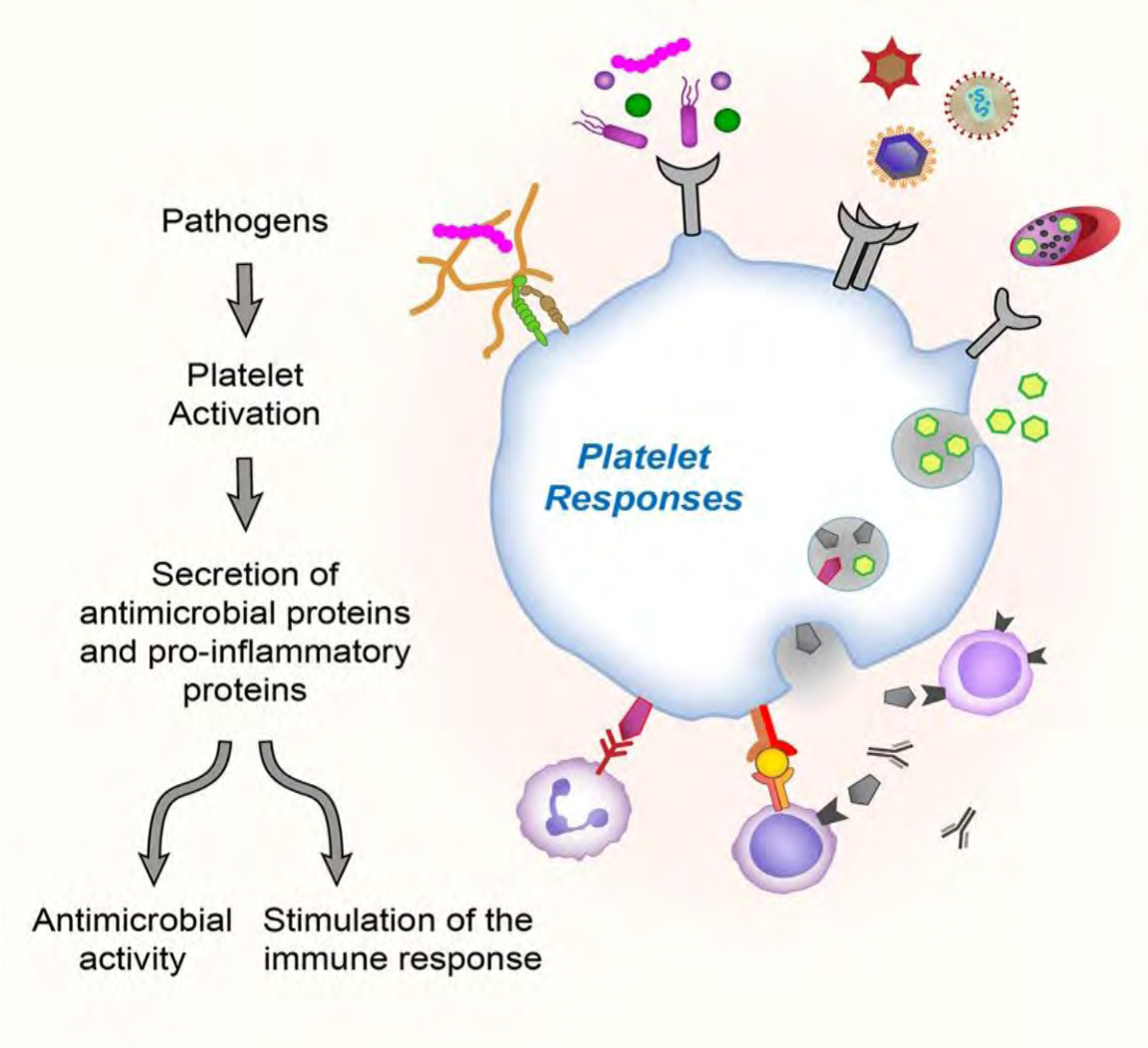

Fig-1.2*: Role of platelets in Inflammation. Platelets recognizes pathogens by immune receptors, making interaction with leukocytes, releases inflammatory and bioactive molecules. Adapted from* (Portier & Campbell, 2021)

1.4. Platelet structural overview

Platelets are anucleate cells with 2-3 micrometer in diameter and remain in blood for about 7 to 10 days. They are produced from megakaryocytes present in the bone marrow and lungs (Lefrançais *et al*.,2017). They circulate at a concentration of about 150,000-400,000 per microliter of blood and totally about 1 trillion platelets are present in whole blood (Daly, 2011). The normal count of platelets is maintained by thrombopoietin- a glycoprotein hormones released by liver or kidney (Alentado *et al*., 2021). Platelets play an important role in hemostasis and having a wide variety of structural components (shown in *figure-1.4*) helping them to perform their duty efficiently.

1.4.1. Plasma membrane

A delineating plasma membrane surrounds the platelets, and it forms a sac like structure by folding inside known as open canalicular system (Selvadurai & Hamilton, 2018). When platelets are activated, it become spiky forming pseudopodia, sticky aggregates, and release their cellular content to perform all these physiological functions (Koessler *et al*.,2018). As a result, plasma membrane changes its shape and facilitate the adhesion and aggregation of platelets by attracting other platelets.

1.4.1.1. Plasma membrane lipids

To understand the mechanism and biology of specific cell, cell's membrane lipids are potential markers. All function of platelets i.e., activation, shape change spreading, degranulation requires membrane lipids which are comprised of amino phospholipids i.e. (phosphatidylcholine 40%, phosphatidyl ethanolamine 28%,) sphingomyelins 18%, phosphatidyl inositol 3%, Phosphatidyl serine 10% (Pollet *et al*.,2018). Neutral lipids such as glycerides cerebroids and free cholesterol make about less than 1% of platelet plasma membrane (O'Donnell *et al*., 2014).

1.4.2.2. Plasma membrane proteins

Based on platelets function, platelet plasma membrane represents four basic groups of proteins.

Table 1.1 Functions and Examples of Plasma membrane proteins

Plasma membrane proteins	Function	Examples
Platelet cell surface markers	Shows a platelet's inactive or activation status	Inactive platelets: CD41 (GP IIaIIIb), CD42a (GP IX), CD 42b (GP Ib) and CD61 Activated platelets: show PAC-1, CD62P (P-Selectin), CD31(PECAM) and CD 63 (Blair *et al*., 2018).
Platelet activation receptors	When a ligand attaches to them it activates platelet	Thromboxane A_2 receptor, Thrombin receptors (PAR1 and PAR 4), ADP receptors (P2Y12, P2Y1), CLEC-2, α6β1, $\alpha_2\beta1$ and other integrin receptors (Swanepoel & Pretorius, 2014).
Platelet spreading and aggregation receptors	After platelets activation they help in clumping and aggregation of platelets at site of injury (Cirillo *et al*.,2020).	GPIa/IIa, GPIb-IX-V, $P2Y_1$ and GPIIb/IIIa (Estevez & Du, 2017a).
Platelets cell surface ion channels	Helps to maintain the level of different ions inside platelet	ATP gated P2X1(Tolhurst *et al*., 2008) ORAI, TRAP6, AMPA, Voltage gated Kv3.1 channel, NMDA glutamate receptors

		etc. (Morrell *et al*., 2008)

1.4.3. Platelet cytoskeletal system

In platelets the normal discoid shape, proper position, symmetry of organelles and the change of platelets shape after its activation is important to perform their work. Spectrin-actin based cytoskeleton helps the platelets to maintain these attributes. Also after maturing from proplatelets the correct position of organelles in platelets are assigned by cytoskeletal components i.e., spectrin, actin and tubulin (Thon *et al*., 2010). Spectrin makes plasma membrane-attached meshwork which helps to support the membrane while actin and tubulin makes the cytosolic cytoskeleton. The distribution of α-granules and dense tubular system are by actin while distribution of dense granules and mitochondria by microtubules (Zaninetti et al.,2020). Dynamic actin, tubulin and microtubule filaments are also essential for demarcation membrane system, cyto-skeleton driven cellular responses, and filopodium production after platelets activation (Go *et al*., 2021).

1.4.4. Dense tubular system

A network of interlinked tubules or in other words a residual of endoplasmic reticulum beneath the plasma membrane is regarded as Dense tubular system (DTS) (Selvadurai & Hamilton, 2018). DTS is involved in the release of calcium into the cytoplasm whenever there is a physiological call for that and takes up the excess calcium from cytoplasm whenever it increases through a pump known as SERCA2b (Yadav *et al*.,2019). Without this organelle a thought of platelets aggregation seems impossible due to its role in calcium storage and sequestration.

1.4.5. α-granules

Platelets upon activation release the content of their granules which facilitate platelets contribution in hemostasis. One of them are α-granules which are 200-500nm, making about 10% of platelet volume and release many types of proteins (Ren et al.,2020). These proteins are involved in coagulation, anticoagulation, inflammation, immunity and much more. Coagulation cascade factors i.e. factor Ⅴ, Ⅻ, , and other important coagulation proteins like fibrinogen, von Willebrand, thrombospondin, PAI-1, fibronectin,

antithrombin tissue factors and many growth factors are stored in these granules (Swinkels *et al*., 2019).

1.4.6. Dense granules

They are derived from endosomes also known as δ-granules having a diameter of 150nm and are present about 3-8 per platelet (Ambrosio & Pietro, 2016). Their inner biochemical content is less diverse than α-granules. The main components of dense granules are serotonin, histamine, adenine nucleotides, polyphosphates, pyrophosphates cations and mainly calcium ions (de Jong & Dekker, 2010)..

1.4.7. Lysosomes

Each platelet contains about 1-3 lysosomes and their platelet's related role is not well known. However a conventional role that they have is associated with the activity of acid hydrolases which are involved in digestion of phagocytosed and cytosolic components (Heijnen & Sluijs, 2015).

1.4.8. Mitochondria

Platelets actively participate in many hemostatic processes. To perform their function platelets need an energy in the form of ATP and a well-known "powerhouse of the cell" i.e., mitochondria serve the function of energy providence very effectively. (Hayashi *et al*., 2011a). There are 5-8 mitochondria per platelets (Hayashi *et al*., 2011b). Platelets gets 60% of their energy from glycolysis while 30-40% is provided by mitochondrial OXPHOS (Wang *et al*., 2017). Besides providing energy to platelets mitochondria are also involved in other cellular functions like reactive oxygen species(ROS) production (Stefanatos & Sanz, 2018), Ca^{+} buffering (Ryan *et al*., 2020), regulation of apoptosis (Bloemberg & Quadrilatero, 2019), response to ER stress (Zharikov & Shiva, 2013).

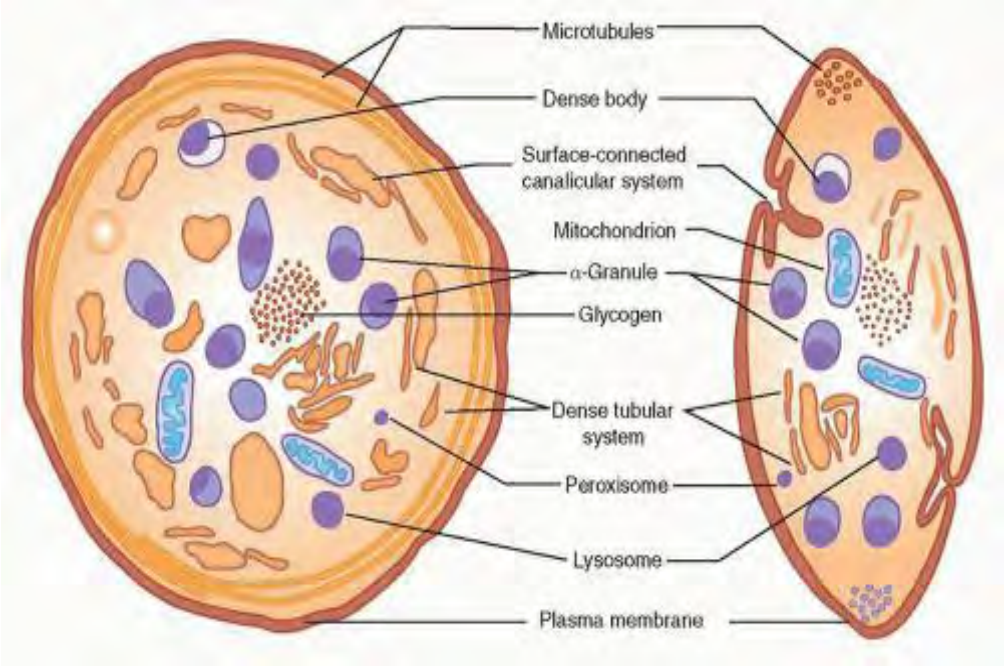

Fig-1.4: *Platelet structure and it's organelles and other components. (Left) frontal view (Right) Lateral view. Platelets without nucleus still have enough structural support like mitochondria, DTS, alpha and dense granules, open canalicular system and a multifunctional plasma membrane to perform its hemostatic role. Adapted from (Platelet Structure and Function in Hemostasis and Thrombosis | Oncohema Key, n.d.).*

1.5. Platelets physiological functions

Platelets are the smaller cells of a circulation and there, they play a role in hemostasis, thrombosis, inflammation and wound healing. In normal circulation vascular endothelium helps them not to activate by the release of prostaglandin (PGI_2) and nitric oxide (NO). when any injury happens

platelets arrive at damaged blood vessels and close them up to restore the blood flow, and then remove the blood clots. (Nieswandt *et al*., 2009). Platelet's ability to form a clot is due to presence of sticky protein that helps them to bind a fibrin meshwork at the site of an injury. After forming a blood clot these little fragments give a big hand in wound healing by releasing many growth factors associated to wound healing like platelets-derived growth factor (PDGF) (directing cell movement), TGFβ (stimulating extracellular

matrix deposition), and vascular endothelial growth factor (VEGF) (stimulating blood vessels regeneration) (Takahashi *et al*.,2019).

