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PLATELET SPECIAL

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Ongoing Research on the Role of Blood Platelets in Cardiovascular Diseases and Depression - The Intriguing Link

Cardiovascular diseases (CVDs) and depression are two prevalent health conditions that have significant impacts on global public health. Despite extensive research, the exact mechanisms underlying these conditions remain incompletely understood. Recently, there has been growing interest in the role of blood platelets in both cardiovascular diseases and depression. This intricate connection has sparked intense research efforts, and recent discoveries point towards a potential culprit: blood platelets.

Cardiovascular diseases, a group of disorders affecting the heart and blood vessels are a leading cause of mortality worldwide. Depression, on the other hand, is a common mental health disorder characterized by persistent feelings of sadness and loss of interest in activities. While these two conditions may seem unrelated, emerging evidence suggests that they may share common underlying mechanisms, including the involvement of blood platelets.

Platelets: Guardians of Hemostasis; Potential Culprits in Diseases

Blood platelets (thrombocytes) are small, disc-shaped cell fragments produced in the bone marrow. They are crucial for maintaining hemostasis, the process of preventing excessive bleeding after injury. However, their role extends beyond blood clotting. Platelets also play a role in inflammation, immune response, and tissue repair. Platelets harbour a diverse array of receptors and signalling molecules, enabling them to interact with various components of the cardiovascular and nervous systems. This intricate interplay has led researchers to explore the potential involvement of platelets in depression and CVDs.

Platelets are known to play a crucial role in the development of CVDs, including atherosclerosis, myocardial infarction and stroke. Atherosclerosis is a condition characterized by the build-up of plaques in the arteries, leading to narrowing and hardening of the arteries. Platelets contribute to the formation of these plaques by adhering to damaged arterial walls and releasing substances that promote inflammation and the accumulation of cholesterol.

In the context of myocardial infarction and stroke, platelets play a central role in the formation of blood clots (thrombi) that can block blood flow to the heart or brain, leading to tissue damage. Antiplatelet medications, such as aspirin and clopidogrel, are commonly used to reduce the risk of thrombosis in patients with CVDs.

In recent years, there has been increasing interest in the role of inflammation in the pathophysiology of depression. Chronic low-grade inflammation has been implicated in the development of depressive symptoms, and several studies have found elevated levels of inflammatory markers in individuals with depression.

Platelets are known to be involved in the inflammatory response. They can interact with immune cells and release inflammatory mediators, such as cytokines and chemokines, which can contribute to the inflammatory process. Furthermore, platelets can secrete serotonin, a neurotransmitter that plays a role in mood regulation. Dysregulation of serotonin signalling has been implicated in the development of depression.

Given these roles of platelets in inflammation and serotonin regulation, researchers have hypothesized that platelets may play a role in the pathophysiology of depression. Several studies have found alterations in platelet function and activation in individuals with depression, including changes in platelet serotonin levels and increased platelet aggregation.

Delving Deeper: Unravelling the Mechanisms

While the initial findings are promising, several areas of research are actively exploring the intricacies of the platelet-depression-CVDs link:

- Dissecting the Mechanisms: Researchers are delving deeper into the specific mechanisms by which depression might influence platelet function. This includes studying the role of various signalling pathways, inflammatory markers, and genetic factors potentially contributing to the observed changes.
- Establishing Causality: Determining the direction of causality is crucial. Does depression directly trigger platelet dysfunction, or do pre-existing platelet abnormalities contribute to the development of depression? Longitudinal studies are ongoing to address this question.
- Identifying Therapeutic Targets: If a clear link between platelet dysfunction and depression-related CVD risk is established, researchers can explore targeting platelet activity as a potential therapeutic strategy. This could involve investigating the efficacy of existing antiplatelet medications or developing novel therapies specifically aimed at modulating platelet function in this context.

Current research is focused on elucidating the precise mechanisms underlying the involvement of platelets in both CVDs and depression. Some of the key areas of ongoing research include:

- **Platelet Function and Activation:** Researchers are investigating how platelet function and activation are altered in individuals with CVDs and depression. This includes studying changes in platelet aggregation, adhesion molecules, and inflammatory mediators.

  Cardiovascular diseases (CVDs) and depression are two prevalent health conditions that often coexist, leading to worse outcomes and increased mortality rates. Platelets, small cell fragments crucial for haemostasis, are intricately involved in the pathophysiology of both CVDs and depression. Altered platelet function and activation have been observed in individuals with these conditions, highlighting the complex interplay between mental health and cardiovascular health.

