Investigation of Adrenergic Receptor-Mediated Mitochondrial DNA Release from Human platelets

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in

Biochemistry/ Molecular Biology

by

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Declaration

I hereby declare that this research study represents my efforts and hard work. The thesis is written and composed by me. No part of this work has been presented previously for any other degree or certificate.

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CERTIFICATE

This thesis, submitted by **Ms. Iraj 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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List of Abbreviations

Abbreviation	Full Name
µg	Micro gram
µl	Micro liter
ACD	Acid-citrate-dextrose
ADP	Adenosine diphosphate
APS	Ammonium persulfate
AR	Adrenergic receptor
ATP	Adenosine triphosphate
Bcl-2	B cell lymphoma-2
BMI	Body Mass Index
BSA	Bovine serum albumin
cGAS	cyclic GMP-AMP synthase
COX 1	Cytochrome oxidase subunit 1
COX 3	Cytochrome oxidase subunit 2
CpG	Cystine-phosphate-Guanine
CRP	C-reactive protein
CVD	Cardio vascular disease
Cyt	cytochrome
DAMPs	Damage associated molecular pattern
D loop	Displacement loop
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FPR 1	Formyl peptide receptor 1
GP Ia	Glycoprotein Ia
GP VI	Glycoprotein VI
GPCRs	G proteins coupled Receptors
HRP	Horse radish peroxidase
IL-1 β	Interleukin 1 beta

1L-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
INF	Interferons
ISG	Interferon Stimulated Gens
KCl	Potassium Chloride
MFI	Mean fluorescence intensity
MgCl2	Magnesium chloride
mitoMPs	Mitochondria containing microparticles
MPs	sub-Micron vesicle
MMP	Mitochondrial membrane potential
mPTP	Mitochondrial Permeability Transition Pore
mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
ND 1	NADH dehydrogenase 1
ND 2	NADH dehydrogenase 2
OH	Origin of replication for heavy chain
OL	Origin of replication for light chain
OMM	Outer Mitochondrial Membrane
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase Chain Reaction
PGI_2	Prostaglandin I2
PKC	Protein Kinase C
PPP	Platelets Poor plasma
PRP	Platelets Rich Plasma
PRRs	Pattern recognition receptors
qRT-PCR	Quantitative Real time Polymerase Chain Reaction
RBCs	Red blood cells
ROS	Reactive oxygen species
rRNA	Ribosomal RNA

SDS	Sodium dodecyl sulphate
sPLA2-IIA	secreted Phospholipase A_2
STING	Stimulator of interferon genes
TBE	Tris Boric EDTA
TLR 9	Toll-like receptor 9
TMRM	Tetramethylrhodamine
TNF-α	Tumor necrosis factor alpha
tRNA	Transfer RNA
vWF	von Willebrand factor
WBCs	White blood cells
WHO	World Health Organization

Abstract

Atherosclerosis, a chronic inflammatory disease, is a major cause of deaths associated with cardiovascular diseases. The strong link between atherosclerosis and inflammation has been observed in several studies. The investigation of the inflammation in atherosclerosis has provided insight to the role of immune cells in the development of this disease. Many inflammatory mediators and cells are involved in pathogenesis of atherosclerosis. Beyond pronounced role in thrombosis and haemostasis, platelets also induce inflammation. Several studies indicated that platelets persuade inflammatory response in neighboring cells such as endothelial cells and leukocytes. Platelets are crucial in proinflammatory surroundings like atherosclerosis, rheumatoid arthritis, allergy and cancer. In atherosclerosis, platelets recruit inflammatory cells towards the lesion site and release cytokines and chemokines in excess. Beside these inflammatory mediators, inflammatory response is activated by endogenous damage associated molecular patterns (DAMPs). The mitochondrial DNA (mtDNA) is endogenous DAMP that may activate immune responses in case of cell damage and death. Platelets have a small number of mitochondria, that is necessary for platelet functioning. Activated platelets release mitochondria and mtDNA in extracellular milieu. Many studies have demonstrated the increase of mtDNA level in several diseases. The stimulation of certain adrenergic receptors (AR) has been linked with platelet aggregation, but the contribution of AR signaling in platelet mtDNA release remains unexplored. This study was conducted to investigate the role of AR signaling in mtDNA release from platelets after treatment with agonists. For this purpose, we enriched platelets by differential centrifugation of healthy participants' blood. Our data confirm the previously published reports that stimulation with AR agonists triggers strong platelet aggregation within seconds of treatment. There was a significant increase in platelets aggregation response in treated samples as compared to controls. In order to answer the main question of this work we extracted mtDNA from the cell supernatants after treatment of platelets with AR agonists at different time points. Our results indicate the release of a significant amount of mtDNA from both treated and untreated platelets. However, there was complete absence of nuclear DNA which confirmed the absence of any nucleated cells in our platelet preparations. After confirmation of the DNA presence in our samples, the mtDNA copy

number was quantified by absolute qPCR. The released mtDNA showed a tendency to increase slightly in samples treated with phenylephrine (alpha AR agonist) after 6 hours of treatment. However, there was no significant difference in the release of mtDNA between two groups. Similarly, a small rise of mtDNA was observed after 16 hour of phenylephrine treatment. In order to assess the possible mechanism behind mtDNA release from platelet, we estimated the mitochondrial membrane potential (MMP) in treated cells using a combination of Tetramethylrhodamine (TMRM) fluorescent probe and flow cytometry. Our data demonstrate that there was more dye accumulation in unstimulated cells and signals disappeared in platelets treated with AR agonists. To further shed light on this pathway we measured caspase 3 activation by western blotting to determine the start of cell death signaling. Analysis of Caspase-3 revealed an unexpected cleavage product which is not associated with apoptosis. However previous studies have linked this caspase 3 cleavage with Calpain-mediated degradation which may be a sign of necrotic mode of cell death. Degradation of tubulin protein in phenylephrine-treated platelets also highlights the probable activation of Calpain in an AR-dependent manner. However future studies are required to confirm the accelerated mtDNA release from platelet upon AR activation and also to further investigate the possible involvement of Calpains in this process.

Key words: Atherosclerosis, Inflammation, Platelets, mtDNA, qRT-PCR, Phenylephrine, PRP.

Introduction

1.1Atherosclerosis

Cardiovascular diseases (CVD) include the disorders associated with heart and blood vessel. Heart diseases and circulatory system diseases are the leading cause of mortality throughout the world (Rafieian *et al.*, 2014). According to world health organization (WHO) 2014 report each year 17.9 million people die from CVDs, an estimate of 31% of all worldwide deaths. More than 75% of CVD deaths occur in underdeveloped and poorly developed countries. Eighty-five percent of deaths from CVDs are due to stroke and heart attack. Atherosclerosis is the underlying cause of CVD-associated death. Atherosclerosis is a condition in which plaque builds up inside the arteries (Ross, 1999). It is the main contributor to coronary artery disease, but it is difficult to identify atherosclerosis at an initial stage (Meurman *et al.*, 2004). Histopathological examination of atherosclerotic tissues has showed that many inflammatory mediators and immune cells play role in atherosclerosis (Galkina and Ley, 2009).

Peripheral arterial disease, ischemic stroke, and ischemic heart disease are a major clinical manifestation of atherosclerosis (Herrington *et al.*, 2016). Atherosclerosis risk factors include dyslipidemia, hypertension, smoking, premature coronary disease, albuminuria and inflammation (Halaria, 2013). These risk factors have been reported in different studies and treatment for these risk factors decrease mortality rate.

1.2. Inflammation and Atherosclerosis

Inflammation is an important biological response that triggers when immune cells detect tissue injury or infection (Newton and Dixit, 2012). Many extracellular mediators regulate the inflammation which include cytokines, eicosanoids (prostaglandins), growth factors, peptides, complement and cell free DNA. Macrophages, mast cells, dendritic cells, fibroblast, circulating leukocytes including neutrophils and monocytes are immune cells that recognize cell damage and pathogens with pattern recognition receptors (PRRs). Pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) are recognized either directly or indirectly by surface receptors or intracellular receptors. The endogenous DAMP and exogenous PAMPs activates

inflammasomes. Inflammasomes are complexes of cytoplasmic proteins which comprise receptors, cysteine protease caspase 1 and adaptor proteins (De Leo *et al.*, 2016). Chronic inflammation plays a major role in the development of atherosclerosis (Conti and Shaik-Dasthagirisaeb, 2015).

1.3. Causes of inflammation

Inflammatory mediators increase during infection, dyslipidemia, hypertension, smoking, and excessive ROS production. These risk factors have been reported in different studies and treatment for these risk factors decrease mortality rate.

1.3.1. Smoking

In smoking the most common substance burned is dried tobacco plant leaves. Twenty percent of world population smoke tobacco (Basu *et al.*, 2011). Tobacco smoke is a toxic chemical source exposed to body. It is considered as greatest contributor towards premature death and illness. Smoking affects the immune-inflammatory system. Smoking increase the circulation of pro-inflammatory cytokines and many other inflammatory mediators like increase in the production of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and interleukin 8 (IL-8) and decrease level of anti-inflammatory cytokines interleukin 10 (IL-10) (Arnson *et al.*, 2010).

1.3.2. Diabetes

Diabetes besides microvascular disease in kidney and eye cause dyslipidemia, high blood pressure, insulin resistance, and prothrombotic tendency, which are pro-atherogenic (Beckman *et al.*, 2002). Hyperglycemia and diabetes generate inflammatory state in our body. Functions of many cells are altered by diabetes including smooth muscle cells, endothelium, and platelets. Diabetic patients have 2-4 folds increase in risk of coronary artery disease (Jousilahti *et al.*, 2011).

1.3.3. Stress

Stress elicits cytokine and chemokine response and triggers sterile inflammation. Repeated stress in the body increases inflammatory response and is linked with many mood disorders. Many life stressors like threat, isolation, and social conflicts increase the level of C-reactive protein (CRP), IL-1β, and IL-6 (Aschbacher *et al*, 2012; Dickerson *et*

al, 2009). Many clinical experiments have studied that inflammatory state increase due to repeated chronic and excessive stressor exposure and is linked with increased risk of mood disorders as depression (Segerstrom and Miller, 2004; Kiecolt-Glaser *et al.*, 2010). It is reported that uncontrollable tail shock in rats increase sterile inflammation (Fleshner *et al*, 2017). This sterile inflammation may contribute in development of atherosclerosis.

1.3.4. Mitochondrial dysfunction

Mitochondria metabolism in cell disturb by inflammatory mediators like TNF-α which upset oxidative phosphorylation and hence increase mitochondrial ROS. Increase ROS production perturb mitochondrial membrane potential (MMP) dynamics and amplify inflammation. This might cause cellular degeneration (Kazak *et al.*, 2012: van Horssen *et al.*, 2017).