1.6. Platelet immune receptors

Platelets exhibit a plethora of (Toll like receptors) TLRs immune receptors to detect the endogenous DAMPs and exogenous PAMPs to play their role in immunity. They have a series of TLRs to detect various PAMPs and DAMPs. For instance mitochondrial DNA which acts as a DAMP is released by a cell through apoptosis, necroptosis and via mitochondrial derived vesicles and that is detected by TLR-9 (G et al., 2019). Similarly, mitochondrial formylated proteins also act as DAMPs and are detected by FPR receptors on cell surface of platelets (He & Ye, 2017). Mitochondrial derived N-formyl proteins bind highly to FPR2/AXL receptor while bacterial derived and synthetic FMLP is reported to bind with FPR 1 receptors with high affinity (Ye, Boulay, Wang, et al., 2009). Neutrophils also release DNA outside the cell and the histones in the form of neutrophil extracellular traps (NETs) which can be sensed by TLR-4 on platelets and thus generating an immune response (Vorobjeva & Chernyak., 2020).

1.7. Platelet priming

Platelet functional responses either its hemostatic, thrombotic, or immunological all depend on its activation status. There is a fact that platelets are heterogenous in size, age, and expression levels of a surface receptors (A et al., 2021). The heterogenous behavior of platelets is explained by several intrinsic factors like signals exposures and their parent megakaryocytes. Each platelet has a different response level to the same agonist, and this is a reason platelets activation have a different stages, status, or populations. Prior to platelets activation, some bio molecules or weak agonists influences the cell's response to a subsequent challenge and this is known as "priming"(Brouns *et al*., 2020).

1.7.1. Positive and negative platelet priming

Platelet priming is categorized as positive (enhancing platelets functionality) and negative (decreasing functionality) priming (Henson *et al*., 2000).By Positive or negative priming, we mean that how quickly platelets respond to any external stimulant after getting primed by negative or positive primers. Positive primers like thrombopoietin (Moore *et al*.,2019), epinephrine (Versteeg *et al*., 2013), IGF-1 (Hers, 2007), Gas 6

(COSEMANS et al., 2010), prostaglandins, N-formyl peptides ,TNF-α (Pircher *et al*., 2012) are known to enhance the platelet responses.

Negative primers like adenosine, NO, prostacyclin, thrombomodulin, insulin decrease the platelet functionality. Due to these primers the threshold potential of platelet activation by active agonists changes. As some DAMPs such as FMLP, mitochondrial DNA, netotic DNA may evoke a platelet for an immune response (Marcoux *et al*.,2019, Petito *et al*.,2018) that can also prime the platelets and can change a platelets hemostatic functional status in a pathogenic or sterile inflammation.

1.8-Platelet activation and signaling

Platelet activation requires several weak or strong agonists. These agonists act on their respective receptors on platelet cell surface. These receptors receive the external environmental signal, internalize them and then a specific signaling pathway begins which eventually ends up on platelets activation.

In a human body, for platelets activation there are several (patho)-physiological agonists like thrombin, ADP, adrenaline, collagen, fibrinogen, serotonin, podoplanin, fibronectin, laminin, vWF and others. The three well-known kinds of receptors through which these platelets activating moieties act are G-protein coupled receptors (GPCRs), Immunoreceptor tyrosine-based activation motif (ITAM) receptors, and integrin receptors (Shown in *figure-1.5*).

1.9. Types of platelet activation receptors

1.9.1. Activation of platelet by GPCRs

G-protein coupled receptors (GPCRs) are the most diverse and specialized group of receptors consisting of a single polypeptide chain which span through a plasma membrane seven times and are coupled with a trimeric G-protein having α, β, and ϒ subunit (Davenport *et al*.,2020). In platelets most of the activation function is mediated by these GPCRs. Common GPCRs present on platelets cells surface and their respective α-subunit along with the signaling pathway they follow are enlisted in table 1.2

Table 1.2 Activation of platelets by GPCRs

Agonists	**GPCRs**	**α-subunit**	**Signaling pathway**
Thrombin	PAR1, PAR4	$G\alpha_q$	PLC-IP_3, DAG Increases cytosolic calcium
ADP	P2Y1, P2Y12	$G\alpha_q$, $G\alpha_i$	Decreases cAMP, Increases cytosolic calcium
Adrenaline/epinephrine	α 2_A adrenergic	$G\alpha_z$	Interacts with adenylate cyclase and lowers cAMP, increases cytosolic calcium, Activates Akt pathway.
Thromboxane A_2	TP	$G\alpha_q$	PLC-IP_3, DAG Increases cytosolic calcium

1.9.2. Activation of platelet by ILRs (ITAM-linked receptors):

Platelet activation via ILRs involves an immunoreceptor tyrosine-based activation motif sequences which by their tyrosine motif phosphorylation or dephosphorylation runs a cascade of signaling by providing a docking site for many Src kinases. GP-Ⅵ is activated by collagen, CLEC2 (C-type lectin) receptors by podoplanin and FcϒRⅡC is activated by low levels of Immunoglobin G (Rayes *et al*., 2019). Activation by these agonist recruits several tyrosine kinases resulting in activation of PLC-ϒ with a subsequent

generation of IP3and DAG which help in the release of calcium from DTS eventually leading to platelet activation (Stefanini *et al*., 2009).

1.9.3. Activation of platelet by integrin receptors:

Platelets exhibit a heterodimeric receptor on their surface which belongs to integrin β1 and β3 families. They exist in a low-affinity state which quickly switch to a high-affinity state during platelet activation. β1 integrin receptors present on platelets surface are α2β1 (activated by collagen), α5β1 (activated by fibronectin), α6β1 (activated by laminin) and β3 integrin receptors are αvβ1 and αIIbβ3 (activated by vitronectin and fibronectin) (Grüner *et al*., 2003). αIIbβ3 is the most commonly or dominantly used integrins which has greater role in platelets aggregation and is activated by fibrinogen (Kuijpers *et al*., 2003). Integrin receptors activate platelets by outside-in and inside-out signaling(Huang et al., 2019).

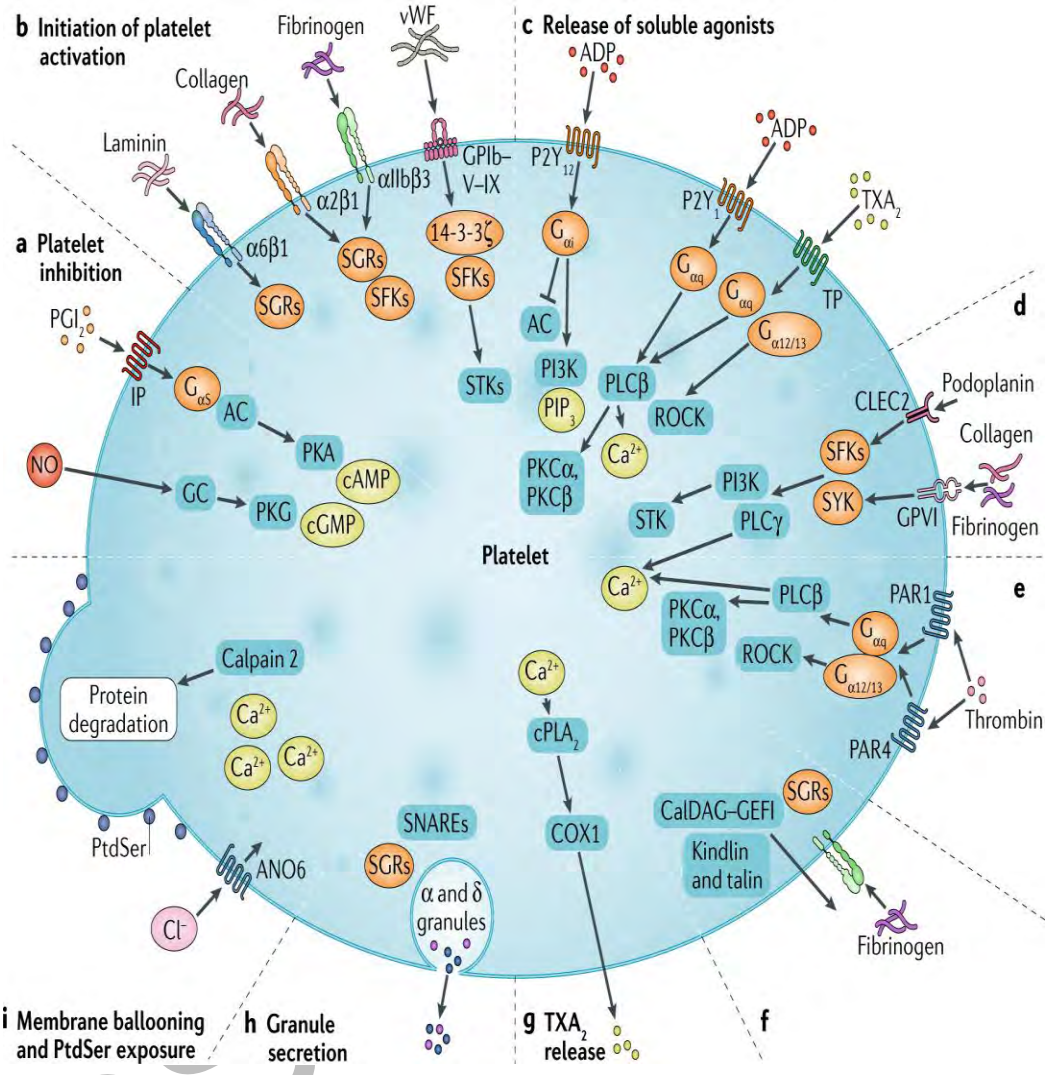

***Fig-1.5**: Platelet receptors and their signaling pathways. Showing GPCRs, ILRs and integrin receptors, their respective ligands and signaling pathways. Adapted from(van der Meijden & Heemskerk, 2018)*

1.10. Pro-inflammatory mediators and their effect on platelet hemostatic role (aggregation)

Platelets are mainly recognized by their property of coagulation, aggregation, and wound healing, and when these attributes are not performed by platelets, this can lead to serious complications(Krishnegowda & Rajashekaraiah, 2015).

Platelets are activated by a variety of (patho)physiological agonists to perform their function. When concentration of the agonists goes beyond a threshold it can cause a thrombus formation. But when an infection happens to a body DAMPs, PAMPs and

some other inflammatory mediators may also activate platelets immunologically as platelets have immune receptors. Now to check whether these intracellular signaling cascades due to these "Immunological activation" brings a change in platelets activation or not. So, this study was designed to explore the priming effect of DAMPs or PAMPs (especially FMLP, mitochondrial DNA, cell free DNA and mitochondrial formylated proteins) on agonist-induced platelet aggregation

AIM AND OBJECTIVES

This study is aimed to link the immunological activation of platelets to the agonist-induced platelet aggregation with the following objectives:

- Evaluation of ADP and Adrenaline-induced aggregation
- Impact of different pre-inflammatory mediators on platelet aggregation
- Analyze the priming potential of different priming agents on ADP and adrenaline-induced aggregation.

2.MATERIALS AND METHODS

2.1. Ethical approval

For the conduction of this research study an ethical approval has already been taken from Bioethical committee of Quaid-I-Azam University Islamabad.,

2.2. Blood collection

Healthy and young volunteers (who have not taken any NSAID for last two weeks) were recruited from different departments of Quaid-i-Azam University for the study by taking their written consent. 10ml venous blood was drawn and mixed with 3.8% trisodium citrate (TSC) (Sigma Aldrich, Germany??) in 9:1(9 parts of blood: 1 part of TSC) in a 15ml falcon tube (SPL Life sciences, Korea)

2.3. Preparation of platelet rich plasma (PRP)

Density-based centrifugation was employed to enrich the platelets in the form of platelet rich plasma (PRP). For this purpose, two-step differential centrifugation was performed in a swing bucket centrifuge (Kokusan Model H-103RS, Japan) at room temperature. First centrifugation was done at 200g for 20 minutes and 2/3 of the supernatant plasma was carefully separated. It was further centrifuged at 100g for 10 minutes for platelet enrichment and removal of RBCs and WBCs. PRP was then separated and transferred into a microcentrifuge tube (1.5ml) for experiments or it was placed in a rotatory mixture to avoid spontaneous aggregation.