  Platelets play a pivotal role in maintaining cardiovascular health by forming clots to prevent excessive bleeding and initiating repair processes in injured blood vessels. However, in individuals with CVDs, platelets can become hyperactive, leading to increased clot formation and the risk of thrombotic events such as heart attacks and strokes. Similarly, in depression, platelet function is dysregulated, contributing to the elevated risk of CVDs in these individuals.

  One of the key mechanisms underlying altered platelet function in both CVDs and depression is the dysregulation of neurotransmitters such as serotonin. Platelets store and release serotonin, which not only regulates mood but also plays a role in platelet activation and aggregation. Reduced serotonin levels, commonly observed in individuals with depression, can lead to increased platelet activation, promoting a prothrombotic state. Conversely, in CVDs, alterations in serotonin signalling pathways can also contribute to platelet hyperactivity, further exacerbating the risk of thrombotic events.

  In addition to serotonin, other neurotransmitters and neuromodulators, such as norepinephrine and dopamine, also play a role in platelet function and are dysregulated in both CVDs and depression. These neurochemical imbalances can lead to abnormalities in platelet activation pathways, including the upregulation of adhesion molecules and the release of pro-inflammatory and pro-thrombotic factors, further promoting a prothrombotic state.

  Furthermore, chronic inflammation, a common feature of both CVDs and depression, can directly impact platelet function. Inflammatory cytokines can activate platelets and promote their aggregation, contributing to the development and progression of atherosclerosis and other cardiovascular complications. Moreover, the chronic inflammatory state in depression can exacerbate platelet activation, creating a vicious cycle that further increases the risk of CVDs.

  The relationship between platelet function, CVDs, and depression is bidirectional, with each condition influencing the others in a complex interplay of biological and psychosocial factors. For example, individuals with CVDs are more likely to develop depression, which, in turn, can worsen cardiovascular

outcomes through its effects on platelet function and activation. Similarly, depression can lead to lifestyle factors such as poor diet and physical inactivity, which can independently impact platelet function and cardiovascular health.

Researchers have noted that platelet function and activation are altered in individuals with CVDs and depression, contributing to the increased risk of adverse cardiovascular events in these populations. Understanding the mechanisms underlying these alterations is crucial for developing targeted interventions to improve outcomes in individuals with comorbid CVDs and depression. Future research should focus on elucidating the specific pathways linking platelet dysfunction, CVDs, and depression, with the ultimate goal of developing novel therapeutic strategies to mitigate the cardiovascular risks associated with these conditions.

- **Platelet Serotonin Levels:** Platelet serotonin levels have long been of interest in the study of depression, as they offer a window into the functioning of the serotonin system, which is known to play a crucial role in mood regulation. Serotonin is a neurotransmitter that is involved in various physiological processes, including mood, appetite, and sleep. It is synthesized in the brain and also found in blood platelets.

  The relationship between platelet serotonin levels and depression is complex and not fully understood. However, several studies have suggested that alterations in platelet serotonin levels may be associated with the development and progression of depression.

  One of the key findings in this area of research is that individuals with depression often have lower levels of platelet serotonin compared to healthy controls. This has led to the hypothesis that decreased platelet serotonin levels may be a biomarker for depression. However, it is important to note that not all studies have found this association, and the relationship between platelet serotonin levels and depression is likely influenced by various factors, including genetic predisposition, environmental factors, and the presence of other medical conditions.

  The role of platelet serotonin in depression is further supported by the fact that many antidepressant medications, such as selective serotonin reuptake inhibitors (SSRIs), act by increasing serotonin levels in the brain. SSRIs work by blocking the reuptake of serotonin, thereby increasing the availability of serotonin in the synaptic clefts between neurons. This increased serotonin signalling is believed to help alleviate symptoms of depression.

  In addition to its role in neurotransmission, serotonin is also involved in platelet function. Serotonin released from platelets plays a role in blood clotting and vasoconstriction, which are important physiological processes. Dysregulation of platelet serotonin levels may therefore not only impact neurotransmission but also contribute to other aspects of physiology that are relevant to depression.