1.4. Mitochondria

These are double membrane organelles found in the eukaryotic cells. Mitochondria originate approximately 200 billion years from α-proteobacterium engulfment by an eukaryotic cell (Friedman & Nunnari, 2004). Their specificity in different cells and tissues play a major role in metabolism. They have their own DNA that make them specific from other organelles. Mitochondrial DNA (mtDNA) encodes proteins of ATP synthase and electron transport chain. During a process of cellular respiration, mitochondria are involved in ROS production. Any trouble in activity of electron transport chain (ETC) cause many diseases (Indo *et al.*, 2007). In Parkinson's disease the complex activity decreases, and complex I activity increase in mitochondria of schizophrenic patient (Ben-Shachar *et al.*, 1999)

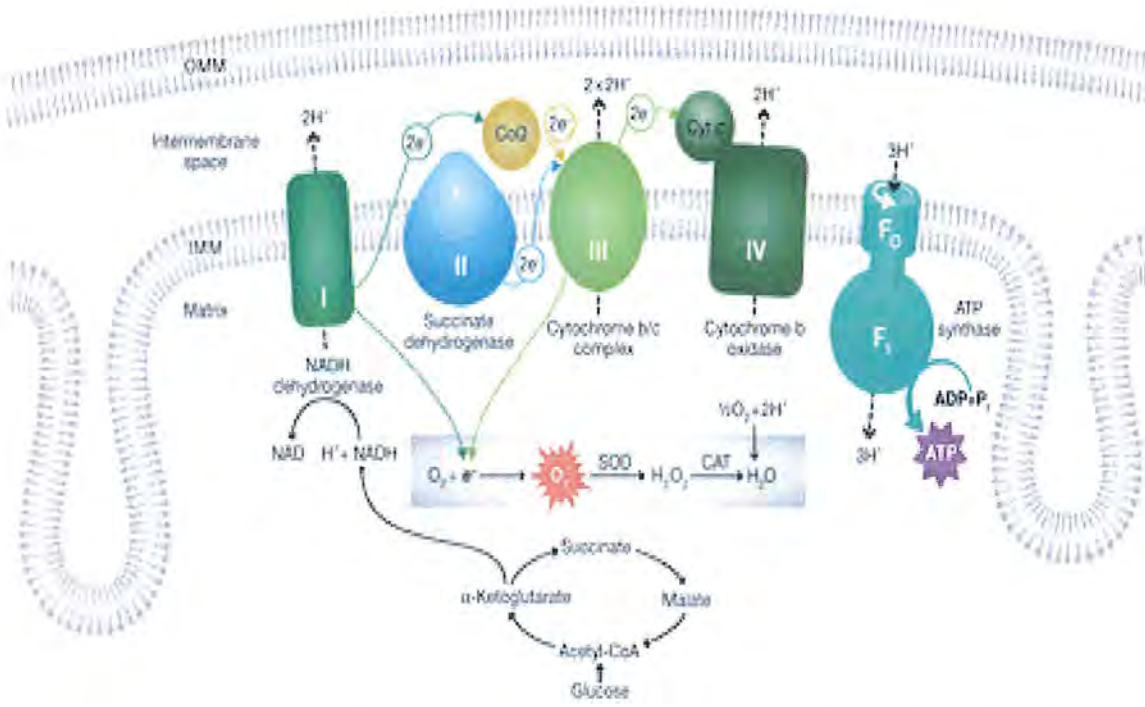


Figure. 1.1 The electron transport chain (ETC) and reactive oxygen species (ROS) production adopted from Dorn GW (2015).

1.5. Mitochondrial DNA and inflammation

Mitochondrial DNA (mtDNA) is double stranded circular structure, present inside the mitochondria. The size of the human mitochondrial genome is 16.6 Kb (Attardi and Schatz, 1988). The mtDNA encodes genes for 22 transfer RNAs (tRNA), 2 ribosomal RNA (rRNA) and 13 proteins that are part of oxidative phosphorylation (Calvo and Mootha, 2010). Twelve polypeptide coding genes are located on the heavy strand. A 1.1 kb region in mtDNA is a displacement loop (D loop). This region plays a key role in transcription and translation. Other 1200 proteins that are also involved in a process of transcription and translation are nuclear encoded. These proteins are first synthesized in cytoplasm and then imported from cytoplasm to mitochondria (Bonawitz *et al.*, 2006). For appropriate cell functioning it is necessary to regulate mitochondrial biogenesis.

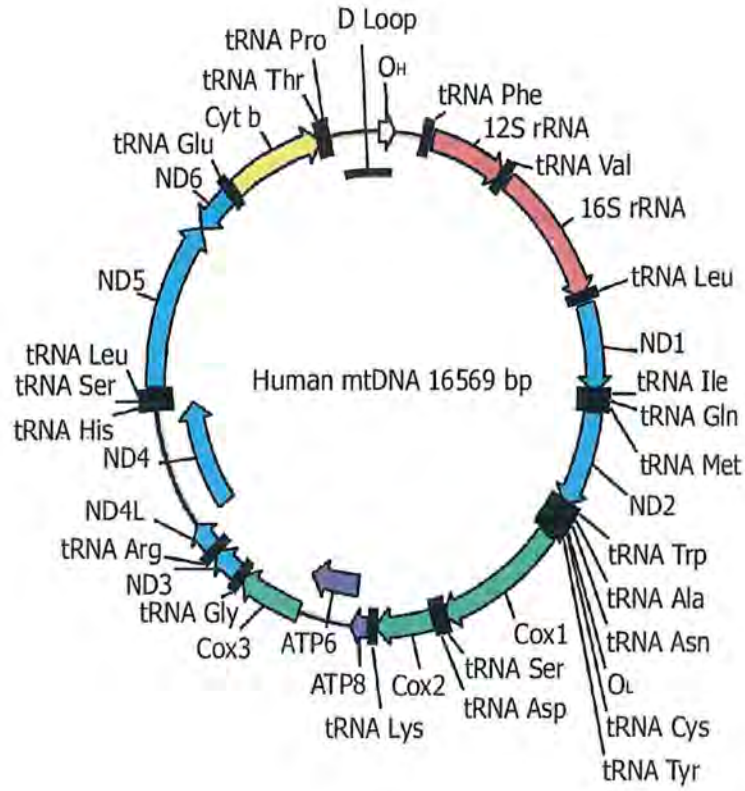


Fig.1.2 The map of human mtDNA, OL and OH represent the origin of replication for heavy and light strand; ETC: Electron transport chain; ND1-ND6: are NADH dehydrogenase subunit; Cyt b: Cytochrome; COX1-COX3: represent cytochrome oxidase subunits 1-3. ATP6 and ATP8: mitochondrial ATPase subunit 6 and 8 respectively. Adopted from (Shokolenko *et al.*, 2014).

Different patterns of methylation on mtDNA make it different from nuclear DNA. It is estimated that CpG methylation in human mtDNA is 2-5 %, but precise CpG methylation is still under debate (Hong *et al.*, 2013). Localization of DNA methyl transferases in mitochondria support the chance of mtDNA CpG methylation (Bellizzi *et al.*, 2013). The mtDNA has hypomethylated CpG motifs that resemble bacterial DNA.

Sterile inflammation is non-pathogen-initiated inflammation. During death of host cells, the endogenous molecules (i.e. DAMPs) are released. The released mtDNA also acts as a DAMPs. The mtDNA provoke the innate immune system. The immune stimulatory response of mtDNA was first reported in 2004 by Collins et., al.

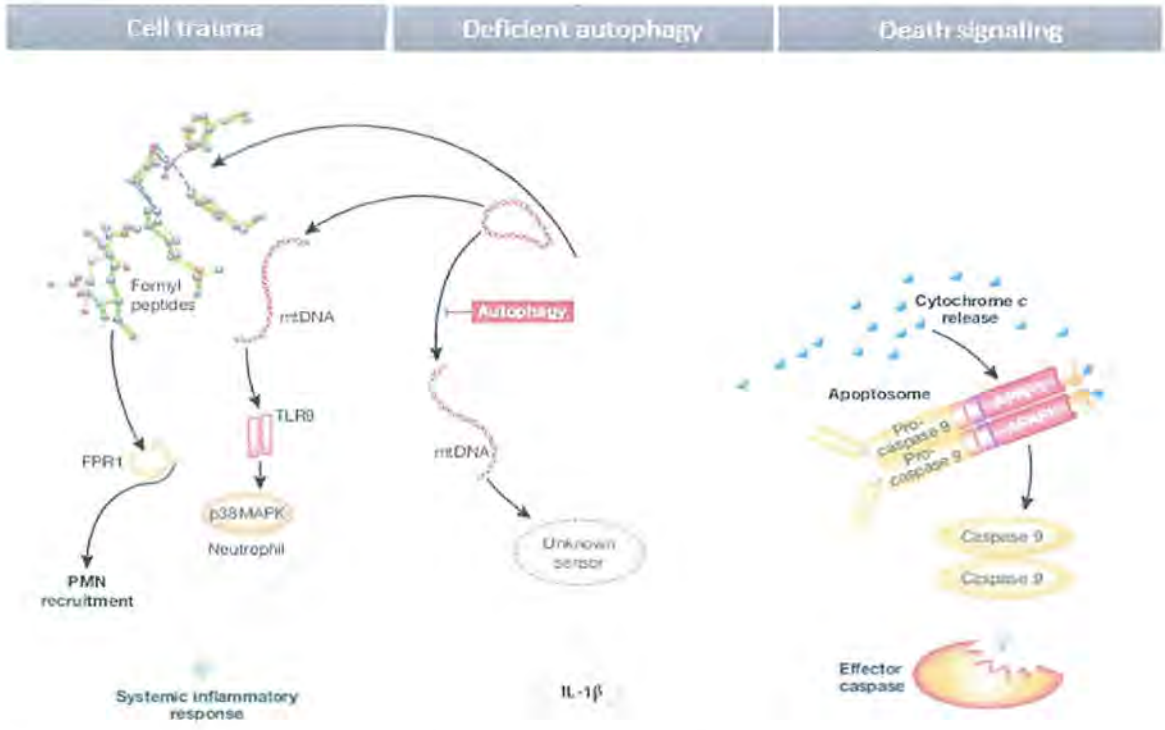


Fig.1.3 Released mitochondrial DAMPs and inflammatory pathways. The mtDNA released by severe trauma. This released mtDNA is recognized by Toll like receptor 9 located on neutrophils and activates p38 MAP kinase pathway in neutrophils. Mitochondria also released N-formyl peptide that bind with formyl peptide receptor 1 (FPR 1) present on neutrophils and recruit more neutrophils. Defective mtDNA released into cytosol by autophagy-deficient cells and activate inflammasomes by cytosolic sensor. Adopted from (Arnoult *et al.*, 2011).