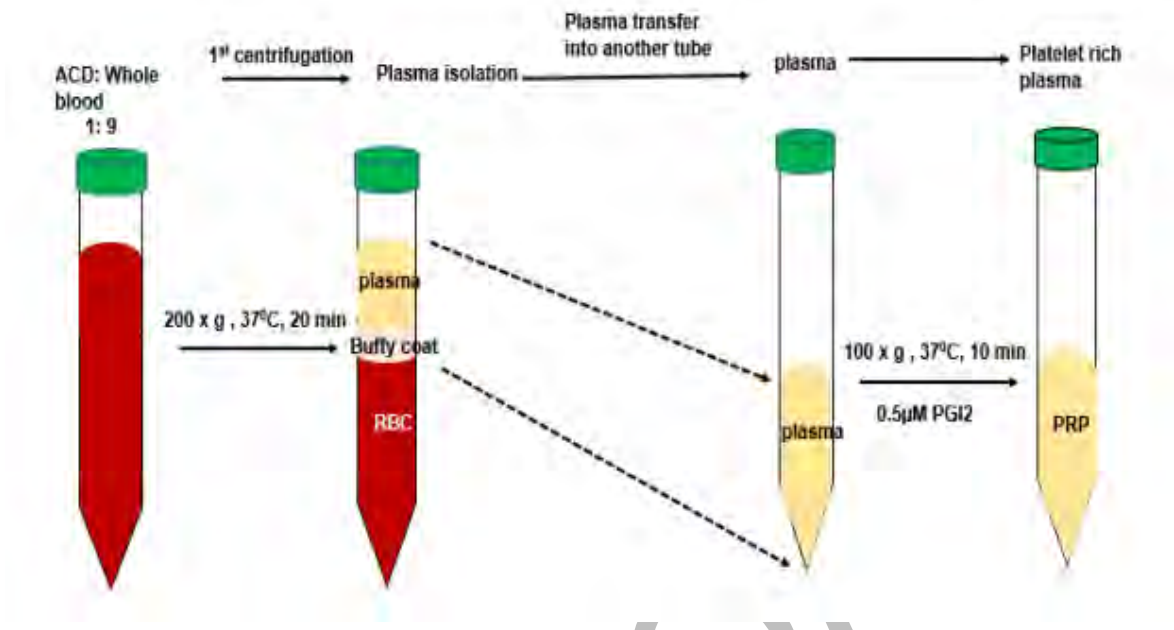

***Fig-2.1**: Platelet's isolation using a two-step differential centrifugation from human whole blood*

2.4. Platelets counting

For homogeneity, an equal number of platelets are necessary to be used in each experiment. For this purpose, we counted platelets manually using an Improved Neubauer counting chamber (Marienfield, Germany. PRP was diluted ten times with Tyrode's buffer which is normal physiological buffer containing in mM ((NaCl 140, Glucose 5, HEPES 10, $MgCl_2$, $CaCl_2$). This diluted PRP was loaded with a volume into the counting chamber without making air bubbles. After allowing the cell settlement for 10 minutes incubation, platelets were counted in 5 small squares located in the central big square on a microscope (IRMECO Germany) using a dry objective lens of 40X. Number of platelets were calculated using the following formula.

$$\text{Platelet Count}/\mu l = \frac{\text{Number of platelets counted x dilution factor}}{\text{No. of squares counted x area of square depth of chamber}}$$

Fig-2.2:*(Left) Neubauer counting chamber (Right) Bright field microscope.*

2.5. Preparation of FMLP-induced blood cell's supernatant

After the first centrifugation of whole blood at 200g for 20 minutes the upper layer of plasma is separated, and the buffy coat and RBCs layer is left behind. In order to use a blood cell' supernatant, we added normal saline to this blood in 2:1 ratio and then a 600u l of this blood was treated with FMLP (50nM) and incubated for almost 60 minutes. After an incubation, this treated blood was centrifuged at 13000rpm for 10 minutes in order to get a cell free supernatant, which contains cellular DAMPs which will be used as for priming agents for platelets.

2.6. Mitochondrial DNA isolation

Platelets are the cells which lack nucleus, which also means that they lack nuclear DNA. The only DNA they have is mitochondrial DNA (mtDNA). So, in order to extract mtDNA, we used platelets. First, we took a venous blood from healthy donors and mixed it with anticoagulant Tri-sodium citrate (TSC) in 9:1 ratio. Then, a two-step differential centrifugation was performed in a swing bucket centrifuge (Kokusan Model H-103RS, Japan) at room temperature. First centrifugation was done at 200g for 20 minutes and 2/3 of the supernatant plasma was carefully separated. It was further centrifuged at 100g for 10 minutes for platelet enrichment and removal of RBCs and WBCs. PRP was then

separated and transferred into a microcentrifuge tube (1.5ml) and 50u l of EGTA (anticoagulant) was added to 1000u l of PRP and a pellet of platelets was obtained by centrifuging this PRP at 2000g for 5 minutes. After obtaining platelets pellet supernatant was discarded and to this pellet, we added 750u l of solution A and after mixing and incubation for almost 15-20 minutes, it was centrifuged at 12000rmp for 1 minute. Supernatant was discarded and the pellet was re-dissolved in 400u l of solution A and was again centrifuged at 12000rmp for 1 minute. The pellet obtained was re-suspended in 400u l of solution B, 12 u l of 20% SDS and 5u l of proteinase K and incubated for 3 hours at 37^{o}C in incubator. After incubation, a mixture of solution C and D of volume 500u l was added and centrifuged at 12000rmp for 10 minutes. After centrifugation, the upper aqueous layer was separated in another tube and DNA was precipitated by adding 55u l of sodium acetate (3M, pH 6) and equal volume of ice-cold isopropanol. After precipitating DNA, the tube was centrifuged at12000rmp for 10 minutes in order to obtain DNA pellet. Supernatant was discarded and 200u l of 70% ethanol was added to DNA pellet and was again centrifuged at800rmp for 7 minutes. After centrifugation ethanol was discarded and the DNA pellet was dried out by keeping the tube open for about 30 minutes in incubator. Then a DNA pellet was dissolved TE buffer and this DNA was stored at -80^{o}C for later use as a priming agent. Before using this DNA, every time it was quantified at Nanodrop (Titertek Berthold, Germany).

Table 2.1: Composition of Solutions

Solution Name	Chemical composition	Concentration
Solution A	Sucrose Magnesium Chloride Tris (pH 7.5) Triton X-100	320 mM 5 mM 10 mM 1% (v/v)
Solution B	Tris (pH 7.5) EDTA (pH 8.8) Sodium Chloride	10 mM 2 mM 400 mM
Solution C	Saturated Phenol	
Solution D	Isoamyl alcohol Chloroform	1 volume 24 volume

2.7. Mitochondrial protein isolation

In addition, with mtDNA, mitochondrial proteins especially formylated proteins also act as DAMPs. Therefore, these mitochondrial proteins were used as a priming agent in our experiments. For this, first we isolated PRP from blood by two-step centrifugation in a swing-bucket centrifuge (Kokusan Model H-103RS, Japan). Then this PRP was separated and transferred into a microcentrifuge tube (1.5ml) and 50u l of EGTA (anticoagulant) was added to 1000u l of PRP and a pellet of platelets was obtained by centrifuging this PRP at 2000g for 5 minutes. To this pellet, an ice-cold homogenization buffer of volume 800u l was added and vortexed (Scilogex MX-2 cat # 821200059999) for 5 seconds. Then this tube was incubated on ice for 2 minutes and again an intermittent vortexing (Scilogex MX-2 cat # 821200059999) was done for about 5 minutes. Centrifugation was

done at 700g for 5 minutes. Supernatant was transferred to another microcentrifuge tube, and this was again centrifuged at 3000g for 5 minutes. The pellet obtained had mitochondria. Now this mitochondria containing pellet was subjected to protein isolation by adding 100u l of radioimmunoprecipitation assay (RIPA) buffer (10mM Tris pH 8, 0.1% SDS, 0.01% sodium deoxycholate, 140mM NaCl, 1% Triton X-100, 1mM EDTA,1mM DTT, 1% Protease Inhibitor, 1% NaF, 1% sodium orthovanadate) and an intermittent vortexing (Scilogex MX-2 cat # 821200059999) was done for about 15 minutes. After vortexing the tube was incubated on ice for 15 minutes and the centrifuged at 12000rmp for 10 minutes. The supernatant was separated and contained proteins.

2.8. Protein quantification by using Bradford assay:

The mitochondrial proteins obtained were quantified by using Bradford assay. The standard solution, bovine serum albumin (BSA) (Carl Roth, Germany) protein was used. BSA stock solution was prepared by taking 40 mg/ml of crystalline BSA and dissolved in distilled water. Then, this BSA stock was diluted twenty times (2mg/ml) in order to prepare the working dilutions. This working dilution was then used for 7 further dilutions ranging from 0 to 1.2 u g/u l. Distilled water was considered as blank. Blank and standards solution of volume10u l each was poured in 96-well microplate (Extragene EL1190-F, South Korea). Bradford reagent(1X) (Roth, Germany) of volume 200u l was added in each well. Then a microplate was kept in dark at 37^{o}C for about 30 minutes. After incubation, at 595nm the reading was taken by spectrophotometer. By subtracting the blank's absorbance value, corrected absorbance of each was obtained. Then the corrected absorbance value of each standard value was used to draw the standard curve. By this standard curve the value of our protein samples was obtained. The protein samples were diluted 10 times and 10u l of this was poured in a microplate and quantified by using Bradford assay. By using the TREND formula in Microsoft Excel sheet, the proteins were quantified.

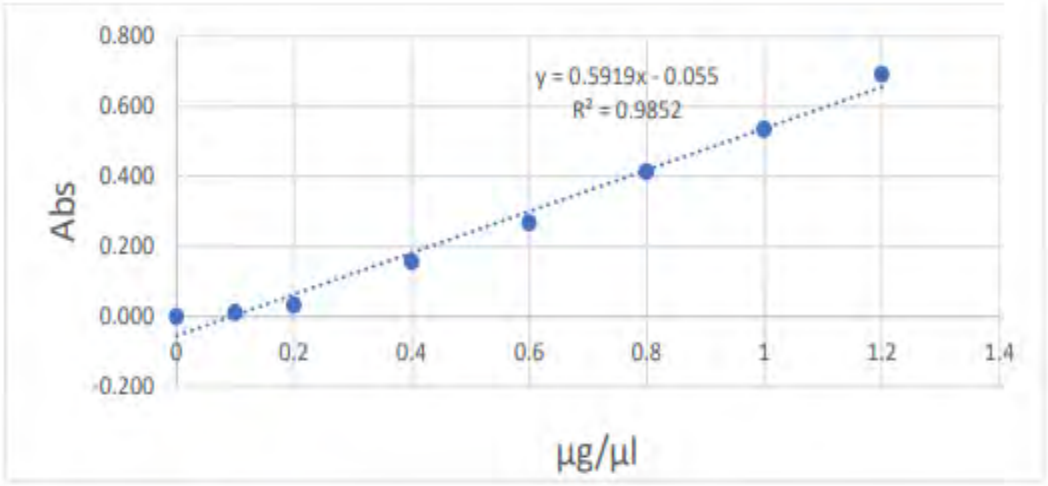

Fig:2.3. *BSA standard curve*

2.9. Platelet aggregation using a time-lapsed assay

To check the priming effect of n-FMLP, cell free DNA, mitochondrial proteins, mitochondrial DNA platelet aggregation was performed by time lapsed based spectrophotometric assay. A 96-wells microplate was used for this assay, and it was substantiated by the help of Multiskan Go (Thermo Fisher Scientific, USA) spectrophotometer, The counted platelets were normalized at 150,000/µl by using a modified Tyrode's buffer. Normalized PRP was treated with priming agents like FMLP (5&15 minutes incubation), Glucose (7&15 minutes incubation), FMLP treated blood cell's lysate (15&30 minutes incubation) and were incubated on a rotary mixer at 37 ^{0}C. Two kinetic loops with constant shaking of plate at 5 seconds of interval was adjusted in Skanit software (version 4.1.0.4.3, for microplate reader) and absorbance was taken at 595nm after each kinetic loop. In each well 200u l of normalized PRP was added and initial reading was taken. Platelets were stimulated with agonists (Adrenaline or ADP) and final reading was taken. Data was normalized on Microsoft Excel (Microsoft Inc, USA) and plotted in the form of curves which was further analyzed to get an amplitude and slope (aggregation/min). Mean and SEM were calculated and statistically analyzed by using one way ANOVA with Bonferroni's multiple comparison post-tests using Graph pad Prism software (version 5.01).

3.RESULTS

3.1. Demographic and Anthropometric parameters

Before conducting a study, the demographic data helps us to Spotify the characteristics of an individual's participating in the study. The given data was collected from participants at the time of sampling with their consent.

Table 3.1. Demographic and anthropometric profile of participating individuals

Characteristics	Mean ± SEM	±SD
Age	23.92± 1.058915	±3.81
Weight (Kg)	56.42± 3.78504	±10.01
Height(cm)	163.5± 4.396638	±11.63
Infection (Yes/NO)	NO	NO

3.2. Platelet enrichment

The key or can say a star component of our experiment were platelets. Moreover, their normal count and purity was also our major concern. Therefore, we checked the platelet count of each individual that either it lies in normal range or not. Additionally, the purity of platelets enriched plasma was checked by observing it under the microscope. We found an average platelet count between 200,000-3,50,000/ul of all our participants.