  It is also worth noting that platelet serotonin levels are influenced by various factors, including diet, stress, and medications. For example, some studies have suggested that dietary factors, such as low levels of tryptophan (an amino acid precursor of serotonin), may be associated with decreased platelet serotonin levels and an increased risk of depression. Similarly, stress and certain medications, such as non-steroidal anti-inflammatory drugs (NSAIDs), have been shown to affect platelet function and serotonin levels.

  Overall, while the relationship between platelet serotonin levels and depression is complex and not fully understood, there is evidence to suggest that alterations in platelet serotonin levels may play a role in the development and progression of depression. Further research is needed to fully elucidate the mechanisms underlying this relationship and to determine the potential clinical implications of these findings.

- **Platelet Biomarkers:** Beyond their traditional role in clotting, platelets have emerged as important players in various physiological and pathological processes, including inflammation, immunity, and cardiovascular diseases (CVDs). Additionally, recent studies have suggested a potential link between platelet function and mental health, particularly depression. This has sparked interest in identifying

platelet biomarkers that could help predict the risk of both CVDs and depression.

Platelets as Biomarkers: Platelets are not just simple cell fragments but are dynamic cells that respond to various stimuli in the body. They contain a range of bioactive molecules, including cytokines, chemokines, growth factors, and microRNAs, which are released upon activation. Changes in the levels or activity of these molecules in platelets could serve as biomarkers for different diseases, including CVDs and depression.

Platelets play a crucial role in the development of CVDs, primarily through their involvement in thrombosis, the formation of blood clots that can block blood flow in arteries. Several platelet biomarkers have been proposed for predicting the risk of CVDs:

1. Platelet Activation Markers: Platelet activation is a key step in thrombosis. Markers of platelet activation, such as P-selectin and CD40 ligand, have been associated with an increased risk of CVDs. Elevated levels of these markers in platelets could indicate a higher risk of thrombotic events.
2. Platelet Aggregation: Platelet aggregation, the clumping together of platelets, is another important process in thrombosis. Abnormal platelet aggregation, measured by aggregometry assays, has been linked to an increased risk of CVDs.
3. MicroRNA Profiles: Platelets contain a variety of microRNAs that can regulate gene expression. Changes in the microRNA profile of platelets have been observed in patients with CVDs and could serve as potential biomarkers for predicting CVD risk.
4. Platelet Membrane Proteins: Proteins present on the surface of platelets, such as glycoprotein IIb/IIIa, play a role in platelet aggregation and are potential biomarkers for CVD risk.

While the exact cause of depression is not fully understood, there is growing evidence suggesting a link between platelet function and depression. Several platelet biomarkers have been proposed for predicting the risk of depression:

1. Serotonin Levels: Platelets contain serotonin, a neurotransmitter that plays a role in mood regulation. Changes in platelet serotonin levels have been observed in patients with depression and could serve as a biomarker for depression risk.
2. Inflammatory Markers: Platelets are involved in inflammation, which has been implicated in the development of depression. Inflammatory markers, such as C-reactive protein (CRP) and interleukin-6 (IL-6), have been associated with depression and could be used as biomarkers.
3. Oxidative Stress Markers: Oxidative stress, an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses, has been linked to depression. Platelet markers of oxidative stress, such as malondialdehyde (MDA) and glutathione peroxidase (GPx), could be potential biomarkers for depression risk.

Platelets are not only important for haemostasis but also play a role in various physiological and pathological processes, including CVDs and depression. Identifying platelet biomarkers for predicting the risk of these diseases could lead to early detection and personalized treatment strategies. Further research is needed to validate these biomarkers and understand the underlying mechanisms linking platelet function to CVDs and depression.

- **Developing Novel Therapies Targeting Platelet Function and Activation for CVDs and Depression:**

  Cardiovascular diseases (CVDs) and depression are two prevalent and burdensome health conditions worldwide. While they may seem unrelated, emerging research suggests a potential link through platelet function and activation. Platelets, traditionally known for their role in haemostasis, have increasingly been recognized for their involvement in inflammation, immune responses, and even mental health disorders like depression. Targeting platelet function and activation opens up a promising avenue for the development of novel therapies for both CVDs and depression.

  Dysregulated platelet activation can lead to pathological thrombosis, a hallmark of CVDs such as heart attack and stroke. Beyond their haemostatic role, platelets are increasingly recognized for their

involvement in inflammatory processes. Activated platelets can release a plethora of inflammatory mediators and interact with immune cells, contributing to the progression of inflammatory conditions, including atherosclerosis, a key underlying factor in many CVDs.