The key role of mitochondria in a pro-inflammatory response was identified when oxidized mtDNA was injected in mice, where it was observed that nuclear genome did not trigger inflammatory arthritis in mice (Garcia-Martinez I et al., 2016). The mtDNA enhances the pro-inflammatory response by directly engaging the pattern recognition receptors of the innate immune system (West *et al.*, 2011). A study has shown that Toll-like receptor 9 (TLR9) drive mtDNA mediated inflammation (Petrasek *et al.*, 2011). In disease conditions it has been reported that mtDNA content fluctuates in body fluid. Freely circulating mtDNA level increase in condition such as trauma, sepsis, acute myocardial infarction, and uncontrollable cell death (Lam NY *et al.*, 2004). In diabetic nephropathy circulating mtDNA reduces in body fluid (Czajka *et al.*, 2015). So mtDNA is used for understanding the cause of mitochondria-associated human diseases and

aging. This is an attractive option because we can easily access the body fluid as compared to body tissues and organs (Malik and Czajka, 2013).

1.6. Mechanism of Mitochondrial DNA release

Cell free mtDNA was detected in many studies during mtDNA quantification by quantitative polymerase reaction (qPCR). During cellular stress or necrosis DAMPs are released in the extracellular milieu and mtDNA is a dominant endogenous DAMP. The mechanism of release of mtDNA in cytosol is not clear.

A weakly selective channel present in the inner membrane of mitochondria is known as mitochondria permeability transition pore (mPTP) (Bernardi *et al.*, 2006) releases mtDNA into the cytosolic environment under mitochondrial stress and apoptosis (García and Chávez, 2007). The mPTP opening allows the free movement of ions across the pore. This free movement leads to ATP depletion and membrane depolarization that results in mitochondria inability to maintain electrochemical potential resulting in organelle swelling. Infarct volume decrease in the heart by directing cyclosporine A, an inhibitor of mPTP (Piot *et al.*,2008). This mean mPTP is the best therapeutic target in ischemic cell death associated with MI and stroke.

Programmed cell death is necessary for proper development, tissue homeostasis and anti-viral defense. Apoptosis, a programmed cell death mechanism, is regulated by Bcl-2 family proteins. Some Bcl-2 family members Bcl-xl and Bcl-2 are effective inhibitors of apoptosis. Beside this other family members promotes apoptosis, these includes Bak and Bax. In a healthy cell Bak is inserted in OMM and 21kDa Bax protein is mainly a cytosolic protein. During apoptosis 6-8 monomer of Bax homo-oligomerized (96-260KD) and translocated from cytosol to OMM (Guihard *et al.*,2004: Gross *et al.*, 1998: Antonsson *et al.*, 2001: Gross *et al.*, 1998). This oligomerization of Bax is important for Bax activation. Bax translocation to OMM is regulated by phosphorylation. A recent study showed that Bax phosphorylation at Ser184 promotes its sequestration to cytoplasm (Gardai et al.,2004: Rathmella et al., 2003).

Bak/Bax, permeabilize the OMM and allow the escape of intermembrane space protein such as cytochrome c to escape into cytosol. Recently the mtDNA release mechanism

was explained by using live-cell lattice light-sheet microscopy. A small amount of cytochrome c is required for mtDNA efflux and mitochondria fragmentation. A large pore at OMM allows the inner membrane herniation; the herniated membrane carries matrix components and mtDNA. When herniated part loses its integrity then mtDNA release into the extracellular environment and this free mtDNA activates many other pathways including cGAS/STING pathway (McArthur *et al.*, 2018). Another study indicated that Bcl2 mediated mitochondrial release engages cGAS-STING pathway, this pathway further induces type 1 interferon response (White *et al.*, 2014).

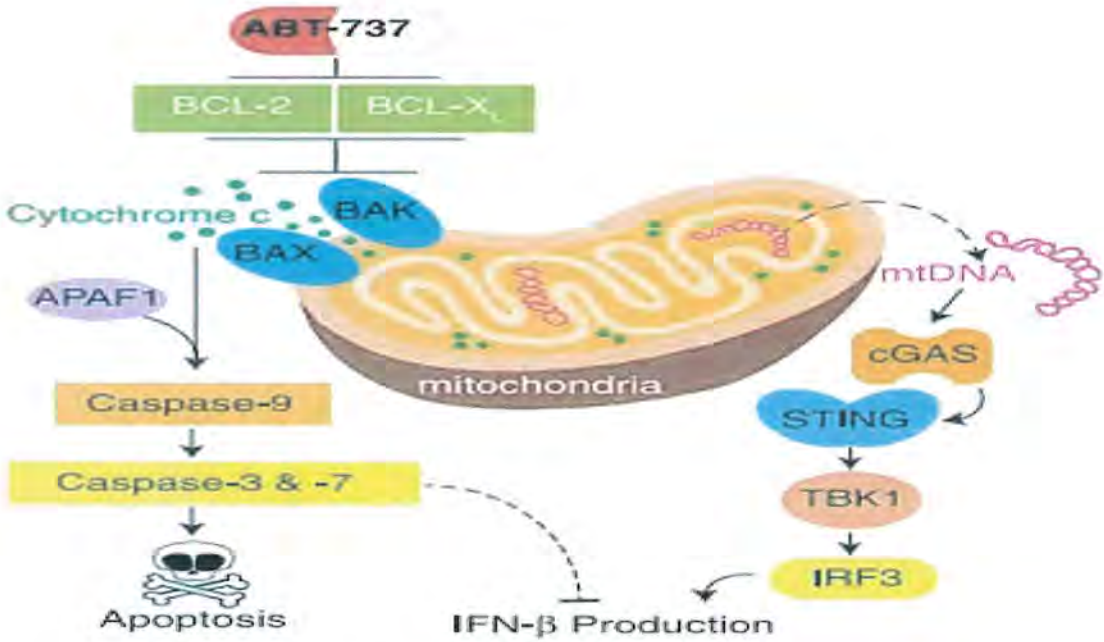


Fig.1.4 Mitochondrial DNA release in response to stress and activation of cGAS STING pathway. Adopted from (McArthur *et al.*, 2018).

The mtDNA released by neutrophils also initiate type1 interferon production by the endosomal TLR9 or cGAS-STING pathway. One such example is systematic lupus erythematosus in which neutrophils release its DNA and other granules in extracellular spaces. These structures are known as neutrophil extracellular traps (NETs). The presence of mtDNA in released content indicates its role in type 1 INF production (McIlroy *et al.*, 2014). Type 1 INF, interferon stimulated genes (ISGs) and many pro-

inflammatory cytokines expression increases by interaction of released mtDNA with dendritic cells (Caielli *et al.*, 2016).

1.7. Mechanism of release of free mitochondria and Mitochondrial DNA from platelets

In the blood circulation platelets are released by megakaryocyte. Platelets are typically discoid in shape and have a thickness of ~1 μm and are ~4 μm in diameter (White, 1968) (Raslova *et al.* 2003). In platelets about seven mitochondria are present but ATP turnover in these cells are more than other cells such as muscle cells. This suggests that platelets are metabolically active. Energy is required for secretion of granules content and activation. Energy is derived from two main processes like oxidative phosphorylation and glycolysis (Akkerman, 1978). The rate of both processes increases during activation and secretion to fulfill energy demand (Akkerman, 1979).

On platelet activation, platelets release granular contents (a, dense, and lysosomes) in extracellular milieu. Platelet release mitochondria by unknown mechanism and play role in platelet activation and responsible for thrombus formation. It was observed that platelet shed its cytoplasmic membrane upon activation in form of submicron vesicle (MPs) (Boilard et al., 2012: Gy ¨orgy et al., 2011). Localization of mitochondria toward cytoplasmic membrane indicate that it is releases in the form of mitochondria-containing microparticles (mitoMPs) (Boudreau *et al.*, 2014). Free mitochondria level also increase in plasma upon activation of platelet with thrombin. The secreted phospholipase A2-IIA is abundant in cellular lineage of platelet (Kramer *et al.*, 1989). The sPLA2-IIA hydrolyze glycolipids and release lysophospholipid and free fatty acid (Lambeau and Gelb, 2008). These mitochondrion act as endogenous substrate for sPLA2-IIA the and its hydrolysis leads to release of many proinflammatory signals like (mtDNA, arachidonic acid, and lysophospholipids) (Zhang *et al.*, 2010: Funk, 2001: Silliman *et al.*, 1998). It has been observed the sPLA2-IIA and mitochondria released from mitoMPs, platelets or other damaged cells are present in inflammatory microenvironment of rheumatoid arthritis (RA) (Boilard *et al.*, 2010). It has been observed the mtDNA level increase with increase of sPLA2-IIA levels in platelets storage bag (Boudreau *et al.*, 2014). Intravenous injection of extracellular mitochondria and sPLA2-IIA amplify inflammation.

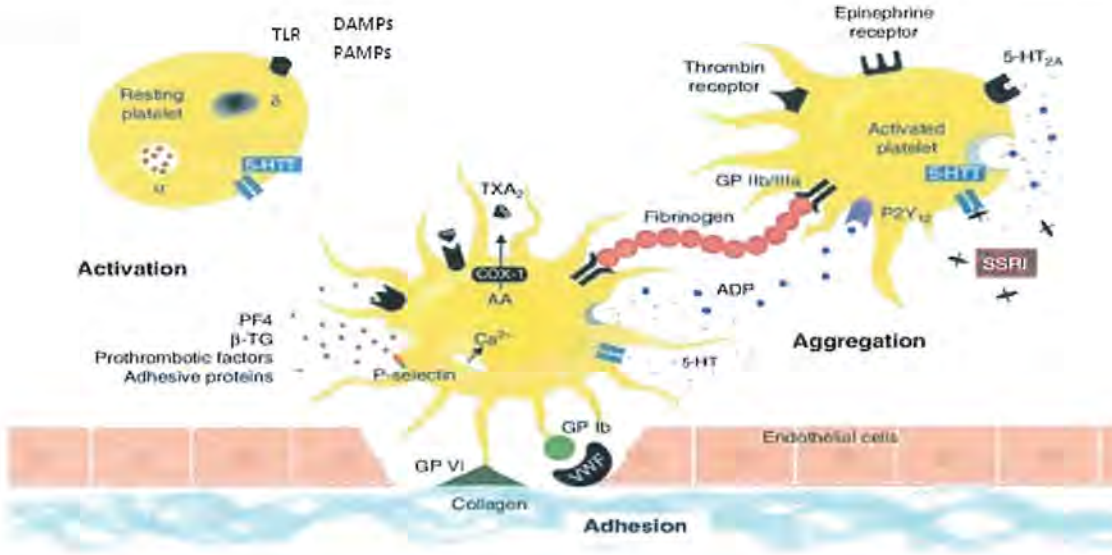


Fig.1.5 Mechanism of platelet activation and thrombus formation: DAMPS and PAMPs at vascular injury site or inflammatory site activate platelet by interacting with TLR. Platelet adhere to the extracellular matrix at a vascular injury site via interaction between platelet receptors (GP Ib/V/IX) and vWF. Subsequent interaction with collagen and (GP VI and GP Ia) receptors stimulates granule release, platelets spreading and activation of integrin. This interaction results in more release of agonists like (ADP, serotonin, thromboxane A2, epinephrine, and thrombin). The soluble granules activate more αIIbβ3 receptors by activating GPCRs. This activation results in stable thrombus formation (adopted and modified from (de Abajo, 2011).