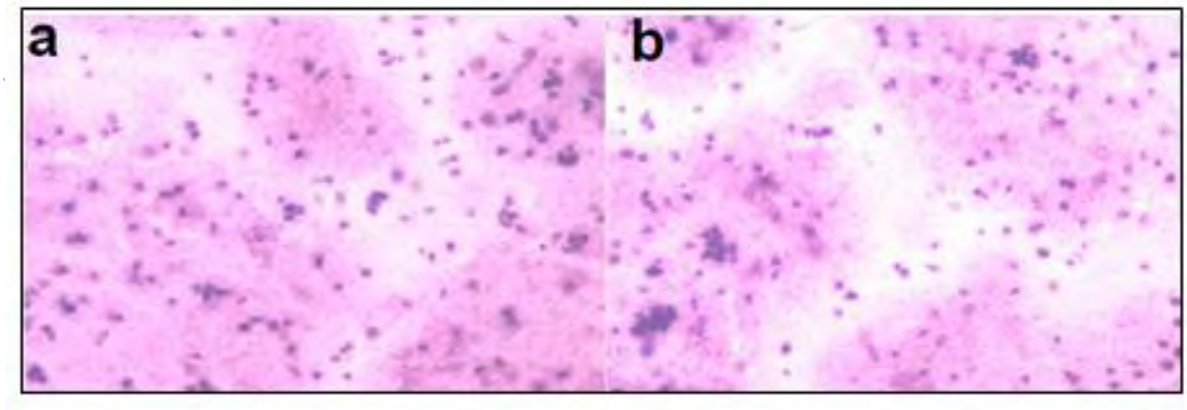

Fig.3.1. *Microscopic image of enriched platelets showing purity of enriched platelets*

3.3. Principle of spectrophotometer-based platelets aggregation study

Platelet rich plasma (PRP) with adjusted platelet count (done by Modified Tyrode Buffer (MTB)) was treated with two agonists separately i.e., ADP (20u M) and Adrenaline (10u M). Additionally, without agonist (ADP & Adrenaline) treated PRP was considered as a control group. Spectrophotometer can tell a concentration of a sample by measuring the scattering of transmitted light. When ADP or Adrenaline is added to PRP, platelets start to form aggregates. Thus, the suspended platelets start reducing which means the sample is now scattering less light as result the absorbance decreases. This decrease in absorbance is detected by spectrophotometer and it gives us an idea of platelets aggregation.

In our experiments, when PRP was treated with ADP and Adrenaline it showed a drop in curve of absorbance which was taken as extent of aggregation, while the straight line of control group show that there is no aggregation without an agonist.

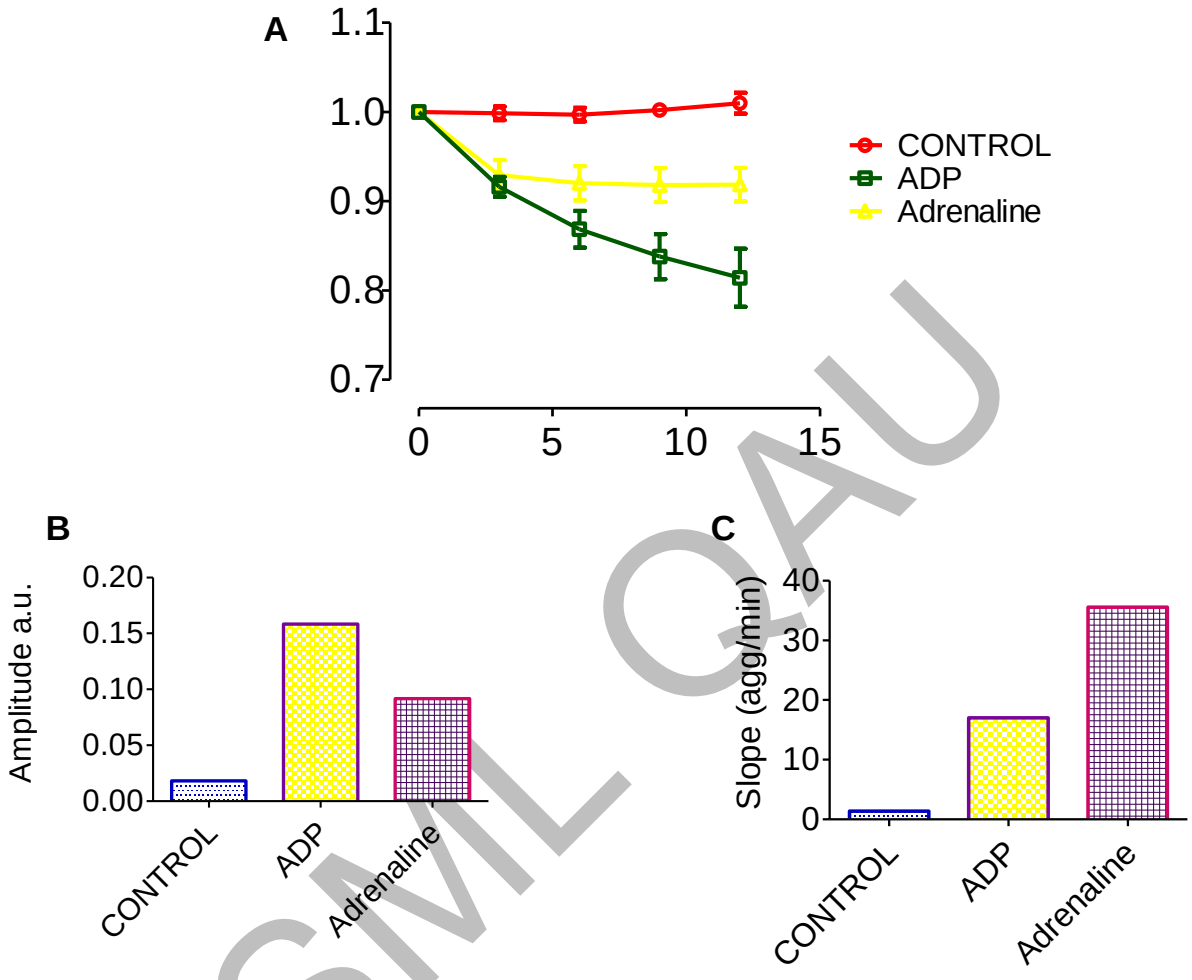

Fig-3.2: 20uM ADP and 10uM Adrenaline induced aggregation: 200ul *of PRP was treated with 20uM ADP and 10uM Adrenaline which was left untreated was used as a "Control". Absorbance was taken at 595nm for 15 minutes with constant stirring. A) The curve represents platelets aggregation at 595nm by ADP and Adrenaline. B) Bar graph represents the Amplitude. C) The slope bar graph represents the rate of decrease in absorbance/min.*

3.4. Effect of PAMPs or DAMPs on agonist-induced platelets aggregation:

The immunological response of platelets when activated by any inflammatory molecule (PAMPs, DAMPs or any pro-inflammatory mediator) results in movement of platelets to the site of an infection, granular secretion, and phagocytosis of the alarmin (Portier & Campbell, 2021b)(Ali et al.,2015). However, a investigate the role of "immunological activations" on agonist-induced platelets activation and aggregation we have used different DAMPs (Glucose, FMLP treated blood lysate, mitochondrial DNA, and mitochondrial proteins) and PAMPs (FMLP).

Table:3.2. List of primers used and their concentrations

Primers	Concentrations
FMLP	20u M
Glucose	20mM
FMLP treated blood cell's supernatant	20u l
Mitochondrial protein	50ng/u l
Mitochondrial DNA	50ng/u l

3.5. Effect of FMLP on Platelet aggregation in response to purinergic signaling

Adenine nucleotides (ATP&ADP) activate platelets when they bind with the specialized purinergic receptors (Cattaneo, 2014). On the other hand, FMLP[(N-formyl-methionyl-leucyl-phenylalanine) (bacterial or synthetic)] when binds to their FPR 1 receptor on platelet's cell surface it induces chemotaxis in platelets and mobilization of intracellular calcium (Czapiga *et al.,*2005). Therefore, a time dependent incubation of platelets with FMLP might have an impact on purinergic signaling (either enhance or decrease). Aiming to investigate the change in ADP mediated aggregation response in platelets by

the pre-treatment of platelets with FMLP, we did a time dependent incubation of platelets with FMLP and then stimulated these platelets by treating it with ADP.

3.5.1. Investigation of short-term FMLP incubation on ADP-mediated platelet aggregation

In order to determine the impact of FMLP on platelet aggregation the PRP (150,000/u l) was pre-treated with commercially purchased synthetic FMLP for 5 mins and then stimulated with ADP. alone treated with FMLP was used as a control group of a FMLP and ADP treated group to check the sole effect of FMLP on platelet aggregation The absorbance taken shows a significant drop in ADP-stimulated platelets as compared to unstimulated platelet (control group) indicating increased platelet aggregation. However, no apparent effect was seen on ADP-induced platelet aggregation between FMLP-treated and vehicle group (Fig 3.1 A). Moreover, when amplitude and rate of aggregation per minute (slope) was calculated from curve, we found no statistically significant difference between FMLP treated and vehicle (DMSO) (Fig:3.3B and C).

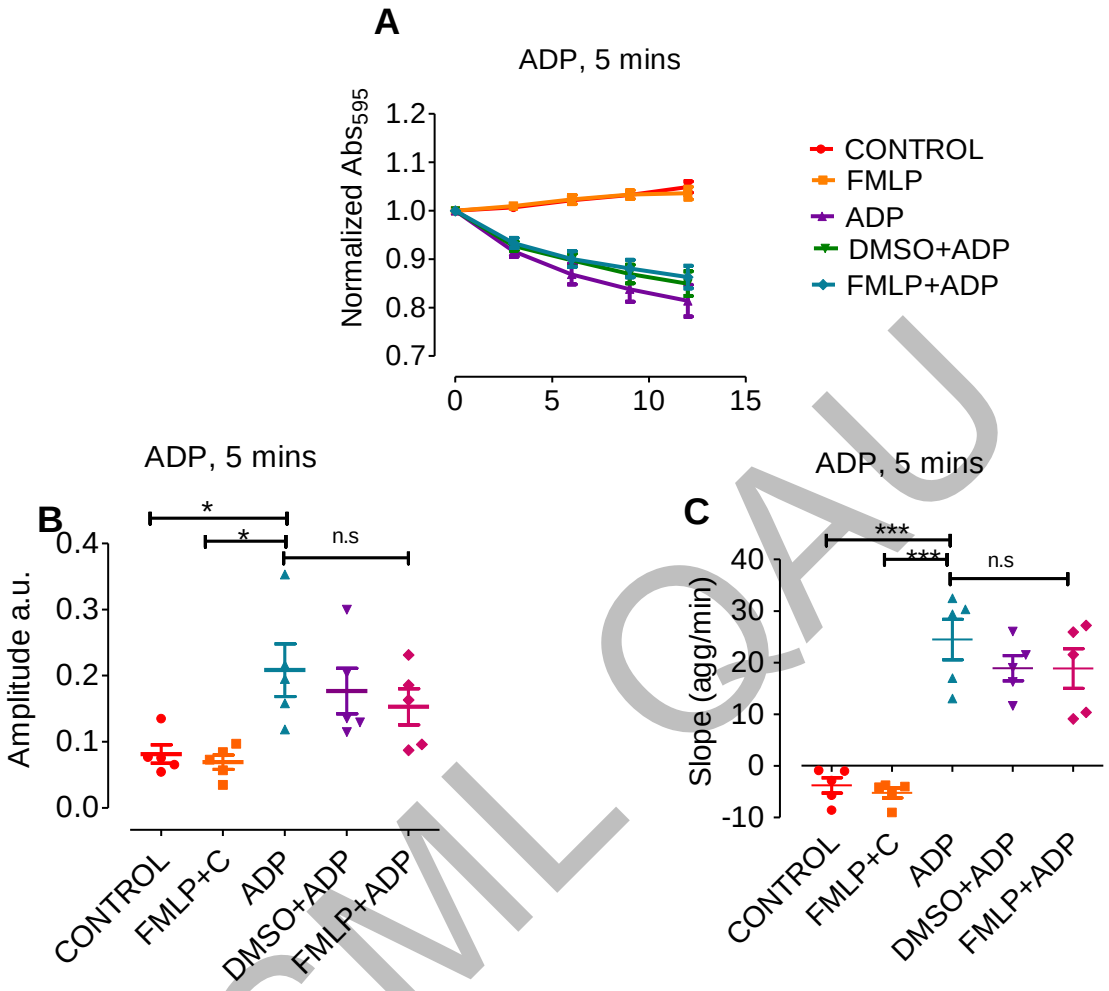

Fig-3.3: ***Effect of short incubation with FMLP on ADP-mediated platelet aggregation:*** *200ul of PRP with adjusted platelet count ($1.5x10^5$/ uL) in MTB was used in the experiment. PRP was pre-treated for 5 minutes with FMLP (20 uM) and DMSO (0.2%). A) Curve showing drop in absorbance indicating platelet aggregation after stimulation with 20uM ADP. (B) Scatter plot showing amplitude calculated from curve (C) Scatter plot showing the rate of aggregation per minute calculated as slope. Data represents mean± SEM, n=4 one-way ANOVA with Bonferroni MGCT, **p<0.05, ***P<0.0001.*

3.5.2. -Effect of prolonged FMLP incubation on ADP-mediated platelets aggregation

As shorter incubation with FMLP showed no difference in ADP-induced platelet aggregation. Therefore, we increased the incubation time of FMLP to determine its impact on platelet aggregation. To check whether an increased incubation time with FMLP gives a priming effect to platelets or not, we incubated PRP with vehicle DMSO (0.2%) and FMLP for 15 minutes. Unexpectedly, our data shows no significant difference in platelet aggregation even after increasing the incubation of FMLP as determined by curve (Fig 3.4 A) amplitude (Fig 3.4 B) and slope (Fig 3.4 C).