The association between platelets and depression may seem surprising at first, but several lines of evidence support this connection. Firstly, platelets contain and release serotonin, a neurotransmitter implicated in mood regulation. Alterations in platelet serotonin levels have been reported in individuals with depression, suggesting a potential role of platelets in the pathophysiology of depression.

Furthermore, platelets from individuals with depression exhibit increased activation and aggregation in response to various stimuli, indicating a state of hyperactivity that may contribute to the development or progression of depressive symptoms.

Potential Therapeutic Approaches: Given the role of platelets in both CVDs and depression, targeting platelet function and activation could represent a novel therapeutic approach for these conditions. Several strategies could be explored:

1. Antiplatelet Agents: Drugs that inhibit platelet activation, such as aspirin and clopidogrel, are already widely used for the prevention and treatment of CVDs. These agents could potentially be repurposed for the management of depression, although more research is needed to establish their efficacy in this context.
2. Serotonin Modulation: Since platelets are a major storage site for serotonin, modulating platelet serotonin levels or function could be a potential target for treating depression. However, the complex interplay between platelet and brain serotonin systems would need to be carefully considered.
3. Inflammatory Pathway Inhibition: Targeting the inflammatory pathways mediated by platelets could be beneficial for both CVDs and depression, given the role of inflammation in the pathogenesis of these conditions. Drugs that inhibit specific inflammatory mediators or pathways could be explored.
4. Platelet-Targeted Therapies: Developing therapies that specifically target platelet activation pathways involved in both CVDs and depression could provide more targeted and potentially more effective treatments.

Despite the potential of targeting platelet function and activation for the treatment of CVDs and depression, several challenges remain. These include the need for a better understanding of the complex role of platelets in these conditions, as well as the development of targeted therapies that can selectively modulate platelet function without causing excessive bleeding or other adverse effects.Future research should focus on elucidating the specific mechanisms by which platelets contribute to CVDs and depression, as well as identifying novel therapeutic targets that can be exploited for the development of more effective and safer treatments. By harnessing the potential of platelet-targeted therapies, we may be able to improve outcomes for patients with these debilitating conditions.

- **Role of Platelets in the Gut-Brain Axis:** There is emerging evidence suggesting a link between the gut microbiota, inflammation, and mental health. Researchers are investigating how platelets may be involved in this gut-brain axis and its implications for CVDs and depression.

  The gut-brain axis, a bidirectional communication network between the gastrointestinal tract and the central nervous system, plays a crucial role in maintaining homeostasis and influencing various physiological processes. Recent research has highlighted the involvement of plateletsin the gut-brain axis. This emerging area of study has significant implications for understanding the pathophysiology of cardiovascular diseases (CVDs) and depression.

  Platelets are not merely involved in hemostasis; they also interact with immune cells, endothelial cells, and the nervous system, making them key players in inflammatory and neuro-inflammatory processes. The gut is a major site of immune activity, and its dysfunction can lead to systemic inflammation, a hallmark of CVDs and depression. Platelets have been found to interact with the gut microbiota, the diverse community of microorganisms residing in the gastrointestinal tract, which plays a crucial role in gut health and immune regulation.

One of the mechanisms by which platelets influence the gut-brain axis is through the release of bioactive molecules such as serotonin. Platelets take up serotonin from the gut, and upon activation, release it into the circulation, where it can act on various tissues and cells, including the brain. Disruption of serotonin signalling has been implicated in both CVDs and depression, highlighting the potential role of platelets in these conditions.

Furthermore, platelets can interact with the gut microbiota through toll-like receptors (TLRs), which are key regulators of the innate immune response. Activation of TLRs on platelets by microbial products can lead to platelet activation and the release of inflammatory mediators, contributing to systemic inflammation. This interaction between platelets and the gut microbiota can have profound effects on both local gut health and systemic inflammatory processes, which are implicated in the development of CVDs and depression.

The implications of these findings are significant for the prevention and treatment of CVDs and depression. Targeting platelet function and the gut microbiota could offer novel therapeutic approaches for these conditions. For example, modulating the gut microbiota through diet, probiotics, or faecal microbiota transplantation could help regulate platelet function and reduce inflammation, potentially lowering the risk of CVDs and improving mood disorders.