1.8. Role of adrenergic signaling in platelet activation and its possible role in atherosclerosis

The activation and inhibition of platelet play a major role in hemostasis. Platelet express many receptors on its surface which includes many integrins ($\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$), Toll-like receptors, Glycoprotein [GP] Ib/IX/V, ADP receptors, TxA_2 receptors, tyrosine kinase receptors, G-protein coupled receptors (GPCR), PAR-1 and PAR-4, thrombin receptors, P-selectin and many other receptors. Many of these receptors are expressed on other cells but some are specific for platelet. Adrenergic receptors class of GPCR are expressed on platelet and are target of norepinephrine, epinephrine and adrenaline. Nine adrenoceptors are identified and classified into three major categories; α_1, α_2 and β-adrenoceptor. These receptors and their downstream signaling pathways play diverse physiological function in central nervous system and cardiovascular system. Phenylephrine is pharmacological agonist of α_1-adrenergic receptor. The α_1-adrenergic

receptor increase diacylglycerol production, inositol phosphate turnover and protein kinase C (PKC) activation through phospholipase C activation and involved in cardiac homeostasis (Dajani *et al.*, 1999). The α-adrenergic mediated signal transduction is not clear in platelets. In platelets alpha-2 adrenergic receptor enhances granule secretion, integrin activation and Thromboxane A2 production by activation of many downstream signaling pathways including PI-3 Kinase, Src family kinase, PKC and G protein pathways (Boulaftali *et al.*, 2013).

Aim and objectives

The main aim of this stud was to investigate the release of mitochondrial DNA from human platelets upon treatment with adrenergic receptor agonist. The study has been designed with following objectives:

- To quantify mitochondrial DNA by using quantitative PCR from platelet supernatants.
- To assess contribution of AR signaling in mtDNA released from platelets upon stimulation with AR agonists.
- To calculate the mtDNA copy number in treated and untreated platelet sample
- To identify the possible mechanism for the mtDNA release from platelets.

Materials and Methods

2.1. Ethical Approval

The present research was approved from the Bioethical committee of the Quaid-I-Azam University Islamabad. The volunteers who participated in this study were from Quaid-i-Azam university Islamabad. Before sampling, informed written consent was obtained from the participants. Volunteers with a history of medication in last two weeks or with a family history of platelet related pathologies were excluded from the study.

2.2. Sample Collection

The venous blood samples were collected from normal healthy individuals by using sterilized 10ml syringes provided with 22G needle. Blood was transferred to 15ml centrifuge tube having ACD (1:9) as an anticoagulant. Samples were handled according to blood safety procedure approved by World Health Organization.

2.3. Platelet Isolation

To obtain platelet rich plasma (PRP), blood sample was immediately centrifuged (Eppendorf Centrifuge 5810 R, Germany) at 200× *g* for 20 minutes at 25-27 degrees Celsius. After centrifugation, 2/3 of PRP was isolated in DNase/RNase free 15 ml centrifuge tube (ExtraGENE, USA). The isolated PRP was again centrifuged at 100× *g* for 10 minutes; this second centrifugation ensures the maximum separation of other cells. 1 ml PRP was transferred carefully to 1.5ml eppendorf tubes. PRP was centrifuged at 2000× *g* for 20 minutes to obtain platelets for further assays. Platelet poor plasma (PPP) was discarded. Platelets were washed by adding 1ml Tyrode's buffer (140mM NaCl, 5mM glucose, 5mM KCl, 10mM HEPES, 1.5mM $MgCl_2$) without calcium and 0.5µl PGI_2. Pallets were centrifuged at 2000× *g* for 1 minute. Again, supernatant was discarded, and platelets were resuspended in calcium free Tyrode buffer.

2.4. PRP Smear Preparation and Field's Staining

15-20 µl of PRP is used to confirm the contamination of WBCs in PRP. Following steps were performed for smear formation.

Frosted microscope glass slides were used for preparing the blood smear. These slides were cleaned before use. 8-10 µl of PRP drop was placed about half inch away from the edge of glass slide. The specimen slide was placed on horizontal smooth surface and spreader slide was held between forefinger and thumb. The smooth edge of spreader slide was placed at a 30°-45° angle on specimen slide and the drop was behind the slide. The spreader slide was pushed gently in forward direction and PRP was allowed to spread till the edge of specimen slide. A monolayer of PRP was formed on the slide and had bullet shape. Participant ID number was labelled at the frosted end of glass slide. Before staining PRP film was dried completely in air. For fixing PRP film, slide was immersed for one minute in absolute methanol. The fixed PRP film slide was dipped for a few seconds in Field Stain B (Eosin). Slide was washed with tap water. Slide was again dipped in second stain i.e Field Stain A (Methylene Blue) for thirty seconds. Slide was aging washed gently with tape water and allowed to again dry in air. The stained slide was examined under a microscope. WBC's contamination and platelets quantification were done at 100x.

2.5. Manual Platelet Counting

PRP sample was diluted (1:10) to prepare suitable concentration for cell counting. This dilution was made by adding 3µl of PRP in 27µl of normal saline. The Neubauer counting chamber was cleaned with ethanol and placed on the microscope stage. 10µl of diluted sample was loaded in chamber and kept for 20 minutes to settle the cells. Platelet counting was done in 5 small square of large central square under 40X objective. Th platelet number per ml was calculated by using formula:

$$\text{Platelets count (per ml)} = \frac{\text{Number of cells counted}}{\text{Volume of chamber µl}} \times \text{dilution} \times 1000$$

2.6. Platelet Aggregation Assay

Platelet aggregation assay was performed with PRP, obtained by centrifugation of anticoagulated blood. Platelet number was counted before treatment with agonist. Aggregation process was carried out in 96 wells plate. To measure change in light absorption 90 µl PRP sample and 10 µl of Phenylephrine (stock 1000 µM) was added in

96 well plate. The entire plate was shaken. The readings were repeated every 15 second and absorbance of each sample was measured for 5 minutes at 37 °C at 595 nm by using a spectrophotometer Multiscan Go (Thermo Ficher Scientific, Japan). To generate smooth aggregation curve, it was ensured that samples in wells were free from air bubbles. The baseline was set with PRP and the percentage aggregation was calculated based on aggregation rate of treated samples relative to control.

2.7. Measurement of Mitochondrial Membrane Potential

To measure mitochondrial membrane potential washed platelet as described above were resuspended in 500 µl of calcium free Tyrode's buffer and 0.25 µl of PGI_2. Sample were loaded with 0.5µM TMRM and incubated for 30 minutes at 37°C. The platelets were washed twice by adding 500 µl Tyrode buffer without calcium and 0.25 µl PGI_2. Sample was centrifuged at 6000 rpm for 15 minutes. Again, platelets were resuspended in Tyrode buffer without calcium and PGI_2. Samples were incubated at 37 °C for 30 minutes. 2mM $CaCl_2$ was added just before samples were measured in flow cytometer.

2.8. Platelet treatment with agonists

Platelets were isolated as described previously. The resuspended platelets were treated with different concentration of agonists: 100µM Phenylephrine (lot# C0613, Santa Cruz), 100µM Nor-adrenaline, 100mU thrombin and 500mU thrombin (lot# 031212D, Thrombin Pacific technology). 4µl of 100mM calcium chloride was added in each sample and final sample volume was adjusted to 200µl. Samples were incubated at 37°C with gentle rotation for 2 hours, 6 hours and 16 hours. After incubation samples were centrifuge at 6000×g for 15 minutes. Supernatant was used for DNA extraction and pellet was processed for protein extraction.

2.9. DNA Extraction

DNA was extracted from platelets supernatant by using NucleoSpin isolation kit (REF 740956.50, MACHEREY-NAGEL, Germany). Following steps were performed for DNA isolation. In washed platelets 195-200µl of Tyrode's Buffer, 4ul of 100mM $CaCl_2$ and agonists were added in respective tubes. Samples were incubated for 2-6 hours. After incubation samples were centrifuged at 4000 g for 10 minutes. Supernatant was

transferred to a new microcentrifuge tube. 150µl of supernatant 600µl of RAV1 solution were mixed and vortexed well. Samples were incubated at 70°C for 5 minutes. 600 µl of ethanol (96-100%) was added for binding and vortexed for 10-15 seconds. NucleoSpin columns were placed in collection tubes (2ml). Samples were loaded in stepwise fashion and centrifuge at 8000×g for 1 minute. The flow-through was discarded. For washing and drying silica membrane 500 µl RAW solution was added to the column. Columns were centrifuged at 8000 ×g for 1 minute and flow-through was discarded. 600 µl of RAV3 buffer was added for second washing. Columns were centrifuged for 1 minute at 8000 ×g and flow-through was discarded along with collection tube. Columns were placed in new collection tubes (2ml) and 200 µl of buffer RAV3 was added and centrifuged at 11000×g for 5 minutes to completely remove ethanolic buffer. Columns were placed in new sterile microcentrifuge tubes and 30 µl of RE buffer (preheated to 70°C) was added. Columns were incubated for 1-2 minutes and centrifuged for 1 minute at 11000×g to elute the DNA which was later stored at -20°C till further processing.

2.10. PCR Amplification

In order to amplify the mitochondrial DNA, we performed conventional polymerase chain reaction (PCR). Following primers were used for amplification of nuclear and mitochondrial genes. Following reagents were added in 200 µl PCR tubes. The final volume of PCR amplification reaction mixture was 25 µl.

Table. 2.1. Primers used for conventional PCR

Sr.#	Primers Name	Primer Sequence (5'-3')	Tm°C	Amplicon Size
1	NADHF	ATACCCATGGCCAACCTCCT	62.7 °	110bp
2	NADHR	GGGCCTTTGCGTAGTTGTAT	59.1°	110bp
3	hB2mF	TGTTCCTGCTGGGTAGCTCT	60 °	187bp
4	hB2mR	CCTCCATGATGCTGCTTACA	60 °	187bp

Table 2.2. PCR reagents and their quantities used in 25 µl of Reaction mixture

Sr.#	Reagents	Quantity
1	10X Taq Buffer with (NH4)2SO4	2.5 µl
2	25 mM $MgCl_2$	1.5 µl
3	dNTPs (10mM)	0.5 µl
4	Taq Polymerase (5 Units/ microliter)	0.2 µl
5	Forward Primer	0.5 µl
6	Reverse Primer	0.5 µl
7	DNA sample	4 µl
8	Ultrapure DNase free water	15.3 µl
	Total Reaction Mixture Volume	**25 µl**

Reaction mixture in each tube was prepared. The tubes were vortexed at 8000 rpm for 25 seconds. Then tubes were placed in T1 Thermocycler (Biometra, Germany) and PCR conditions were set for gene amplification.