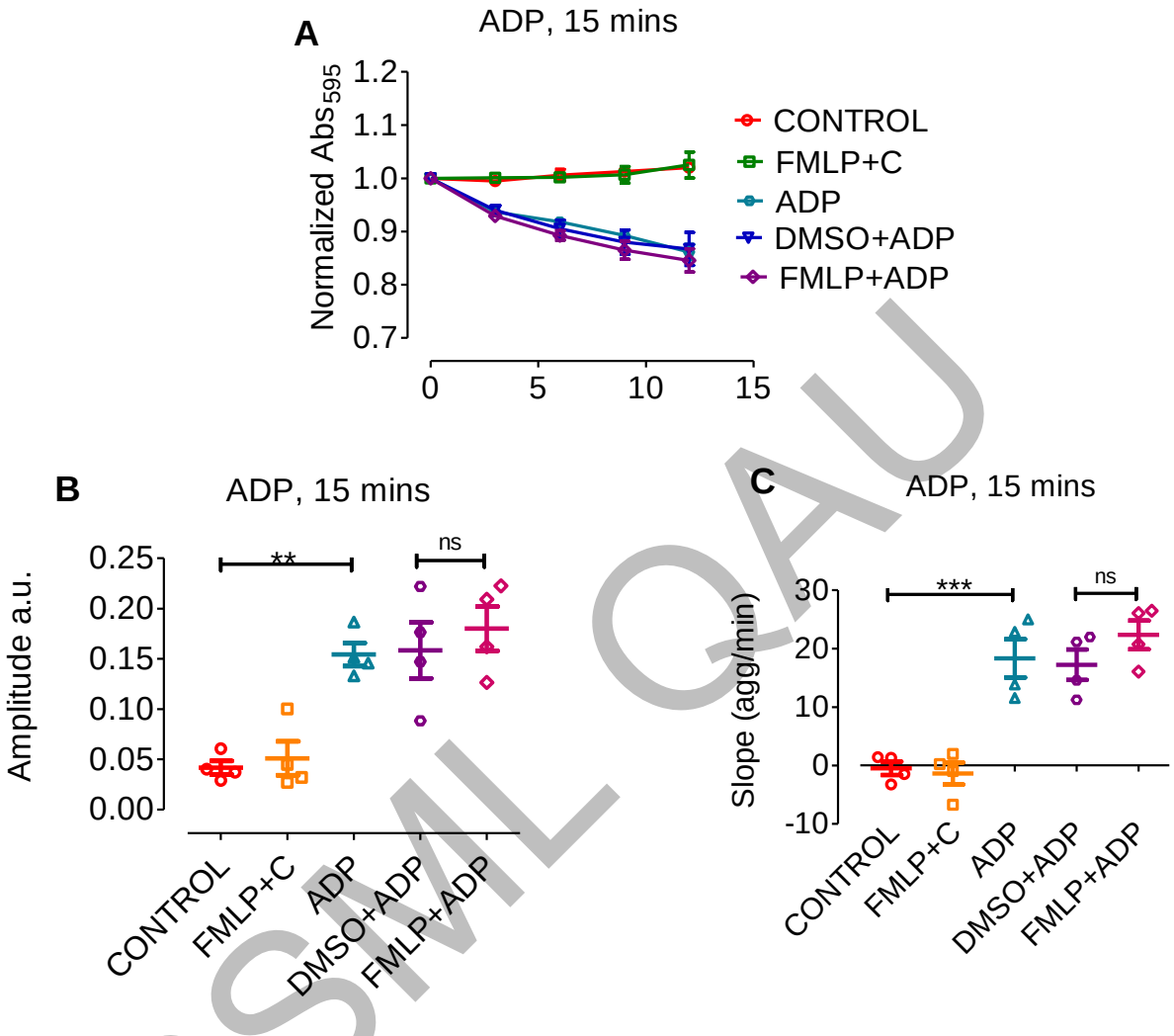

Fig-3.4: ***Effect of prolonged FMLP incubation on ADP-mediated platelets aggregation:*** *200ul of PRP with adjusted platelet count (150,000/ ul) was treated with 20uM ADP after pre-treatment with FMLP (20uM) and DMSO (0.2%) and the aggregation was noted at 595nm.A) Drop in curves showing the extent of aggregation. (B and C) Scatter plot showing the Amplitude and Slope calculated from aggregation curve. Data represents mean +SEM, n=5, one-way ANOVA with Bonferroni MGCT ***P<0.05, ***P<0.0001.*

3.6. Effect of FMLP on platelet aggregation in response to adrenergic signaling

Adrenaline is a weak soluble agonist that activates platelets through adrenergic receptor. With the intention to check FMLP priming effect on platelets aggregation we incubated PRP with FMLP for 5 minutes and 15 minutes and then stimulated it with Adrenaline.

3.6.1. Investigation of short-term FMLP incubation on adrenaline-mediated platelet aggregation:

In order to investigate the short-term incubation of FMLP on Adrenaline induced platelets aggregation we incubated PRP with FMLP and DMSO (0.2%) for 5 minutes. Only FMLP treated PRP was used as a control group of FMLP + Adrenaline treated group. Notably, we found a decline in absorbance in adrenaline stimulated group as compared to untreated PRP, only FMLP and vehicle control group. But unexpectedly no increase in adrenaline mediated platelet aggregation due to FMLP, and no change in aggregation was spotted between FMLP and vehicle groups, after analyzing the data by calculating amplitude and slope from aggregation curve (Fig.3.5 B & C).

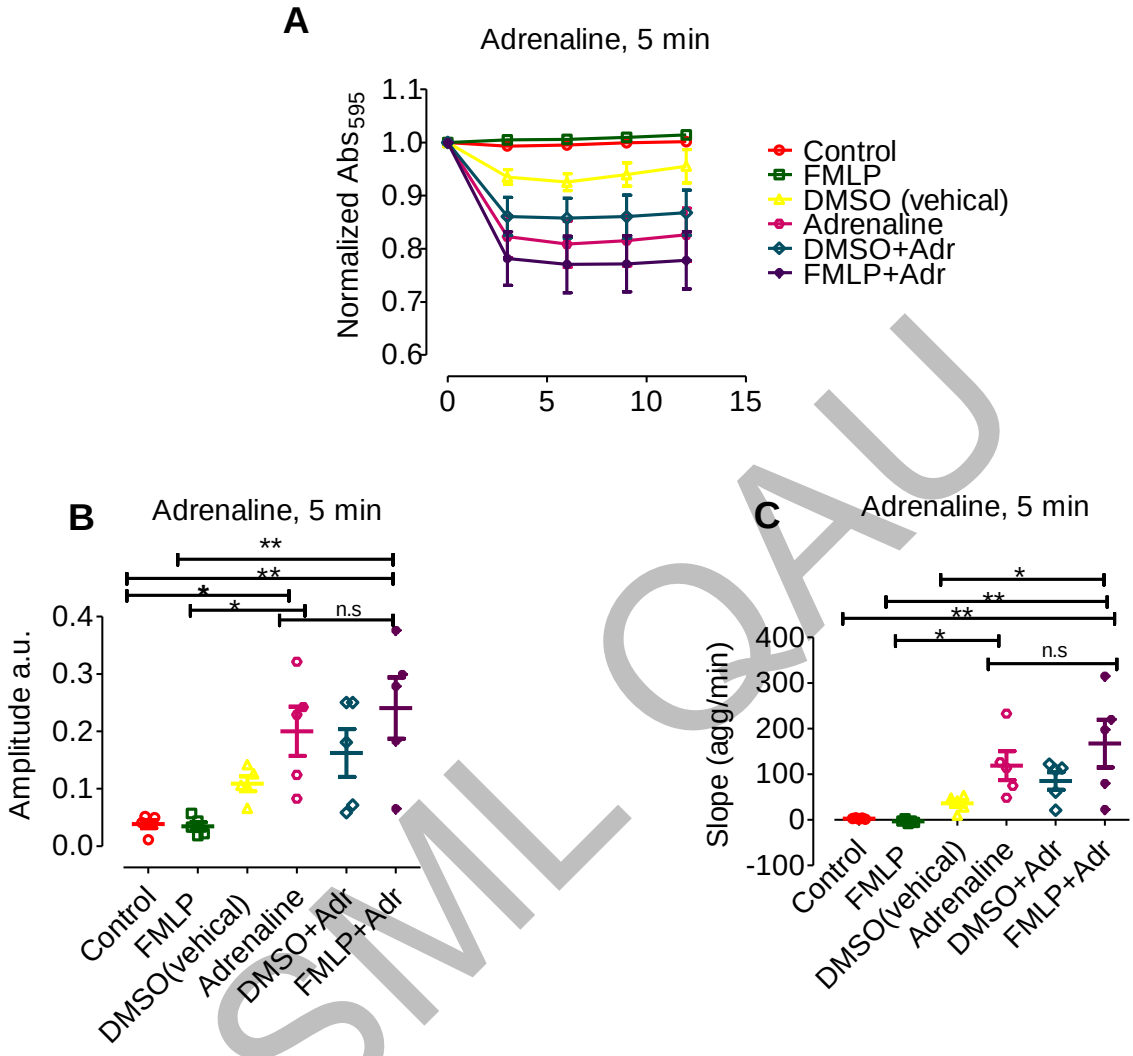

Fig-3.5: ***Investigation of short-term FMLP incubation on adrenaline-mediated platelet aggregation:*** *200ul of PRP with adjusted platelet count ($1.5x10^5$/ uL) was pre-treated with FMLP (20µM) and DMSO (0.2%) and stimulate by 10uM adrenaline. Aggregation curve was obtained at 595nm. A) The curve represents the extent of aggregation at 595nm. B) Shows a scatter plot of Amplitude. C) A scatter plot of Slope. Graph (A-C) shows mean ±SEM, n=5, one-way ANOVA with Bonferroni MGCT ***P<0.05, ***P<0.05.*

3.6.2. -Investigation of prolonged FMLP incubation on adrenaline-mediated platelet aggregation

FMLP short-time incubation demonstrated no significant effect on an adrenaline-induced platelets aggregation. Thus, to check whether an increase in incubation time has an impact we next incubated PRP with FMLP for 15 minutes and then stimulated with Adrenaline. Untreated PRP, FMLP alone and solely DMSO (vehicle control of adrenaline) treated groups were considered as a control groups. Adrenaline showed a drop in curve of absorbance but similar to short term preincubation with FMLP, it did not bring any increase in platelets aggregation on stimulation with Adrenaline. Moreover, there was no change in drop of absorbance in FMLP and vehicle treated groups. Furthermore, no significant difference was observed when an amplitude and slope was calculated from curve between FMLP and DMSO (vehicle control) treated groups. (Fig.3.6 A-C)

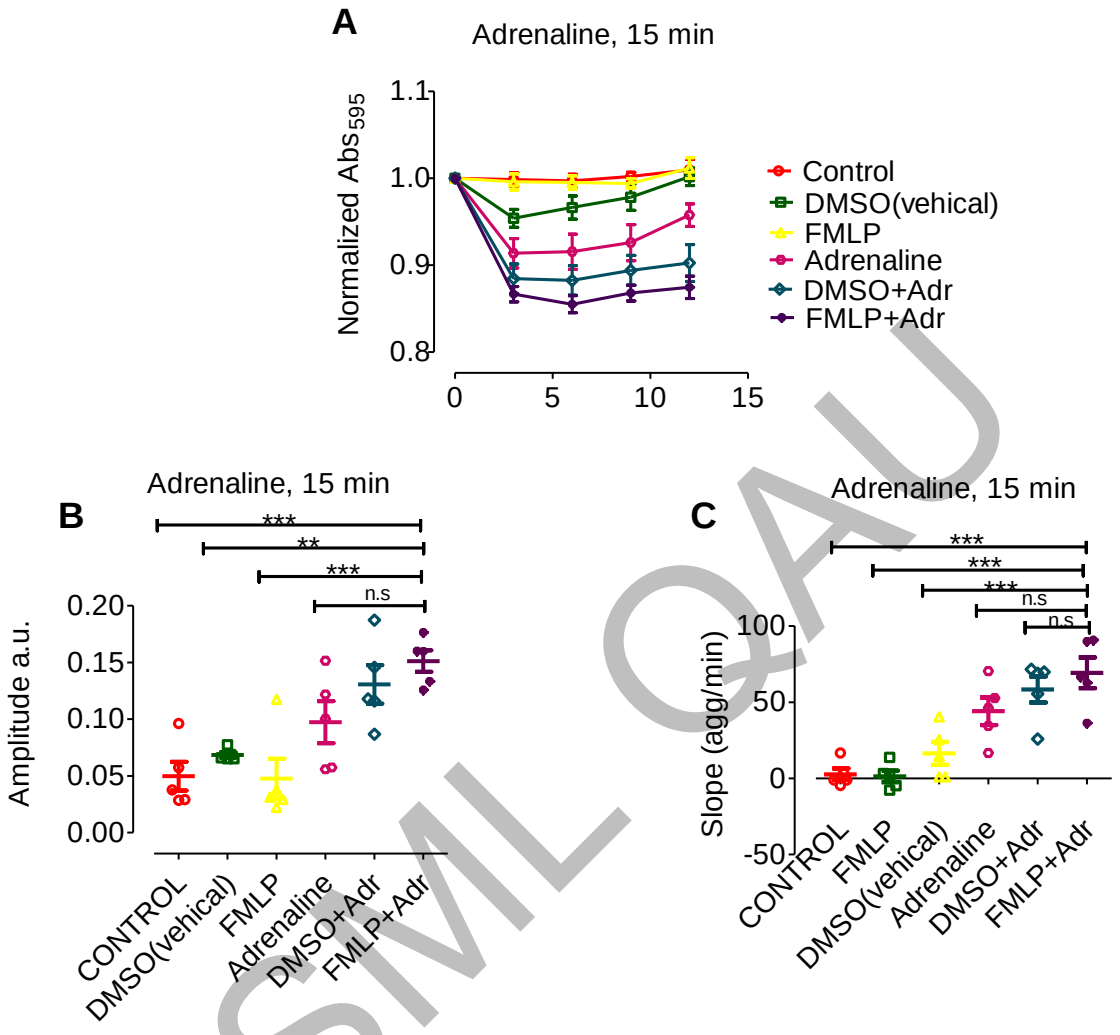

Fig-3.6: Investigation of prolonged FMLP incubation on adrenaline-mediated platelet aggregation: *200ul of PRP with adjusted platelets count (150,000ul) was taken as control group and a pre-treatment of FMLP (20uM) and DMSO (0.2%) was given for 15 minutes. A) At 595nm the aggregation extent was noted-B) Amplitude scatter plot shows extent of aggregation along with significant and non-significant groups. C)Scatter graph shows rate of kinetic per minute(slope). Graph(A-C) shows mean ±SEM, n=5, one-way ANOVA with Bonferroni MGCT ***P<0.05, ***P<0.05.*

3.7. Priming effect of DAMPs on platelet aggregation

DAMPs (Damage/Danger associated molecular patterns) are components released from dead or damaged cells which the body process them as immune alarmins. With an eye on their role in affecting platelets aggregation we used few DAMPs (Glucose, FMLP treated blood cell lysate, mitochondrial DNA, mitochondrial proteins), in order to check their effect on platelet aggregation after stimulating them with a weak soluble agonist i.e., Adrenaline.