Blood platelets are emerging as key players in the pathophysiology of both cardiovascular diseases and depression. Ongoing research is shedding light on the intricate roles of platelets in inflammation, serotonin regulation, and thrombosis, and how these processes may intersect in the context of CVDs and depression. Further understanding of these mechanisms could lead to the development of novel diagnostic and therapeutic strategies for these prevalent and debilitating conditions.

By unravelling the underlying mechanisms and identifying potential therapeutic targets, the ongoing research has the potential to improve cardiovascular/ depression outcomes in individuals and contribute to the development of more personalized treatment strategies. However, further research addressing the aforementioned challenges is crucial to solidify the evidence base and translate these findings into tangible clinical benefits.

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(Ravindra Bangar)
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October to December 2023 Issue (Volume 5, Issue 4)

PJMHS
PACIFIC JOURNAL OF MEDICAL AND HEALTH SCIENCES

INDEX

Research Paper

Review

October to December 2023 Issue (Volume 5, Issue 4)

PJMHS
PACIFIC JOURNAL OF MEDICAL AND HEALTH SCIENCES

INDEX

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PJMHS
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Research Paper

Effect of Eugenol – An Active Component of Clove Oil - On Platelet Aggregation and Lipid Peroxidation in Healthy Individuals

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ABSTRACT

The present study was designed to observe the effect of different doses of eugenol on platelet aggregation and lipid peroxidation in healthy individuals.

The study was conducted on 30 healthy individuals divided in two groups. In group I (N = 15), the effect of single dose of eugenol was observed. The group was further divided into three sub-groups of five each and given plain eugenol in 10 mg/kg, 15 mg/kg and 20 mg/kg of body weight respectively. The effect of plain eugenol observed was compared with the effect of placebo given one day prior to eugenol on platelet aggregation and lipoprotein oxidation susceptibility. Group II (N=15) was similarly subdivided and eugenol was administered in 10, 15 and 20 mg/kg of body weight in single dose. But in this group eugenol was administered with 25 gm of butter and the effect was compared with butter and placebo given one day prior to the fat and eugenol. In both groups blood samples were collected initially and ninety minutes after ingestion of eugenol or placebo with or without fat and analysed for platelet aggregation and lipid peroxidation.

It has been observed that administration of eugenol in different doses to healthy volunteers variably affect platelet aggregation induced by ADP and epinephrine. The magnitude of responsiveness increases as the dose is increased from 10 mg/kg to 20 mg/kg body weight and at higher dosage (20 mg/kg) it is statistically significant ($P<0.05$). However, lipid peroxidation remains unaltered even in higher doses.

Similarly, in group II, when fat was administered with eugenol, the response of platelet towards aggregating agents remained same as in plain eugenol group. Here also the response is dose related and statistically significant ($P < 0.05$) at 20 mg/kg body weight dose. Lipoprotein oxidation susceptibility however remains significantly unaltered in all three doses schedules.

In both the study groups, therefore one observation is common, that eugenol has some potential of lowering platelet aggregation, but the effect is greatly modified by the doses administered. Secondly, as the effect is more or less same in both the groups the absorption of eugenol is practically same and not affected by fat.

KEYWORDS: Acetyl eugenol, Clove oil, Spices, Antioxidant

INTRODUCTION

Herbs have been extensively used in Ayurvedic, Unani and Chinese system of medicine for centuries. The Great Indian Physician Charak had described the medicinal value of more than 1500 plants. Voluminous literature on herbal medicine is available in books like Charak Samhita, Sushruta Samhita, Dhanvantri and Indian materia-medica. Incidentally many of these plants in some form or other are in common use. Few of them are used as condiments while others for the purpose of treating various diseases. Clove has been used as a spice and medicine from early times by Chinese and Indians for various ailments[1].

Spices have been consumed in mostly all the cultures over centuries. It is common assumption that their consumption is related to taste and aroma. However, there is more to spices consumption than mere taste or smell.

In recent times many spices have made cross cultural penetrations which in turn has generated curiosity about their biological effects, mode of action and isolation of active components. Garlic, onion, ginger, clove and curcumin are the few examples. They were used by ancient civilizations millenniaago and were known to their various medicinal effects in these cultures. Yet scientific evidence confirming their medicinal properties have been provided only recently[2-8]. Spices are now known to possess antithrombotic, anti-sclerotic, hypoglycemic, hypolipedemic, anti-inflammatory, anti-arthritic and platelets aggregation inhibiting properties[2-9].