Table. 2.3. PCR conditions for gene amplification

Steps	Temperature	Duration	
Initial denaturation	95°C	10 min	
Denaturation	95°C	1 min	39 cycles
Annealing	60°C	1 min	
Extension	72°C	45 Sec	
Final Extension	72°C	10 min	
Hold	4°C	∞	

After completion of reaction the amplified PCR products were visualized on 2% agarose gel. To further confirm amplicon size, we used 3 µl of 100bp Gene Ruler (Lot.No.00335424, Thermo Scientific).

2.11. Agarose gel Electrophoresis

For visualizing PCR product 2% agarose gel was prepared. To prepare 2% agarose gel, 1g agarose powder was dissolved in 50ml of 1X TBE (Tris Boric EDTA) Buffer. This mixture was heated for 2 minutes in microwave oven. The mixture was heated until agarose solubilize completely. The mixture was allowed to cool down below 70 °C and then 5µl of 1% ethidium bromide dye (EtBr) was added and mixed. The mixture was poured in casting tray and allowed to solidify for 30-40 minutes. Gel was transferred to running tank (Biometra, Germany) having 1X TBE running buffer and combs were removed carefully. A mixture of 4 µl of PCR product and 4 µl of loading dye was loaded into the wells and 110V was applied for 35-40 minutes. Gel documentation system (SYNGENE, UK) was used for visualizing of gel and GeneSnap software was used to acquire images.

2.12. DNA extraction from gel

The gel was visualized briefly under UV to excise the band. DNA was purified from excise gel by using GeneJET Gel Extraction Kit (Thermo Scientific, catalog# K0691). For purification all steps were performed at room temperature. The gel was weighed in a microcentrifuge tube. Binding buffer was added in sliced gel in 1:1 (volume: weight). The gel was incubated at 60°C for 20 minutes to completely dissolve the gel. As the DNA fragment was < 500bp, 1gel volume of 100% isopropanol was added. The mixture was thoroughly mixed. 800 µl of solubilized gel solution was transferred to GeneJET purification column. The column was centrifuged at 12,000rpm for 1 minute. The flow-through was discarded and column was placed back in the same collection tube, this step was repeated until entire volume has been applied to the column membrane. 700µl wash buffer was added in column and column was centrifuged for 1 minute at 12,000 rpm. The flow through was discarded and column was placed back in same collection tube. The column was centrifuged additionally for 1 minute to remove residual wash buffer. The column was transferred into a clean 1.5 ml eppendorf. 30 µl of elution buffer was added to the center of column and again column was centrifuged at 12,000rpm for 1 minute. The eluted DNA was stored at -20°C.

2.13. Preparation of Standards for Absolute Quantification

The platelets mitochondrial DNA was amplified with NADH primer and reaction mixture was prepared as discussed above in table 2.2. The PCR product was checked by electrophoresing 4µl of PCR product and 4µl of loading dye. The PCR product was expected size. Then 50µl PCR product was electrophorized at 110V for 30 minutes. The concentration of eluted DNA was confirmed by using nanodrop. The copy number per purified DNA is calculated (For NADH: Copy number = Concentration × 8.29 × 10^{10} /µl). The sample was diluted to get 1× 10^{9} copies/ µl. Dilution standard was prepared in tenfold dilution for 4-8log. 4 eppendorf were labelled with 1× 10^{4} to 1× 10^{8} each containing 90 µl water. 10µl sample was transferred from 1× 10^{9} copies/ µl stock solution to tube labelled with 1× 10^{8}. Tube was mixed and 10µl sample was transferred from 1× 10^{8} to 1× 10^{7}. The step was repeated until the 10 µl aliquot was transferred to eppendorf labelled with 10^{4}. Real time PCR was performed to determine the amount of mtDNA in the sample.

2.13. Real Time PCR

Real quantification of eluted DNA was done on MyGo real-time PCR using the recipe mentioned in table 2.4

Table2.4. The Reagents and their quantities used for qRT-PCR

Sr.#	Reagents	Quantity
1	DNA	4 µl
2	Forward primer	0.5µl
3	Reverse primer	0.5 µl
4	Eva Green	2µl
5	Nuclease free water	3 µl
	Total Reaction Mixture Volume	**10µl**

The qPCR reaction was performed in real time qPCR machine and conditions were set before use as given in table 2.5.

2.5. Real time PCR conditions for gene amplification

Cycle step	Temperature	Duration	Cycles
Initial activation	95°C	12 min	1
Denaturation	95°C	15 s	40
Annealing	60°C	20s	
Elongation	72°C	20 s	

2.14. Protein Extraction by Lysis Buffer or RIPA Buffer

Protein was extracted from platelet pellet by using lysis buffer. The lysis buffer composed of: 10mM Tris-Hcl (pH=8), 140mm NaCl, 1% Triton X-100, 1mm EDTA, 0.1% SDS, 0.01% sodium deoxycholate 1% protease inhibitor, and 1% phosphatase inhibitor. 80ul of buffer was added in the pellet. The cells were lysed by brief vortexing during an incubation period of 15 minutes. Protein was collected by centrifuging the samples at 12000 rpm for 15 minutes at 4°C. The supernatant contained proteins. 60ul of supernatant was transferred to a new microcentrifuge tube and stored at -20°C for performing western blot analysis.

2.14.1. Protein Quantification by Bradford Assay

Bradford assay was performed to quantify protein concentration in the supernatants. The standard protein used for quantification was bovine serum albumin (BSA) (Cat No: 39222.01, SERVA Electrophoresis, Heidelberg Germany). BSA stock solution was prepared by dissolving 40mg of BSA in 1ml distilled water. Stock was diluted 20 times, for this purpose 25ul of BSA from stock dilution was added in 475ul of distilled water. Eight different dilution of standard were prepared, and concentration ranged from 0µg/µl to 1.2 µg/µl. The final volume of dilution was 100µl. Distilled water was used as blank. 10 µl from BSA dilution and protein samples was loaded in 96-well plate. Each sample was loaded in triplicate. 5x Bradford reagent (Cat No: 39222.01, Rot Electrophoresis,

Heidelberg Germany) was diluted in distilled water to make 1x working reagent. 200 µl of 1x Bradford was added in each well. The plate was incubated at 37°C for 30 minutes and absorbance of samples was measured at 595nm by using visible spectrophotometer (MultiSkan Go, Thermo). Example of an experiment is shown in table 2.6 and figure 2.1

Table 2.6 Absorbance of BSA Standards at 595nm

Sr.#	Concentration (µg/µl)	Distilled H20 (µl)	BSA (µl)	Absorbance (nm)
1	0	100	0	0
2	0.1	95	5	0.0875
3	0.2	90	10	0.2285
4	0.4	80	20	0.3175
5	0.6	70	30	0.4405
6	0.8	60	40	0.576
7	1	50	50	0.759
8	1.2	40	60	0.845

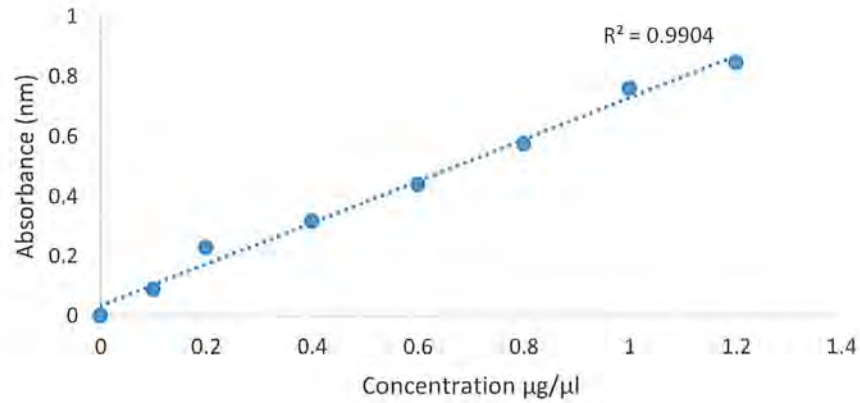


Fig.2.1. BSA Standard Curve

The protein concentration in samples was calculated by using TREND formula in excel sheet and 10 µg/µl sample was prepared.

2.15. Western Blot Analysis

2.15.1 SDS-Gel Electrophoresis

Gel apparatus was cleaned before preparing the gel. Glass plates were assembled carefully in gel casting frame. Following ingredients were used to prepare 12% separating gel.

- 2ml of 30% Acrylamide
- 1.25ml of 1.5M Tris-cl (pH=8.8)
- 0.05ml of 10% SDS
- 0.05ml of 10% Ammonium per sulphate (APS)
- 2ul of TEMED
- 1.65 ml Distilled water

The solution was poured between glass plates and 1ml isopropanol was layered at the top to keep the gel surface smooth. The gel was polymerized within 30 minutes. Isopropanol layer was removed, and gel surface was washed with distilled water and dried by filter papers. 2ml stacking gel solution (5%) was poured on the top of the separating gel. A comb was inserted in the gel to allow the well preparation. The stacking gel was composed of:

- 0.33ml of 30% Acrylamide solution
- 0.25ul of 1M Tris-cl (pH=6.8)
- 10ul of 10% SDS
- 10ul of 10% Ammonium per sulphate
- 2ul of TEMED
- 1.4ml of distilled water

After polymerization the gel was removed from frame and clamped in the gel electrophoresis tank. Protein samples were prepared by adding 5x laemmli buffer (Cat No: 42526.01, SERVA Electrophoresis, Heidelberg, Germany) and water was added to raise volume to 20 ul. The samples were heated at 95°C for 10 minutes. This heating step denatured the protein. The electrophoresis tank was filled with running buffer (3mM SDS, 191mM glycine, and 25mM Tris-base (pH=8) dissolved in distilled water) up to

required mark. The comb was removed from gel and wells were washed with buffer. 3ul of protein ladder was loaded in the first well and samples were loaded in the remaining wells. The gel was run by applying 90V for 30 minutes and then 110V for 1 hour and 30 minutes

2.15.2 Transfer of proteins to nitrocellulose membrane

After electrophoresis the plates were removed, and gel was immersed in transfer buffer (1M Tris-cl [pH: 8], 20% methanol and 383mM of glycine dissolved in distilled water) for 1 minute. Nitrocellulose membrane (CAT No: 10600001, GE Healthcare, Life Sciences) and filter papers were also dipped in transfer buffer. Nitrocellulose membrane was kept between gel and filter papers and bubbles were removed by using glass roller. A 10 V current was supplied for 30 minutes. To confirm the protein transfer from gel to membrane, membrane was stained with Ponceau stain (Cat No: 33429.01, SERVA Electrophoresis, Heidelberg Germany). Membrane was washed with distilled water.