3.7.1. Impact of short-term incubation of high glucose on adrenaline-induced platelet aggregation:

Glucose body concentration is about 5-7mM. But in high glucose condition in extracellular milleu it causes its higher consumption by platelets and thus leading to excessive ROS production and platelets hyperactivity (Fidler *et al*., 2019). So, higher concentration of glucose may act as DAMP. Therefore, in order to check the effect of higher glucose on platelet aggregation we have pretreated the platelets with 20mM glucose for almost 7 minutes and stimulated with adrenaline. Surprisingly, higher concentration of glucose showed increased tendency towards platelet aggregation in comparison to stimulation of adrenaline treatment as indicated by curve (Fig 3.7 A). However, we did not find a statistically significant difference between the two groups (Fig 3.7 B and C).

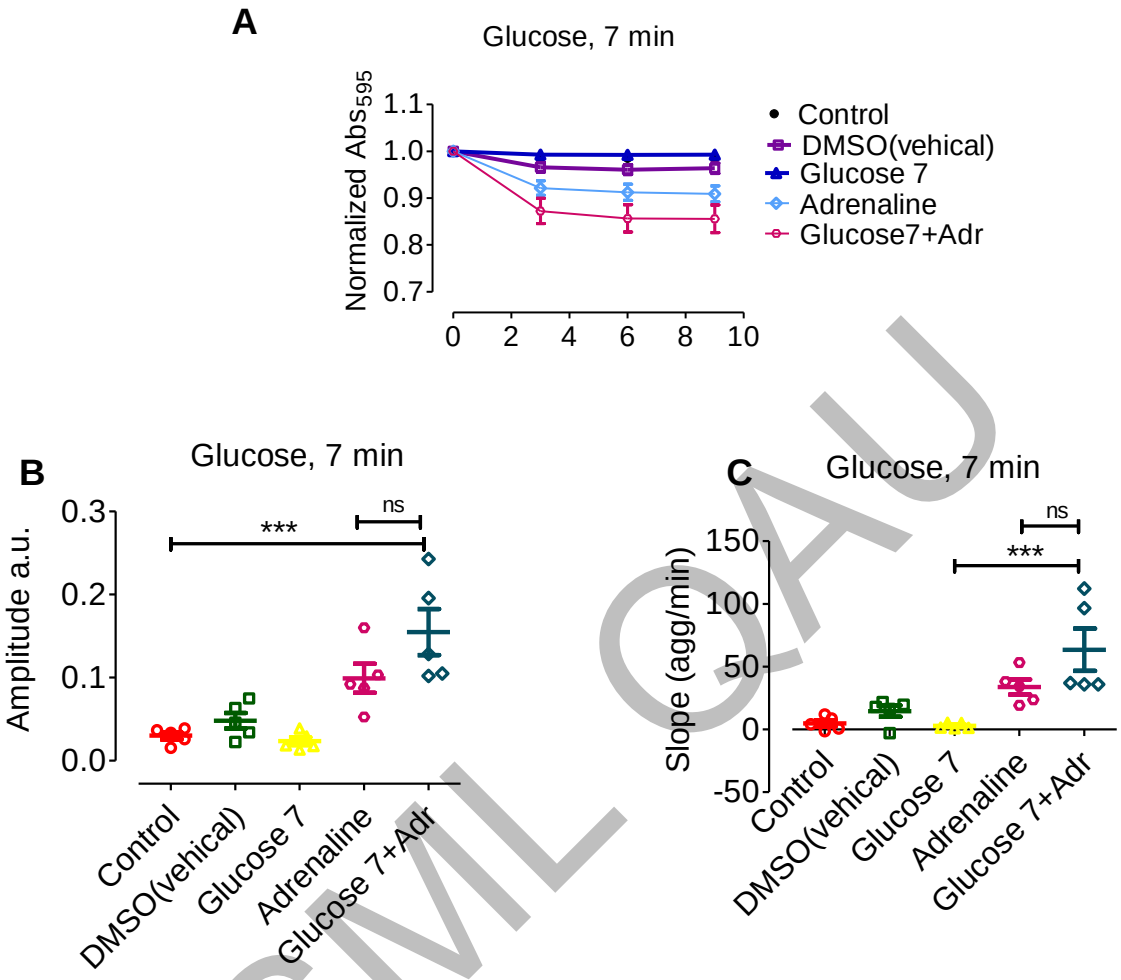

Fig-3.7: ***Impact of short-term incubation of high glucose on adrenaline-induced platelets aggregation:*** *PRP of volume 200µl with adjusted platelet count (150,000µl) was pre-treated with high glucose(20mM) for 7 minutes and then stimulated with Adrenaline(10µM) to check aggregation. Untreated PRP DMSO [vehicle (0.2%)] and only glucose treated groups were considered as control groups. A) At 595nm aggregation curve was obtained. B) shows a scatter plot of amplitude estimated from curve. C) Scatter plot of rate kinetics(slope). Graph(A-C) shows mean ±SEM, n=5, one-way ANOVA with Bonferroni MGCT *P<0.05, *P<0.05.*

3.7.2. Impact of prolonged incubation with high glucose on adrenaline-induced platelets aggregation

As we have noticed an increased trend in platelet aggregation after shorter incubation of glucose, so in the next experiment we have increased the incubation time with glucose to check its priming effect on platelet aggregation. PRP was pretreated with high Glucose (20mM) for 15 mins and then stimulated with adrenaline. Surprisingly, significant difference in drop of absorbance was shown by adrenaline and the treated group compared to the control (PRP), vehicle (DMSO) and alone glucose(20mM) treated group. But no significant difference noticed between glucose treated and adrenaline treated groups. The extent of amplitude and the rate of kinetics(slope) was calculated from curve (Fig:3.8 A-C)

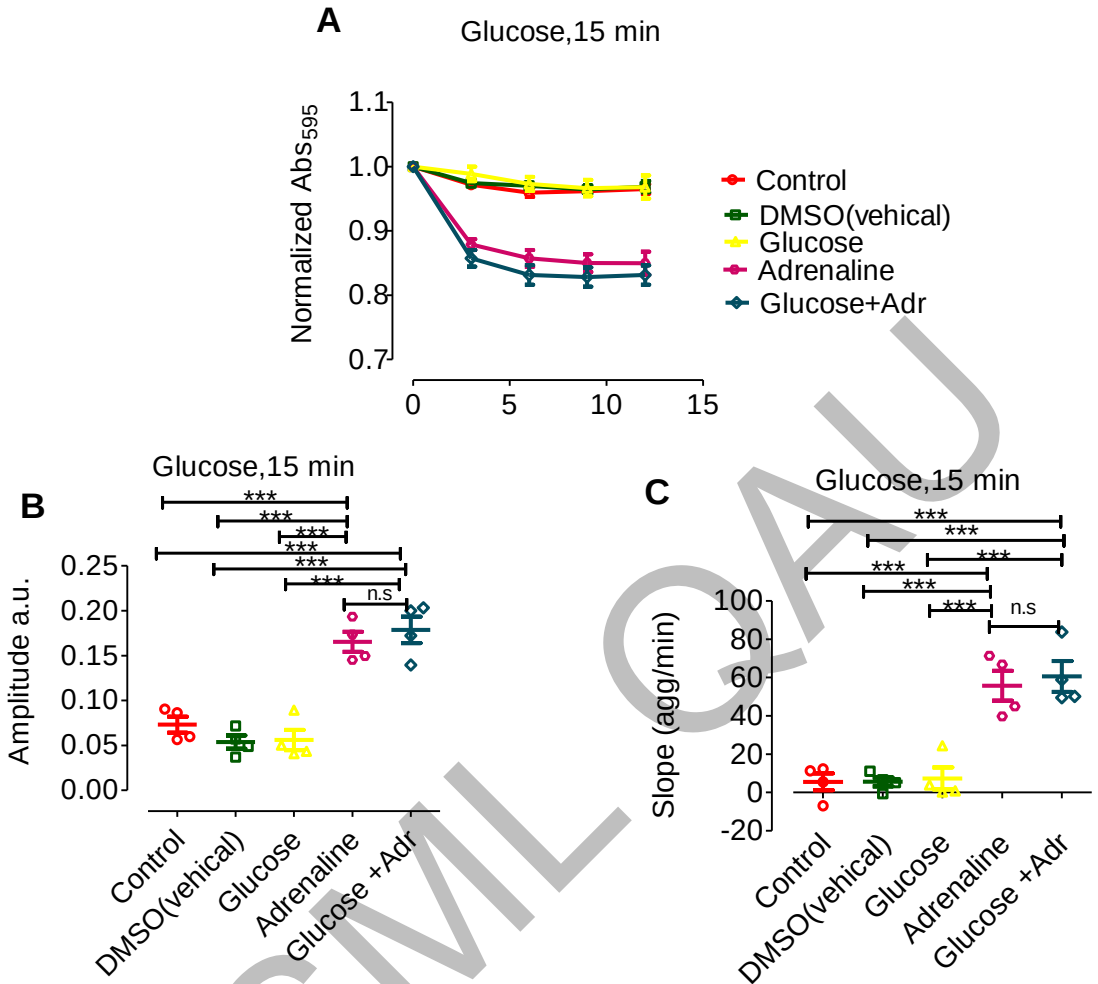

Fig-3.8: Impact of prolong incubation of high glucose on adrenaline-induced platelets aggregation *High glucose (20mM) treated 200µl of PRP (150,000µl) was stimulated with Adrenaline(10µM) after 15 minutes of incubation and a curve was obtained at 595nm.A) Aggregation curve at 595 showing extent of aggregation. B) Calculated Amplitude from curve. C) Shows a scatter plot of slope. Graph(A-C) shows mean ±SEM, n=4, one-way ANOVA with Bonferroni MGCT ***P<0.05, ***P<0.001*

3.7.3. Impact of FMLP treated blood cell's supernatant on adrenaline-mediated Platelet aggregation

FMLP when sensed by leukocytes especially by neutrophils through their cell surface receptors, it forces neutrophils to undergo degranulation and NETosis. In these processes cell release DNA, histones, heat shock and many other proteins (Brinkmann *et al*., 2004) acting as DAMPs. DNA and histones are well known for their role by acting as a DAMP when present in extracellular environment (Roh & Sohn, 2018b). To investigate the priming effect of these cellular release products on adrenaline-mediated platelet aggregation, we first treated blood cell's with FMLP (50nM) and then after 1 hour incubation we used a supernatant of these blood cells as a priming agent for PRP.

PRP was pretreated with blood cell's supernatant for 15 minutes and then stimulated by adrenaline. Adrenaline treated group showed an increase in aggregation compared to control (untreated PRP) and to solely FMLP (50nM) treated blood cell's supernatant treated group. Unexpectedly, no significant difference in aggregation was noticed between blood cell's supernatant treated group and only adrenaline treated group(Fig3.9).