The spices form a part of daily diet in the Indian subcontinent and the Far East. As they were found to influence the oxygenation of arachidonic acid, their consumption might have preventive effect in diseases where elevated levels of ecosanoids have been reported. The spices contribute in preventing the occurrence of diseases mediated by peroxidation of lipids as low incidence of cardiovascular diseases. Inflammatory disorders etc. is incidentally observed in regions where spices are consumed regularly. Dietary intake of natural antioxidants some of which might originate from spices could be an important aspect of body's defence mechanisms against the above mentioned diseases in such populations. Many anti-oxidants have been lately identified as anticarcinogens. Plant xenobiotics may be important in inhibiting carcinogen induced tumorigenesis in humans. Eugenol may be one such compound[10].

Clove (*Syzygium aromaticum*) is rich in volatile compounds and antioxidants such as eugenol, B-caryophyllin and alpha-Lumulene. Eugenol is the major compound accounting for at least 50% while remaining 10-40% consists of eugenol acetate, B-caryophyllin and alpha-Lumulene. Besides its application in the perfume, cosmetic, flavouring, as a food spice, it does have biological activity relevant to human health such as antimicrobial, antioxidant, insecticidal, anti-inflammatory, analgesic, anticancer etc.11 (Haro-Gonjalez.Molecules 2021; 26:6387-6411)

The antiplatelet components eugenol and acetyl eugenol have been isolated and identified. They were potent in inhibiting the platelet aggregation. Their inhibitory effect was reversible. They inhibited arachidonate, adrenaline and collagen induced platelet aggregation[12].

Eugenol (4 Allyl-2 methoxy phenol), is a naturally occurring phenolic compound. It is a major component of clove oil and is also present in oils of cinnamon, basil and nutmeg. It is used mainly as flavouring agent up to a level of 0.01 per cent in foods such as baked products, beverages, sweets and frozen dairy products. It is extensively used along with zinc oxide as a component of several dental materials. [Joint F.A.O./W.H.O. Expert Committee on food additives has established a conditional acceptable daily intake (ADI) of eugenol up to 5 mg/kg for humans[13]. It is considered non-mutagenic, non-carcinogenic and generally recognized. as a safe (GRAS) and is approved by FDA[14]]

Eugenol and acetyleugenol are the known substances present in the clove. Though clove has traditionally been used as aromatic and stomachic, its pharmacological study is meagre. It has been shown that clove and its active principle possess cholagogue effect[15]. The inhibition by eugenol of glucuronic acid conjugation in the stomach of rats and guinea pigs and of dogs may have some bearing on reported mucinogenic activity of eugenol and its beneficial effect in gastric ulcer formation[16-17]. Eugenol also inhibited tumour promotion caused by Anthracene derivatives in mice[18].

Lately, eugenol has been subjected to some more scientific scrutiny. It was found to be anti- aggregatory in ex-vivo experiments. It abolishes arachidonate induced aggregations and second phase of epinephrine induced aggregation. Inhibition of aggregation seemed to be mediated by a reduced formation of thromboxane and increased formation of 12-hydro- peroxy-eicoso-tetraenoic acid (HPETE). It was inferred that possibly some enzymes of arachidonic acid cascade was inhibited by eugenol[10].

In animal experimental study eugenol and acetyl eugenol (100 mg/kg) given orally to rabbits, decreased significantly formation of thromboxane B2 during blood clotting. It has more pronounced inhibiting action 92±6 per cent and biological half life 72±30 minutes. Acetyl eugenol is less active. Eugenol remains most active compound and has reversible action on plateletfunction[19].

Antioxidant activity of eugenol has also been evaluated in vivo, at a concentration similar to recommended level of dietary intake (2.5 mg/kg body weight)[20]. Eugenol inhibited superoxide formation and lipid peroxidation and the radical scavenging activity may be responsible for its chemopreventive action[18]. Lipid peroxidation is known to be initiated by reactive oxygen species[21].

On the basis of above in vitro and ex-vivo studies, it is evident that eugenol is an important naturally occurring phenolic compound to possess strong antioxidant and platelet aggregation inhibiting property. However there is no human study available so far as the literature is concerned regarding the effect of eugenol on platelet aggregation and lipoprotein oxidation susceptibility.