2.15.3. Blocking

Membrane was blocked with 5% skimmed milk (Cat No: 42590.02, SERVA Electrophoresis, Heidelberg Germany) solution. Membrane was incubated with blocking buffer for 1 hour on a shaker

2.15.4. Washing

Membrane was washed three time with TBST (1M Tris 25ml, 1.5M NaCl 50ml and 1ml Tween-20 dissolved in 1000ml of distilled water) for 30 minutes.

2.15.5. Incubation with Primary Antibody

The primary antibody was diluted in 5% BSA and 0.5% sodium azide (Cat No: 30175.01, SERVA Electrophoresis, Heidelberg Germany) dissolved in TBST. The membrane was incubated overnight with primary antibody dilution at 4°C and after incubation primary antibody was removed and membrane was washed with buffer.

Table 2.7 List of Primary Antibodies

Primary Antibodies	Molecular weight	Dilution	Cat. # /Company
α-Tubulin	~50 KDa	1:1000	T5168 /Santa Cruz U.S. A
Caspase 3	34, 17 and 19 KDa	1:1000	CST-9662
Tom 20	20 KDa	1:1000	sc-11415/ Santa Cruz

2.15.6 Secondary Antibody Incubation

After washing, the membrane was incubated with secondary antibody diluted in 5% skimmed milk solution prepared in TBST. The membrane was incubated at room temperature for 1 hour. After incubation membrane was washed thrice with TBST buffer for 30 minutes.

Table 2.8 List of Secondary Antibodies

Secondary Antibodies	Dilution	Catalog#/Company
Mouse anti-rabbit IgG	1:5000	2357/Santa Cruz U.S.A
m-IgGk BP-HRP	1:5000	Sc-516102

2.15.7 Protein Visualization by Chemiluminescence

The hydrogen peroxide and luminal (Cat# sc-2048, Santa Cruz Biotechnology, Inc) in 1:1 to prepare horse radish peroxidase (HRP) substrate solution. The substrate solution was spread over a surface of membrane and the membrane was visualized by gel documentation system (Alpha View SA Version 3.4.0.0). Intensity of bands was measured by using analysis tool of Alpha View software.

2.16. Statistical Analysis

Different statistical tools were used for data analysis. Results were presented as mean ± SEM. Statistical significance was calculated by applying student's t-test. A p-value of less than 0.05 was considered significant.

Results

3.1. Demographic parameter of individuals

The age, height and weight of individuals were recorded, and BMI was calculated by using formula (weight of individual in kg/ height in cm^2). Experiment include blood sample from both male and females volunteer.

Sr.#	Age	Height (cm)	Weight (Kg)	BMI
1	24	167.64	50	18.8
2	24	167.64	54	19.8
3	24	167.64	67	22.7
4	24	167.64	67	22.7
5	24	182.88	86	25.6
6	23	170.69	50	17.6
7	24	170.69	60	21.2
8	25	176.75	69	23
9	24	169.54	55	20.7
10	24	167.64	55	20.2

Table.3.1: The table represents the demographic parameter of volunteer. No difference is observed in BMI of volunteer.

3.2. Confirmation of contamination free platelet preparation

The blood was collected from non-medicated volunteer and these volunteers had no disease record like diabetes and cardiovascular disease. The blood was centrifuged in 3 steps. To prevent platelet contamination samples were handled carefully. WBCs contamination in PRP was confirmed by microscopy of PRP smear and cell counting by automated cell counter which confirmed that maximum number of WBCs and RBCs were removed after second centrifugation. A microscopic screening of multiple PRP

smears demonstrated presence of no WBCs or RBCS. Similarly, PCR of DNA samples extracted from platelets in preparations in our lab has revealed an absence of the nuclear DNA specific PCR product which further confirms the lack of nucleated cells.

Sr.#	Platelet in whole blood	WBCS in whole blood	Platelets number after 100 ×g	WBCS number after 100 ×g
1	338×10^3	6.7×10^3	416×10^3	0.0×10^3
2	244×10^3	6.3×10^3	283×10^3	0.0×10^3
3	278×10^3	6.0×10^3	312×10^3	0.0×10^3

Table.3.2 Data from hematology counter shows that WBCs are abundant in whole blood and WBCs number in platelets rich plasma decrease significantly after second centrifugation at 100×g for 10 minutes and n=3.

3.3. Platelet counting

Platelets counting was performed in the Neubauer counting chamber (Lot# 20795 Marienfeld, Germany) 10 µl of PRP dilution (1:10) loaded in chamber and cells were allowed to settle for 20 minutes. Platelets were counted in central square of chamber under 40 x objective.

Platelets counting by Neubauer counting chamber

Sr.#	Sample	Platelet count per µl
1	Female	767×10^3
2	Female	675×10^3
3	Male	447×10^3

Table. 3.3 manual platelet counting the platelet number in PRP of volunteer calculated by Neubauer counting chamber. The counted platelet number in PRP was higher than the normal range 150,000 to 450,000 platelets per µl of whole blood.

3.4. Aggregation of platelet from healthy individuals and the effect of phenylephrine

Before analyzing the AR-mediated mtDNA release from platelet we performed the platelet aggregation testing using a microplate based spectrophotometric assay. To check the aggregation of platelets, 10µl of phenylephrine (100 µM), a synthetic agonist of α_1-adrenergic receptor, was added in 95 µl of PRP in a 96 well plate and 100 µl of PRP without agonist was taken as control. A rapid rise of curve as compared to baseline shows that there was a significant increase of aggregation in platelets upon treatment with 100µM phenylephrine (Figure 3.1). The experiment was repeated in three biological replicates with consistent results. Unpublished data from our lab demonstrated that the rate of phenylephrine-mediated increase in platelet aggregation was even faster than 100 mU of thrombin which is considered a very strong natural platelet agonist.

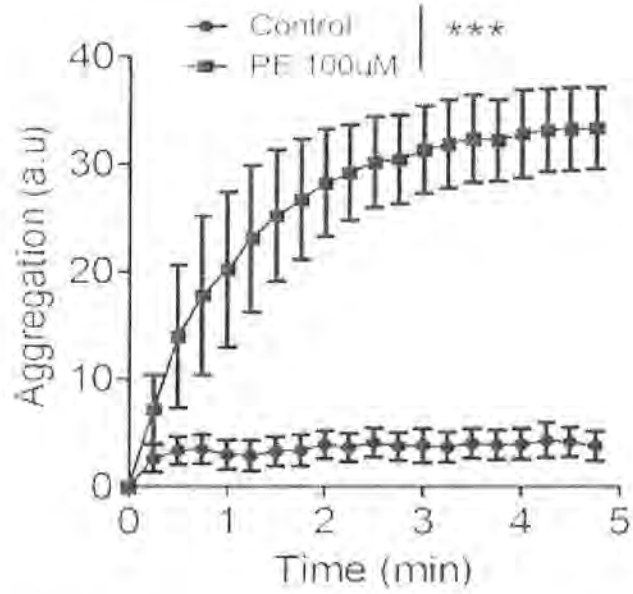


Fig.3.1. Platelet aggregation was measured for 5 minutes according to the final protocol at 37°C. The graph represents the mean platelet aggregation with 100µM phenylephrine as compared to control. n= 3, ***P value is <0.0005.

3.5. Mitochondrial DNA release from activated platelet

Activated platelet release mitochondrial DNA in extracellular environment. To confirm mtDNA release from activated platelet, the samples were treated with agonists; 100µM

phenylephrine, 100 µM nor-adrenaline and 100mU thrombin. The treated and control samples were incubated at 37°C for 2 hours, 6 hours and 16 hours. The mtDNA was eluted from samples by using a kit and PCR amplification of eluted DNA was performed with mtDNA specific NADH primers. The purity of mtDNA was confirmed by amplifying eluted DNA with nuclear DNA specific B2M primers. Agarose gel electrophoresis of PCR product confirms the presence of mitochondrial DNA and absence of nuclear DNA (Figure 3.2 (a) and (b).

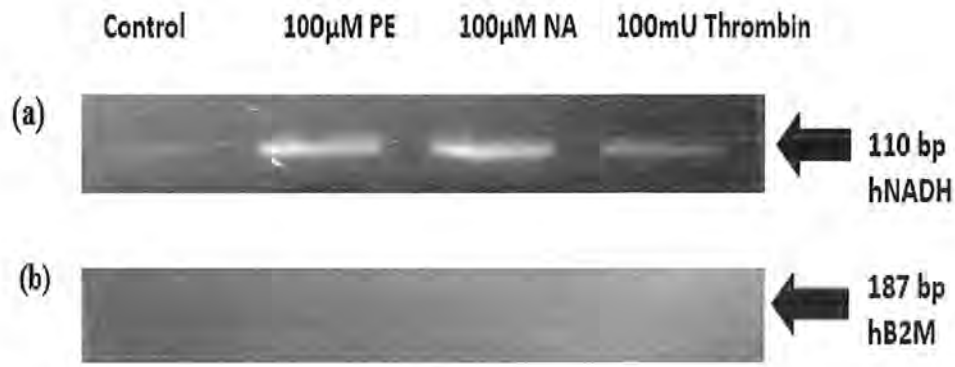


Fig.3.2 PCR-based assessment of mitochondrial DNA purity and quality by agarose gel electrophoresis (a) PCR product obtained from mtDNA specific primer NADH (b) PCR product obtained from nuclear DNA specific hB2M primers. The presence of only 110bp NADH PCR confirm the mitochondrial DNA and absence 187kb hB2M product in treated samples confirm the absence of nuclear DNA.

3.6. Mitochondrial DNA copy number calculation through standard curve

The quantification of mtDNA by conventional PCR is difficult. We aimed to calculate the copy numbers of mtDNA released after treatment of platelets with agonists. The copy numbers of samples were calculated by using a standard curve. For standard curve formation, mtDNA was amplified using mtDNA specific NADH primers. After DNA purification and quantification, the mtDNA copy number was calculation using the formula given in section 2.13. Copy number was calculated in 1 µl and dilutions were prepared from 1×10^4 to 1×10^8 as discussed above. The qPCR of standard dilution and all samples were preformed, and Cq values of standards and samples were recorded.

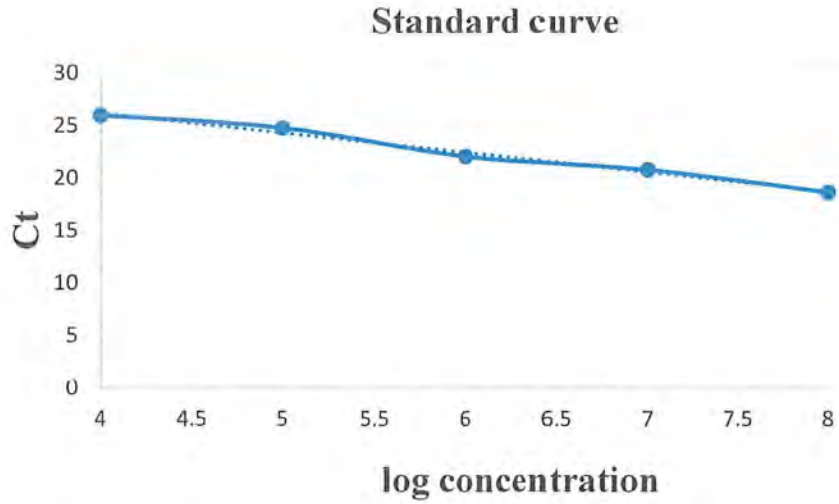


Fig.3.3 represent the standard curve showing the Cq values for each dilution plotted against log concentration.