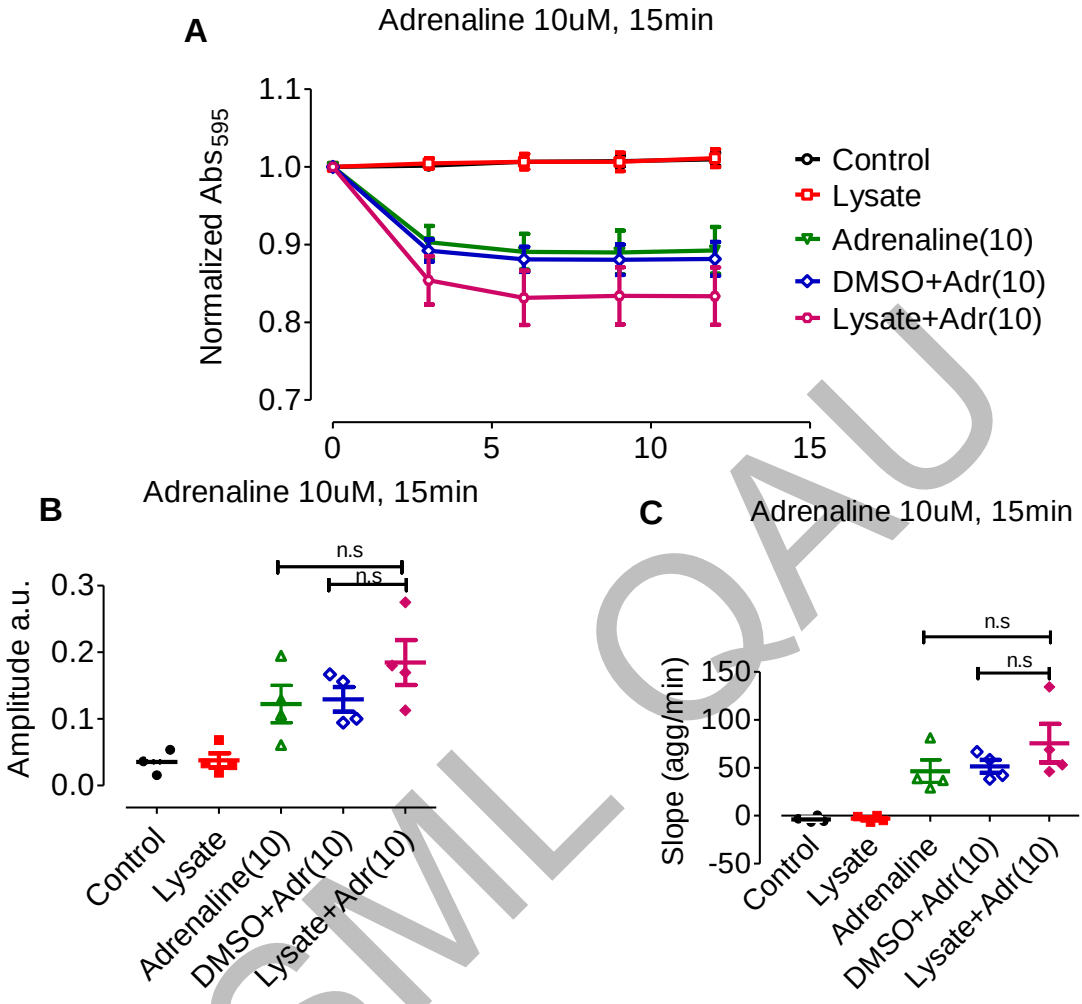

Fig-3.9: Impact of FMLP treated blood cell lysate on adrenaline-mediated platelet aggregation: *30 million platelets were pretreated with FMLP (50nM) treated blood cell's lysate and then after 15 minutes stimulated by Adrenaline. Untreated and alone lysate treated PRP (150,000/u l) and treated vehicle (DMSO 0.005%) group was used as control groups. A) Drop in aggregation curve was obtained at 595nm. B) scatter plot of an Amplitude .C) rate of kinetics (slope) plot. Graph(A-C) shows mean ±SEM, n=4, one-way ANOVA with Bonferroni MGCT ***P<0.05, ***P<0.05.*

3.7.4. Impact of prolonged-FMLP treated blood cell's lysate on platelet aggregation Induced by sub-optimal adrenaline

Agonist working is detected by their extent of platelet aggregation. May be the optimal dose of the adrenaline is masking the priming effect of DAMPs so in aiming to deduce the extent of priming by a FMLP treated blood cells lysate we used a suboptimal dose of adrenaline (2.5u M), and incubation time of FMLP treated blood cell lysate was increased from 15 to 30 minutes.

Pre-treatment of PRP was done for about 30 minutes with FMLP-treated blood cell lysate and were then subjected to stimulation by adrenaline (2.5u M). Untreated PRP and only lysate treated PRP was used as a control group. Unexpectedly, there was no change in aggregation after pretreatment of platelets with lysate. However, adrenaline showed a significant increase in amplitude than control groups. While no significant increase was there between vehicle (DMSO 0.005%) and lysate treated groups.(Fig 3.10)

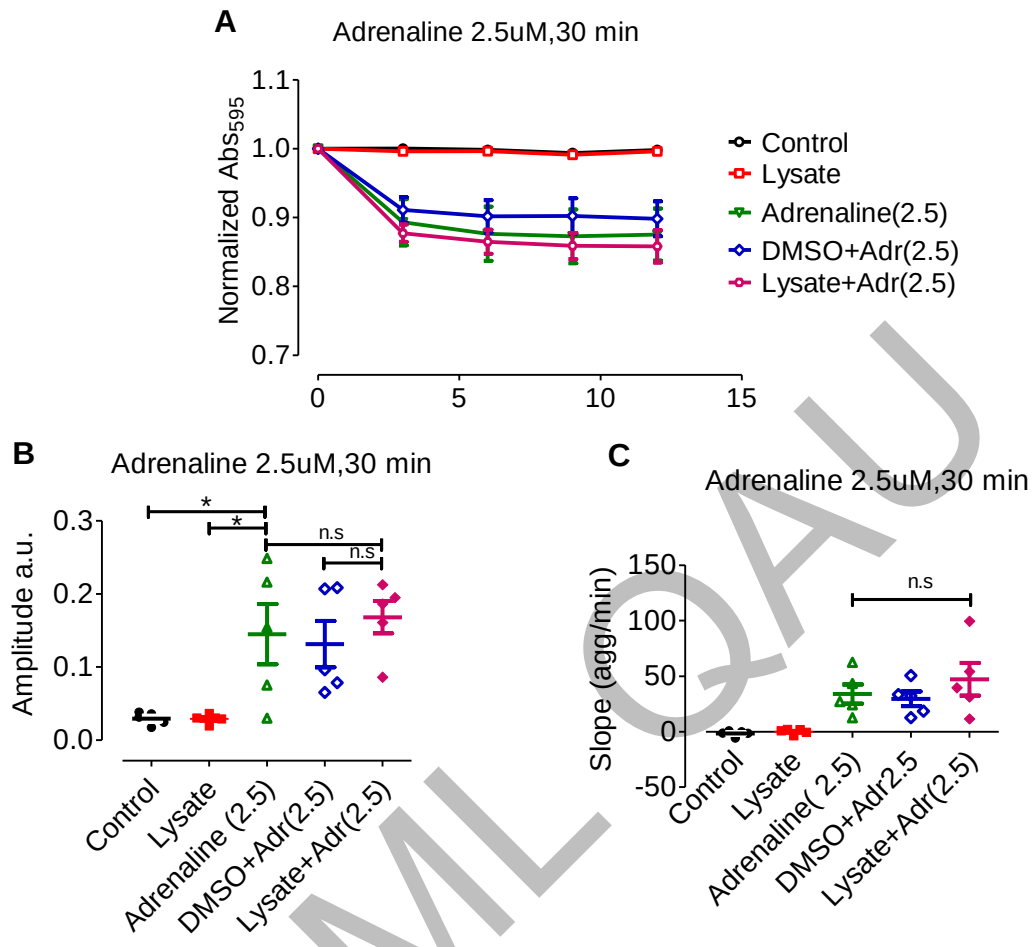

Fig-3.10:Impact of prolonged-FMLP treated blood cell lysate on sub-optimal adrenaline-induced platelet aggregation: *200u l of PRP (30 million) was subjected to pretreatment with FMLP (50nM) treated blood cell's lysate and after 30 minutes of incubation an aggregation curve was obtained at 595nm.A) Shows an extent of aggregation shown by a curve. B) scatter plot of an Amplitude. C) rate of kinetics(slope). Graph(A-C) shows mean ±SEM, n=5, one-way ANOVA with Bonferroni MGCT **P<0.05, **P<0.05.*

3.7.5. Impact of short-term mitochondrial DNA incubation on adrenaline-mediated platelet aggregation

Mitochondria have several molecules which act as DAMPs such as succinate, N-formyl peptides, cardiolipin and mitochondrial DNA (Nuevo &Zorzano,2019). To investigate the effect of mitochondrial DNA on adrenaline-induced platelets aggregation, we incubated PRP with mitochondrial DNA with two time periods i.e.,1&3 hour.

First PRP was treated with mtDNA for about 1 hour and then stimulate with adrenaline. Untreated PRP, only mtDNA treated and vehicle (DMSO) treated groups were kept as control groups. Adrenaline showed a significant increase in aggregation compared to all control groups. Surprisingly, mtDNA showed a significant increase in aggregation after stimulation with adrenaline as compared to only adrenaline treated group. From curve graph slope and amplitude was estimated.(Fig 3.11)

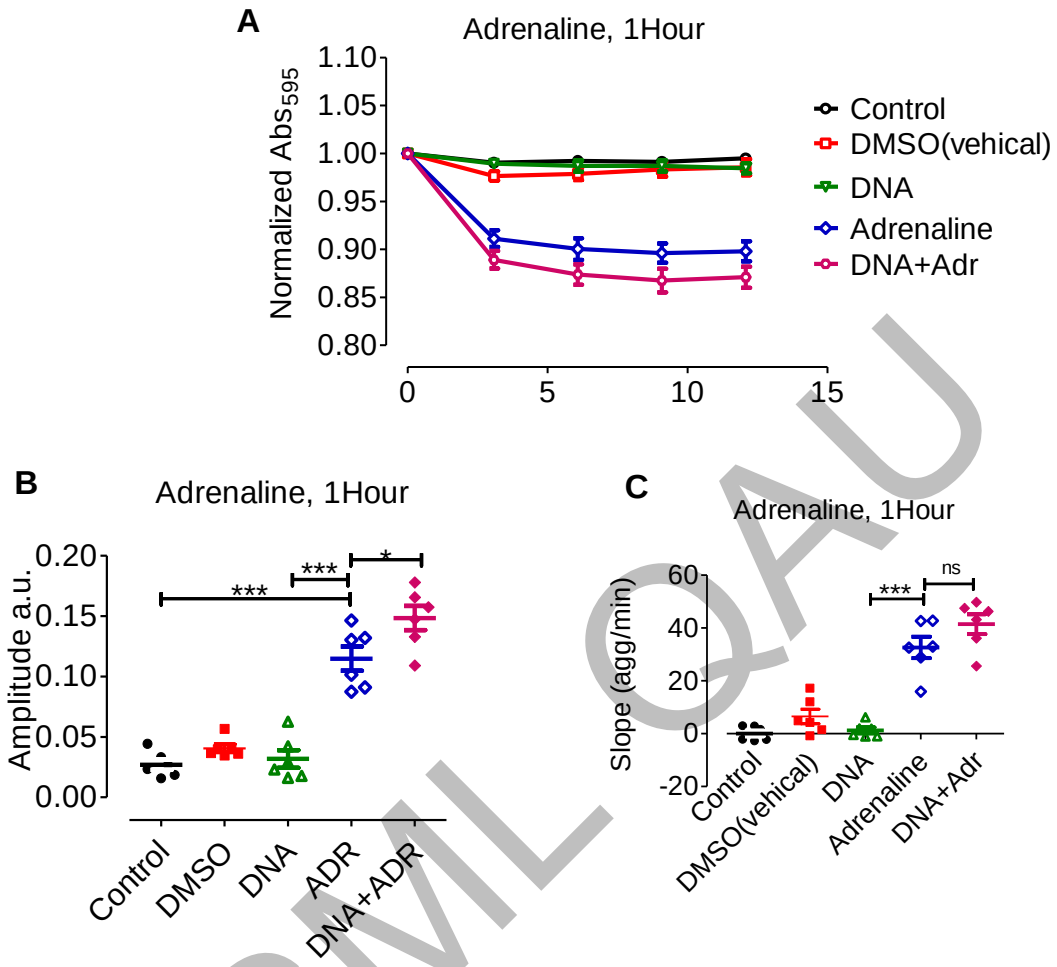

Fig-3.11: Impact of mitochondrial DNA on adrenaline-mediated platelet aggregation: *30 million platelets were pretreated with mtDNA(50ng/u l) and then after 1 hour it was stimulated by Adrenaline. Untreated, alone mtDNA treated PRP (150,000/u l) and vehicle (DMSO 0.2%) groups were used as control groups. A) Drop in aggregation curve was obtained at 595nm. B) scatter plot of an Amplitude .C) rate of kinetics (slope) plot. Graph(A-C) shows mean ±SEM, n=6, one-way ANOVA with Bonferroni MGCT ***P<0.05, ***P<0.05.*

3.7.6. Impact of prolonged mitochondrial DNA incubation on adrenaline mediated platelet aggregation:

We have noticed a significant increase in platelets aggregation by 1 hour incubation with mtDNA. Now to check a long-term incubation of PRP with mtDNA we pre-treated PRP with mt DNA for about 3 hours and then stimulated by adrenaline. Untreated PRP, only mtDNA treated, and vehicle (DMSO) treated groups were considered as control groups. Adrenaline showed a significant increase in aggregation compared to control groups and surprisingly mtDNA also showed a significant increase in aggregation after stimulation with adrenaline compared to only adrenaline treated group(Fig 3.12).