In view of these observations it is considered pertinent to observe the effect of eugenol on platelet aggregation and lipid peroxidation in healthy individuals.

MATERIALAND METHODS

The present study was conducted on 30 male volunteers between age 25-40 years who were non-smoker, nontobacco chewer and not taking any non-steroidal anti-inflammatory drugs for last 15 days. Hypertension, diabetes mellitus and coronary artery disease were excluded by detailed clinical history and relevant laboratory investigations. The study was approved by institutional ethical committee and after informed consent the selected individuals were divided into two groups of 15 each.

Group I (N=15) - Eugenol group

Group II (N=15) - Fat + Eugenol group.

Group I (Eugenol group):

After an overnight fast first blood sample was collected. They were then randomly divided into three subgroups of 5 each and received eugenol on one occasion and placebo on other occasion in different doses.

Group IA (N = 5) - Eugenol 10 mg/kg body Wt. orally

Group IB (N = 5) - Eugenol 15 mg/Kg body wt. orally

Group IC (N=5) - Eugenol 20 mg/kg body wt. orally.

Second blood sample was collected 90 minutes after administration of eugenol or placebo.

Group II (Fat + Eugenol group):

This group was also randomly divided into 3 sub-groups of five each as in group 1 and also received eugenol in same three different doses schedules. But this group in addition received 25 gms. of butter with four pieces of breads with eugenol.

Group IIA (N = 5) - fat + 10 mg/kg body wt. eugenol orally.

Group IIB (N = 5) - fat + 15 mg/kg body wt. eugenol orally.

Group IC (N = 5) - fat + 20 mg/kg body wt. eugenol orally.

On the first day, initially fasting blood sample was collected and then 25 gms. butter with four pieces of bread was fed. Second sample was collected after 90 minutes. On the second day same procedure was repeated but in addition of different doses of eugenol with bread and butter.

All the blood samples were subjected for platelet aggregation using ADP and Epinephrine as aggregating agent and lipid peroxidation.

Preparation of eugenol capsules:

In present study eugenol supplied by Sigma (USA) was used. The calculated amount of eugenol was mixed with lactose powder filled in gelatine capsule. The placebo contained only lactose powder and was given in the same doses.

Platelet aggregation

Platelet-rich plasma (PRP) and Platelet-poor plasma (PPP) was obtained by differential centrifugation. Platelet aggregation was measured turbidimetrically on ELVI-840 aggregometer and Omniscribe chart recorder. The measurement of aggregation was performed exactly after 30 minutes of sample collection to avoid differences of the aggregation due to altered status of the platelet resulting from ex-vivo conditions. After 10 minutes of equilibrium at 37°C and constant stirring at 1,000 rpm, the aggregation of PRP was induced by ADP or epinephrine (Sigma), the response was recorded for 5 minutes, and the results were expressed as percentage aggregation[22].

Lipoprotein Oxidation Susceptibility Test (LOS):

The LOS was measured based on the principle that the hydrolysis of plasma lipoperoxide forms MDA (Malondialdehyde) which reacts with thiobarbituric acid to form a red 2:1 Thiobarbituric acid Malondialdehyde adduct, this coloured complex absorbs maximally at 532 nm and optical density will be measured by spectrophotometry [23].

Reagents used:

1. Dextran sulphate
2. Copper chloride
3. Magnesium chloride
4. Bovine albumin
5. Barbituric acid
6. Trichloracetic acid
7. Hydrochloric acid
8. Sodium chloride
9. n-Butanol.

Reagents:

1. **Dextran sulphate:** 1.0 gm of dextran sulphate was dissolved in 100 ml of distilled water and 0.2 mM of solution of dextrans sulphate was prepared.
2. **Copper chloride:** 8.52 mg of copper chloride dissolved in 100 ml of distilled water. 0.5 nm of copper chloride was prepared.
3. **Magnesium chloride:** 10.2 mg of magnesium chloride dissolved in 100 ml of distilled water and 0.5 M magnesium chloride solution was prepared.
4. **Bovine albumin:** 3 gm of bovine albumin dissolved in 50 ml of distilled water thus 6.0 per cent of bovine albumin solution was prepared.
5. **Hydrochloric acid:** 2.2 ml of HCL dissolved in distilled water and 0.25 N of HCI solution was prepared.