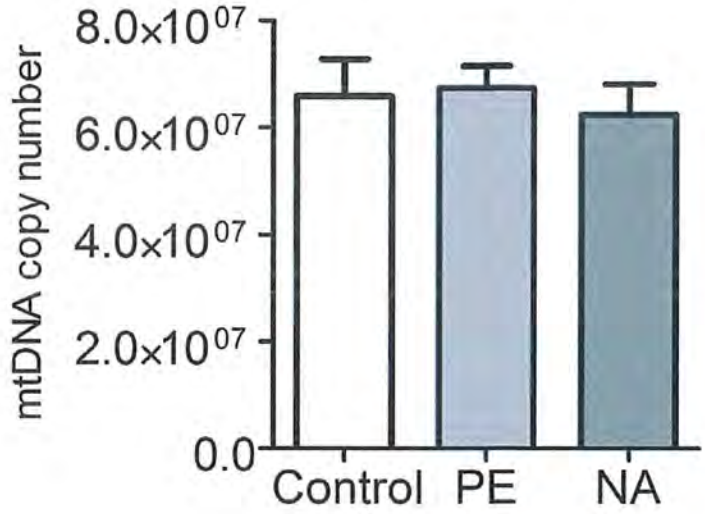


Fig.3.4. The mtDNA copies number in supernatant calculated by using standard curve. The graph shows there is no significant difference in released mtDNA copy number in control and platelets incubated for 2 hours with 100μM PE and 100μM NA. n=3

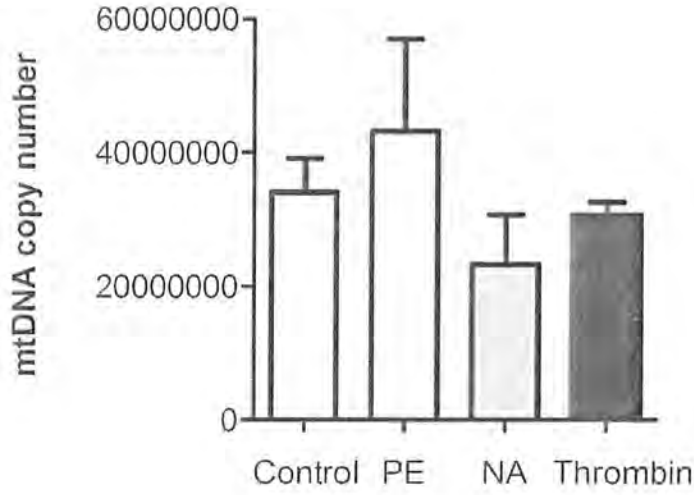


Fig. 3.5. The mtDNA copies release from platelets sample incubation with 100μM PE after 6 hours is slightly higher than control. Less mtDNA copies released in platelets incubated with 100μM NA. 100mU thrombin used as control group. The unexpected results of 100 μM NA and 100mU thrombin need further investigation. n=3.

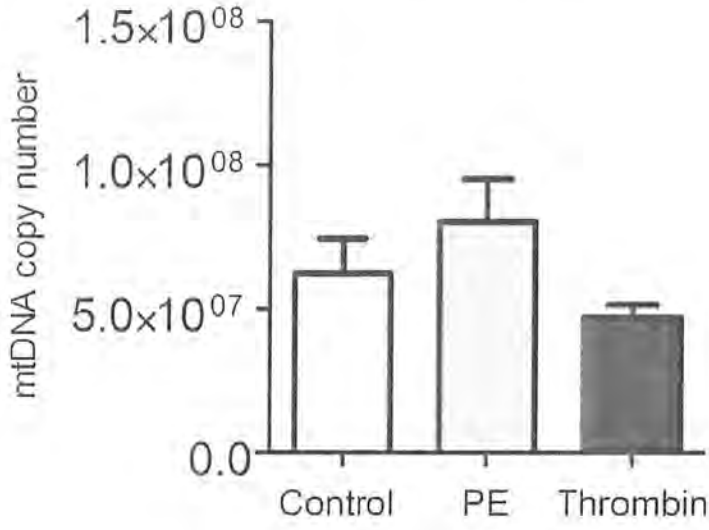


Fig.3.6 The graph shows the mtDNA copy number increase in supernatant of platelets after 16 hours incubated with 100 μM PE. The released mtDNA copies decrease in 500mU treated thrombin. This decrease in mtDNA copies requires consideration. n=3.

3.7. Platelet mitochondrial membrane depolarized with the AR agonist

We further determine the mitochondrial membrane potential disturb by AR agonists. The washed platelets were loaded with TMRM and platelets were treated with 100 μM PE and 1U thrombin. Mitochondrial membrane depolarization of stimulated and unstimulated platelets was measured at 1 minutes, 15 minutes and 30minutes by flow cytometer. The mean fluorescence intensity (MFI) of samples was recorded. The MFI of unstimulated platelets were more than those treated with 100μM PE and 1U thrombin. After 15 minutes MMP was disturbed in platelets stimulated with AR agonist as a result the MFI of TMRM was decreased and the decrease in florescence intensity was more obvious after 30 minutes of stimulation with AR agonist. The MFI of dye decreased in platelets sample stimulated with Thrombin, that was used as positive control.

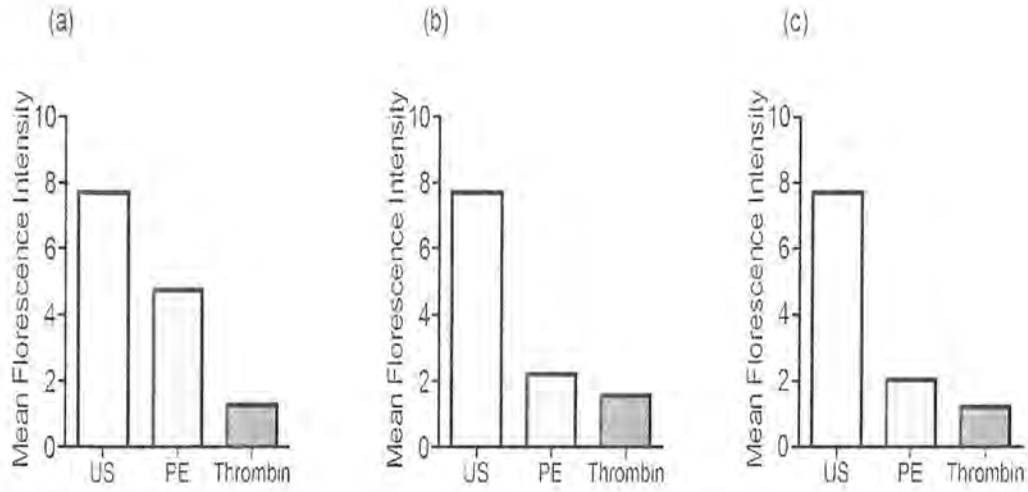
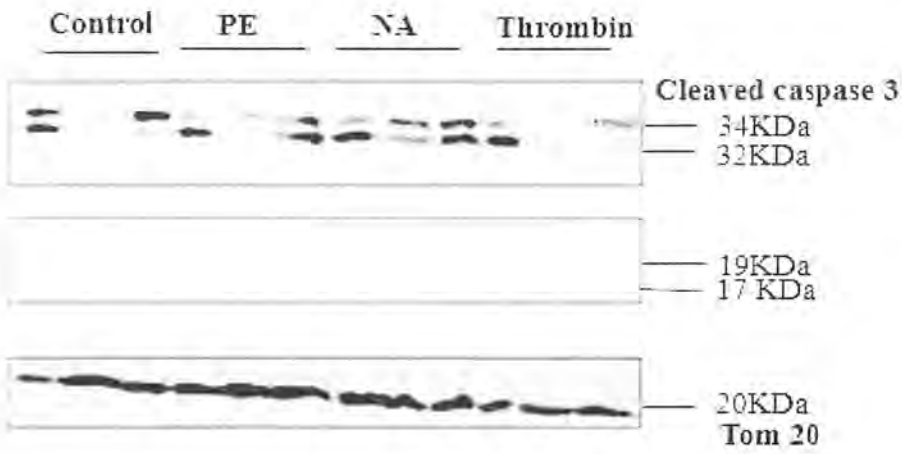


Fig 3.7. Mitochondrial membrane potential by flow cytometry. The AR receptor agonist increase the mitochondrial membrane depolarization. The platelets were stimulated for 1 minute, 15 minutes and 30 minutes. (a) represent the mean florescence intensity soon after treatment with agonist. The TMRM dye accumulate in unstimulated platelets and TMRM accumulation decrease in platelets treated with 100 μM PE and 1U thrombin. (b) after 15 minutes stimulation the MFI decrease in platelets treated with 100 μM PE compared with control (c) the loss of MFI in stimulated cells shows more depolarization of mitochondrial membrane with 100μM PE and 1U thrombin. The MMP depolarized in case of 100 μM PE increase in time dependent manner and membrane depolarization by 1U thrombin remain unchanged in all condition.

3.8. Platelet activation and caspase-3 cleavage

To investigate the mechanism behind the mtDNA release from activated platelet, the samples were treated with different agonist: 100 µM Phenylephrine, 100 µM Noradrenaline and 100mU thrombin and at 6 hours we checked the positive impact of Phenylephrine, so we investigated the caspase 3 level of platelets after 6 hours incubation. Protein was extracted from these samples and caspase-3 expression was checked by western blot. The results showed that no cleaved caspase 3 was found at 17 and 19 KDa which is an indication of apoptosis. Unexpectedly we found a cleaved product of caspases 3 just below the pro-caspase around 34 KDa. The intensity of this cleaved product of caspases 3 was higher in PE treated platelets (Figure 3.5). However, there was no change in the Tom 20 level which was used the loading control.