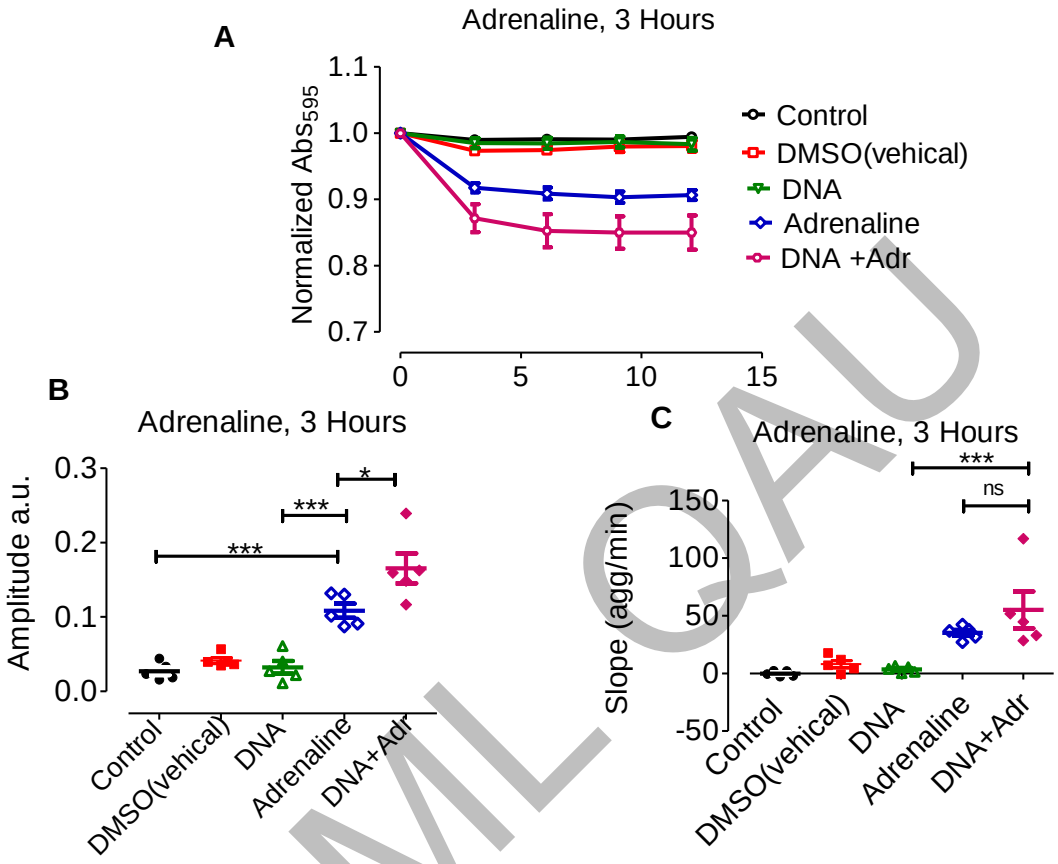

Fig-3.12: Impact of prolonged mitochondrial DNA incubation on adrenaline-mediated platelet aggregation: *30 million platelets were pretreated with mtDNA(50ng/u l) and then after3 hour it was stimulated by adrenaline. Untreated, alone mtDNA treated PRP (150,000/u l) and vehicle (DMSO 0.2%) groups were used as control groups. A) Drop in aggregation curve was obtained at 595nm. B) scatter plot of an Amplitude .C) rate of kinetics (slope) plot. Graph(A-C) shows mean ±SEM, n=6, one-way ANOVA with Bonferroni MGCT ***P<0.05, ***P<0.05.*

3.7.7. Impact of mitochondrial protein on adrenaline mediated platelet aggregation:

Mitochondrial formylated protein which are also known as N-formyl peptides are considered as mitochondrial DAMPs. To check their effect on platelets aggregation we pre-incubated PRP with 50ng of mitochondrial protein and then stimulated it with adrenaline. Untreated PRP, only mitochondrial protein treated (control of mitochondrial protein + adrenaline treated group), and vehicle (DMSO) treated PRP were used as control groups. We found that adrenaline showed a significant increase in aggregation compared to control groups. Unexpectedly, we found no significant difference between mitochondrial protein pretreated group and adrenaline treated group.

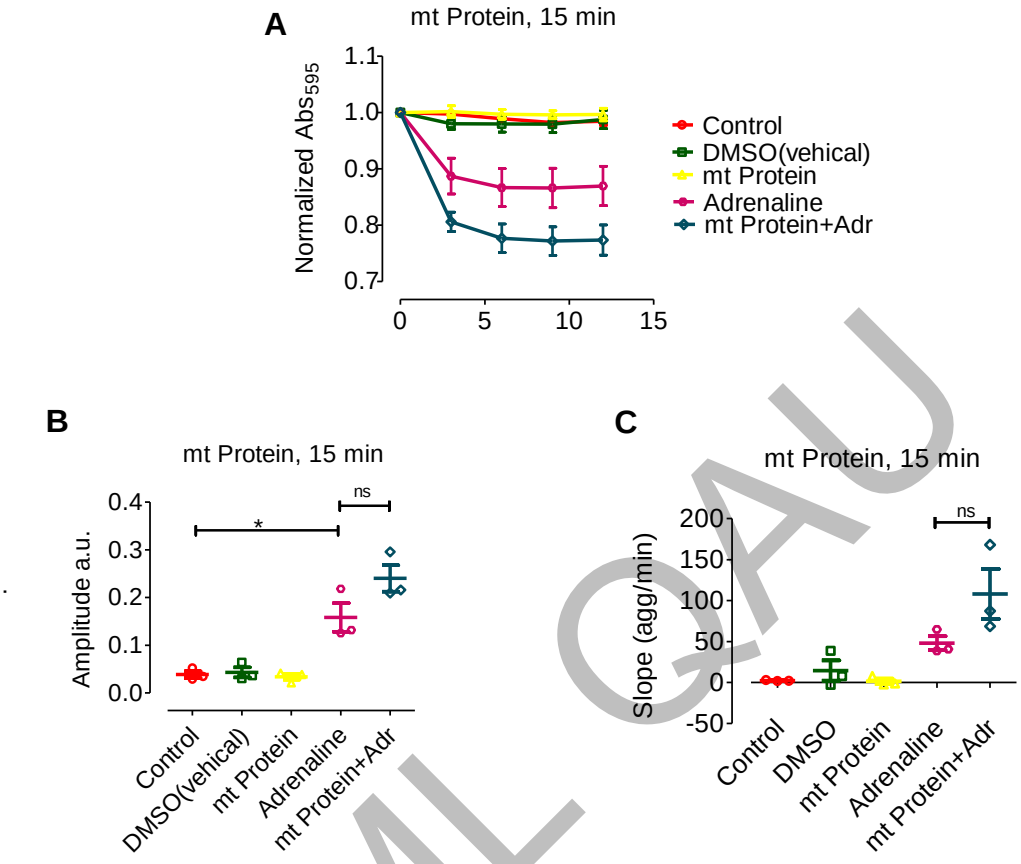

Fig-3.13: Impact of mitochondrial protein on adrenaline mediated platelet aggregation:*200ul of PRP (30 million) was subjected to pretreatment with mitochondrial proteins(50ng/ul) and after 15 minutes of incubation an aggregation curve was obtained at 595nm.A) Shows an extent of aggregation shown by a curve. B) scatter plot of an Amplitude. C) rate of kinetics(slope). Graph(A-C) shows mean ±SEM, n=3, one-way ANOVA with Bonferroni MGCT **P<0.05, **P<0.05.*

4. DISCUSSION

Besides its hemostatic function platelets also have immune receptors which detect PAMPs and DAMPs and react to them by their granular secretion, phagocytosis, chemotaxis and by making interaction with leukocytes (Cognasse *et al*., 2019, Janssen *et al*.,2016).

Platelets have got this ability of detecting many inflammatory molecules due to plethora of immune receptors on their cell surface, and after detecting them, a signaling cascade bring changes in platelets which we call as platelets inflammatory response (Yeaman, 2009)(Speth *et al*., 2013). These immunological responses may bring an impact on platelets hemostatic role. This study was aimed to scrutinize, the so far not fully explored, the effect of pro-inflammatory mediators on agonist-mediated platelet aggregation. Our objective was to find a role of PAMPs (FMLP) and DAMPs (Glucose, FMLP treated blood cell lysate, mitochondrial proteins, and mitochondrial DNA) in agonist mediated microplate based spectrophotometric aggregation of platelets.

ADP and adrenaline both are weak soluble agonists which activate the platelets through their respective receptors on platelets surface i.e., purinergic, and adrenergic receptors (Stalker *et al*., 2012). We have used these two soluble weak agonists to check the response of PAMPs and DAMPs on platelets aggregation.

PAMPs (Pathogen-associated molecular patterns) are infectious molecules that belongs to pathogens. Whenever a body is exposed to PAMPs they are detected by immune cells through PRRs (Pattern recognition receptors) and exhibit their immune response. Platelets are also found to detect PAMPs and participate in inflammation. Keeping this in view, we have used one of the PAMPs i.e. FMLP (*N*-formyl-methionyl-leucyl-phenylalanine) which is synthetic mimic of bacterial formyl peptides that is detected by FPR-1 receptor (Ye, Boulay, Ji, *et al*., 2009). Synthetic FMLP and its time dependent incubation was used to check its priming effect on ADP and Adrenaline mediated platelet aggregation. Literature study have reported the augmentation role of FMLP in thrombus formation when platelets were activated (Salamah *et al*., 2019). Unexpectedly, we found no significant increase in aggregation at different time points (5 & 15 minutes), in case of

both ADP (20u M), and adrenaline (10u M)) mediated aggregation. The possible reasons might be that ADP and adrenaline may not trigger a strong intracellular event (e.g., intracellular calcium mobilization) which has to be enough to show a significant increase in aggregation between treated (FMLP) and non-treated group (only agonist). However, it is also possible that FMLP may show different effect on different soluble (adrenaline, ADP, or thrombin) and non-soluble agonist (collagen and fibrinogen.) mediated aggregation. Additionally, platelets may have an unexplored pathway like inside out signaling to nullify the effect of these PAMPs so that it cannot bother platelet hemostatic function.

DAMPs (Danger/Damage associated molecular patterns) are intracellular physiological molecules whose presence in extracellular milieu or its over-concentration generate an immune response detected by immune cells as alarmins (Murao et al., 2021). DAMPs also generate inflammatory responses and are detected by PRRs, so we have used few DAMPs with an aim to study their effect on platelets aggregation.

Glucose is an energy giving molecule of cell, whose normal concentration in blood is about 3.8-5.8mM /L. High glucose concentration in blood (as in case of diabetes mellitus) induces platelets to become hyperactive due to activation of protein kinase C inside platelets (Assert *et al*., 2001). However, glucose as a DAMP and generate inflammation associated with diabetes(Gurijala, 2019).Therefore, we used a high glucose concentration (20mM) with two different incubation time sets (7 &15 minutes) to check its priming effect on platelet in Adrenaline mediated platelets aggregation. But we have not found a significant difference in platelet aggregation between treated and untreated groups. It might be due to fact that increased concentration of glucose (>20mM) and longer incubation might be required to prime the platelets (Sudic *et al*., 2006).Moreover, inside the body hyperglycemia causes vascular inflammation, increased fibrinolysis and elevation of clotting factors (Carr, 2001). These factors (collagen, fibrinogen & clotting factors) are strong agonists for platelets aggregation. But in vitro we have used a very weak soluble agonist i.e., Adrenaline which may be unable to cause that much aggregation to show a significant difference between two groups.

As discussed earlier several intracellular molecules when present in extracellular milieu act as DAMPs. FMLP induces in leukocytes especially in neutrophils might cause NETosis, in which cellular contents come out along with DNA and protein, which collectively act as DAMPs. Therefore, FMLP (50nM) treated blood cell lysate was used to check its priming effect in adrenaline-induced platelet aggregation. Surprisingly, we found no significant difference in aggregation. Additionally, we also change its incubation time and the concentration of adrenaline (from 10µM to 2.5µM) but our data did not show any significant difference between the two groups. This might be due to the fact that in addition to positive primers there are also negative primers (e.g., adenosine) which increases the activation threshold of platelets (Veninga *et al*., 2021). Thus, the negative priming may reverse the aggregation status of platelets. Moreover, platelets may require a much more incubation time or concentration of this cellular lysate to show its priming influence effectively.

Mitochondria, besides playing their other important cellular roles, in the immunological field are also considered as a source of DAMPs. Here we used mitochondrial DNA (mtDNA) and mitochondrial formylated proteins (mt proteins) to check their effect on platelets aggregation.

When mitochondrial DNA was used as a priming agent on two different incubation time sets (1&3 hour). Surprisingly, we found a significant difference in aggregation between mtDNA and only adrenaline treated group, and these results are compatible with our hypothesis that mtDNA besides playing a role as DAMP may also activate platelets.

Besides mtDNA we also used mitochondrial proteins (mt proteins) as a priming agent to check its effect in adrenaline-mediated platelet aggregation. Unexpectedly, we found no difference in aggregation. The reason might be that, in the mixture of mt proteins we may have a protein/s having a role of negative priming. Additionally, may be the incubation time we are giving (15 minute) might not be sufficient to work as a platelet activator.

Platelets differ from each other in size, shape, and their activation status thus, we can say that platelets are heterogenous in nature. This heterogeneity depends upon their environmental stimuli and their parent cells i.e. megakaryocytes (Kempton *et al*., 2005).

In addition, there are lots of environmental stimulus inside the body like many DAMPs are released inside the body whose individual estimation is difficult. So, these different stimuli change the activation status of platelets by responding differently to the same concentration of an agonist. Another likely scenario may be the platelets heterogenous behavior and individual to individual difference in priming agents like negatively priming which may act as obstacles to counter the impact pro-inflammatory mediators in agonist induced platelets aggregation. Moreover, platelets show a different extent of aggregation with different agonists. In our case we used soluble weak agonists while a priming effect of different PAMPs is reported in collagen and thrombin induced platelets aggregation. Therefore it might be a possible change in the agonist which could yield a significant increase in aggregation.

5. Conclusion

Platelets though being small and anucleate, still plays a vital role in immunity and hemostasis, and in our study, we tried to connect the two physiological compartments together i.e., immunology and hemostasis. Although, except DNA we did not find any increase in platelet's aggregation in primers incubated platelets in ADP and Adrenaline mediated aggregation, but this finding can't be generalized for other pro-inflammatory agents and agonists. This area of study still requires a defined research approaches keeping in view all the limitations of other priming agents, individuals' health status, exposure time of these agents to platelets, and nature and type of agonist.

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