(a)



(b)

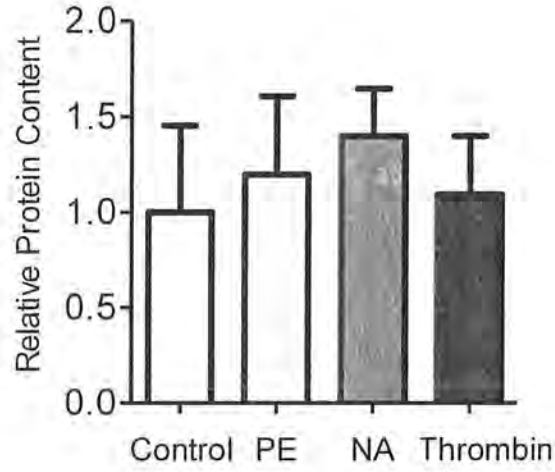
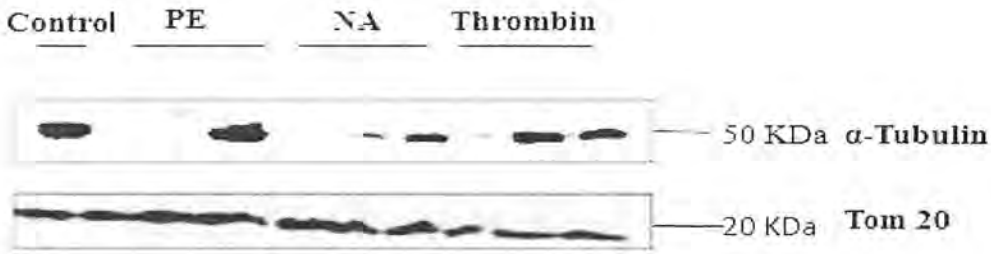


Fig.3.8. (a) Western blot image showing the cleavage of caspase-3 in treated and control samples at 34 KDa and 32 KDa that may be due to calpain mediated cleavage. No cleaved caspase 3 products found at 19 and 17 KDa (b) Graphical representation of caspase-3 cleavage shows that relative protein content is more in platelets treated with PE and NA.

3.9. Calpain dependent alpha tubulin degradation

Based on the results of caspases 3 upon AR agonist stimulation and available literature we hypothesized that the unexpected cleaved product of pro-caspase (Figure 3.5) can be due to action of calpain, which are calcium-dependent proteases often associated with necrotic cell death. For this purpose, we checked the degradation of alpha tubulin in the same samples as described in figure 3.5 because tubulin has been previously reported to be the target of calpains. Interestingly we found that platelets sample collected after treatment with AR agonists showed a decrease level of α-tubulin protein in most of the sample along with some inconsistencies. However further investigation is required to clarify the role of AR agonists in calpain activation and necrotic cell death in platelets.

(a)



(b)

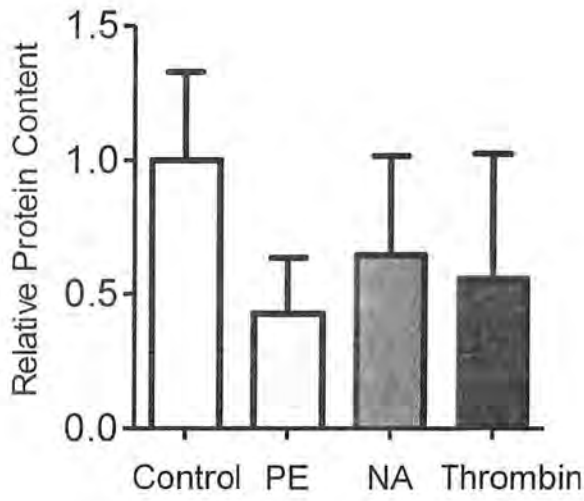


Fig.3.9 (a) Western blot image showing expression of alpha-tubulin in platelets samples after 6 hours treatment with agonists. Calpain mediated α-Tubulin cleavage is more in platelets incubated with 100μM PE and 100μM NA. α-Tubulin degradation is more in control sample **(b)** Graphical representation of expression pattern of alpha-tubuli

Discussion

Globally cardiovascular diseases are the leading cause of death. Cardiovascular disease includes blood vessels and heart diseases. The diseases development is slow, and the risk factors include hypertension, high cholesterol level in serum, diabetes, obesity, stress and smoking. Atherosclerosis is the major cause of CVD death. Past studies accelerated research to link inflammation with atherosclerosis. These studies provide the evidence that inflammation is linked with atherosclerosis.

Inflammation is a defense process against pathogens. Many cells contribute in immunity. Infection and autoimmune reaction contribute to activation of T-cells, macrophages, dendritic cells, leukocytes and mast cells. These cells infiltrate at lesion site at early and late stage of disease. Cytokine secretion and immune cell activation increase inflammatory effector mechanism (De Leo., 2016)

Platelets are ~4µm anucleate cells that are released by megakaryocytes in vasculature and circulate freely throughout the body. In haemostasis, platelets adhere at injury site, became activated and cover the damaged vessel. platelets perform their function by either extrinsic or extrinsic pathways and platelets express many receptors on their cell surface (Mackman *et al.*, 2007). The activated platelets release alpha and dense granules contents (Behnke, 1969). The released granular content recruits more and more platelets and other leukocytes (Clark *et al.*, 2007). The platelets aggregate is stabilized by thrombin-dependent fibrin deposition and other coagulation factors (Andrews and Berndt 2004). Beside its role in thrombus formation and haemostasis, platelets have non-haemostatic role such as in inflammatory response, in cancer metastasis, rheumatoid arthritis, tumor angiogenesis and atherosclerosis (Erpenbeck and Sch€on 2010; Boilard *et al.* 2010; Klement *et al.* 2009).

On average, 5-7 mitochondria are present in each platelet. Mitochondria provide energy to platelet during activation and release of granular content. ROS production during ETC prove to be dangerous for cell. This excessive ROS production change mitochondrial membrane potential and activated platelets release mitochondrial DNA via vesicles and splice phospholipase digest these vesicles and release mtDNA (Zhang *et al.*, 2010) and

elevate mtDNA level in plasma. As a result, there is great interest to use mtDNA as a biomarker (Malik *et al.*, 2013).

As soon as platelets were removed from blood circulation there is increase chance of platelet activation. Therefor PGI_2 was added in blood to prevent platelet activation during experimental platelet isolation and centrifugation (Fukaya and Ito, 2014: Nugraha *et al.*, 2012: Weiss and Turitto, 1979). For performing different assays, it was ensured that PRP is free from RBCs and WBCs contamination because the presence of these cells change the final concentration of mitochondrial DNA and protein concentration. The microscopy of PRP smear revels a complete removal of WBCs. After this we count platelets number by using Neubauer counting chamber (Harrison *et al.*, 2004). The data showed that platelet number is different in different individual.

Many receptors are expressed on platelets surface. Adrenergic receptors expressed on many cells where they perform different role. In platelets GPCRs enhance platelets response by activating PKC, Src family kinase and PI-3 Kinase and release TXA_2 and activate integrins. As we know uncontrollable platelets aggregation cause many diseases and also responsible for CVDs (Weyrich *et al.*, 2003). Different agonists were used to test the platelets activation and aggregation in many studies. Based upon study conducted by Walkowiak in 1997 and many other aggregation-based studies we checked the effect of phenylephrine and other AR agonist in platelets aggregation. We observed there was significant increase in phenylephrine mediated platelets aggregation.

Many studies demonstrate that platelets release mtDNA upon activation. It was observed that metformin inhibitor of complex 1 prevent mtDNA release from platelets that induce platelets activation in DC-SIGN dependent manner (Xin *et al.*, 2016) and previous studies showed the mtDNA copy number was calculated by qPCR (Venegas et al., 2012). Then we hypothesized that mitochondrial DNA copy number elevated in treated samples. We treat the washed platelets with agonists and incubate treated and control sample for 2, 6 and 16 hours. That data showed that Cq value increase when copy number decrease. The mtDNA copy number was calculated from these samples and data showed that mtDNA copy number increase in sample treated with phenylephrine.

There are many factors that contributes to mitochondrial DNA release. Mitochondrial membrane potential has role in proper cell regulation. Dysregulation of MMP in platelets contributes to atherosclerosis and aging (Kaplan and Jackson, 2011: Nicholls, 2004). Mitochondrial membrane potential change in activated platelets and mitochondrial membrane potential depolarize in cells. It was studied that FCCP treatment collapse MMP of rat cortical (Joshi and Bakowska, 2011). They observed that MFI of TMRM decrease as soon as MMP collapse. To detect the change in membrane potential across mitochondrial membrane, the platelets were treated with different agonists and then loaded with TMRM. The results were analyzed as median florescence intensity (MFI) by using flow cytometry because there is chance that level of expression may vary between individual. Our data in line with previous studies and it was observed that dye accumulate in cells that have healthy mitochondria (Perry *et al.*, 2011: Distelmaier *et al.*, 2008). Our data showed that MFI increase in unstimulated sample because these cells have healthy mitochondria and more dye accumulate in mitochondria and decrease due to loss of MMP (Floryk and Houštěk, 1999).

Activated platelets share some common biological feature with apoptotic cells. we found that platelets have pro-apoptotic caspases. Platelet have caspase 3 and activated caspase-3 cleaved products are at ~17 and 19kDa (Shcherbina and Remold-O'Donnell,1999: de Botton *et al.*, 2002). We failed to detect activation of caspase after treatment with nor-adrenaline, thrombin, and phenylephrine. We concluded that platelets activate independent of caspase activation that goes in line with already published data (Wolf *et al.*, 1999)

Surprisingly we found two cleaved bands of pro-caspase as reported in previous study (Wolf *et al.*, 1999). These cleaved band at 34KDa and 32 KDa from that previous study we can conclude that calpain cleaves procaspase-3 without activating them. These two bands are due to full or partial deletion of pro-domain of caspase 3 (Wolf *et al.*, 1999). Calpain mediated platelets activation is responsible for diabetes associated CVDs (Randriamboavonjy and Fleming, 2010). The loading control used was α-tubulin. The western blot shows that We found that α-tubulin protein content is different in different samples this was later confirmed from previous literature that calpain also target α-

tubulin (Huang and Xiaoping, 2016) and in our results we observed that α-tubulin also cleaved in control samples.

By applying pharmacological blockers against AR, we can confirm the mtDNA release from platelets and the possible underlying mechanism.

In summary, mtDNA is a hot and interesting topic in clinical biochemistry for diagnosis and assessment of disease risks including cardiovascular diseases. In our study we found that platelets activate upon treatment with different agonists and there was significant increase in platelets aggregation. We investigated mtDNA in platelets supernatant and did not found any significant difference in mtDNA copy number in control and treated group. However, there was tendency towards higher mtDNA release. We further report that AR agonists cause rapid collapse of mitochondrial membrane potential. Moreover, our data highlight the fact that apoptosis executioner caspase 3 is not activated by AR. Interestingly AR agonists generated a different cleaved product of caspase 3 which may be linked to calpain-dependent degradation often found in necrotic cell death. Degradation of the platelet tubulin upon stimulation with AR agonists also support this hypothesis. To authenticate our data, we need to increase the sample size which is also a shortcoming of our study. In future by increasing sample size and keeping platelets number constant we can answer these questions in a better way.

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