6. **TBARS reagent:** This reagent contained 26 nm of Thiobarbituric acid (TBA) and 0.92 M trichloroacetic acid in 0.25N HCI. The TBA was added first, heated and stirred. After it was dissolved, the TCA was added to the solution and was brought to volume with HCI. This reagent was stored in a dark bottle at room temperature.
7. **Sodium chloride:** 4 gm of sodium chloride dissolved in 100 ml of distilled water to give 4.0 per cent of sodium chloride solution.

Procedure:

Step I: LOS test serum (not plasma) was used in this test. A 500 µ lit serum sample was treated with 50µ lit solution containing 0.2 nm dextran sulphate (MW = 50,000 Genzyme, Cambridge MA) and 0.5 M $MgCl_2.H_2O$ to precipitate the apo. B containing lipoproteins (LDL and VLDL).

Step II: After centrifugation at 3,000 rpm at 20°C for 10 minutes. The supernatant was removed.

Step III: 1 ml of 6% bovine serum albumin (BSA) and another 50µ lit. of dextran sulphate magnesium solution was added.

Step IV: Solution was vortexed and recentrifuged as above to wash away any residual serum proteins and HDL.

Step V: The supernatant was again removed and the remaining precipitate (containing LDL and VLDL) was dissolved in 4% NACI.

Step VI: A volume of redissolved precipitate containing 100 mg of non-HDL cholesterol was combined with sufficient 4 % NaCl to give a total volume 500µ lit.

Step VII: 50µ lit of 0.5 mM CuCl2.2H2O solution was added (Final Copper Concentration was 46 µM).

Step VIII: Samples were incubated at 37°C in a shaking water bath for 3 hours.

Step IX: Next 2 ml of TBARS reagent was added to each tube.

Step X: The mixture was heated at 100°C in a water bath for 15 minutes.

Step XI: After removing and cooling the tubes 2.5 ml n-Butanol was added. The tubes were vortexed and then centrifuged for 15 minutes at 3,000 rpm at room temperature.

Step XII: The pink upper layer was removed and the optical density was determined in a spectrophotometer at 532 nm.

The coefficient of variation of method was 4% intra assay and 9% inter assay.

Normal value: 66±22 nmol. MDA/mg. of non HDL-C.

Statistical Analysis

All data are expressed as mean ± SE. The results were analyzed with Student's t test for paired data. p value of <0.05 was considered statistically significant[24].

RESULTS

TABLE-1
EFFECT OF PLACEBO ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS
GROUP I-A

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after placebo	Initial	90 minutes after placebo	Initial	90 minutes after placebo
Mean	62.00	64.25	60.25	60.50	51.00	53.00
% Change	-	3.62	-	0.4149	-	3.92
SD ±	10.9807	8.7766	8.5421	9.6257	8.9442	4.4721
SE ±	4.9108	3.9251	3.8202	4.3048	4.0000	2.0000
p		NS		NS		NS

P = As compared with initial

NS = Not significant

Administration of placebo in 5 healthy individuals led to alter ADP induced platelet aggregation from mean level of 62± 10.98 to 64 ± 8.77 per cent and was statistically not significant. Similarly the platelet aggregation induced by epinephrine was also remained unaffected (P=NS). Administration of placebo in 5 healthy individuals led to change in lipid peroxidation from a mean of 51±8.94 to 53±4.7 nmol MDA/mg non-HDL-C which was statistically not significant. (Table 1)

TABLE-2

EFFECT OF SINGLE DOSE (10 mg/kg.) OF EUGENOL ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS

GROUP I-A

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after Eugenol (10mg/kg)	Initial	90 minutes after Eugenol (10mg/kg)	Initial	90 minutes after Eugenol (10mg/kg)
Mean	66.75	67.75	60.75	61.25	47.00	47.40
% Change	-	1.4981	-	0.8230	-	0.8510
SD ±	5.9027	4.1963	4.2019	5.5198	8.3666	6.4265
SE ±	2.6398	1.8766	1.8792	2.4686	3.7417	2.8741
p		NS		NS		NS

P = As compared with initial

NS = Not significant

The effect of single dose (10 mg/Kg BW) of eugenol on platelet aggregation and lipid peroxidation on healthy individuals showed that the platelet aggregation induced by ADP and epinephrine did not change significantly. Similarly, lipid peroxidation did not alter to any significant extent. (Table 2)

TABLE-3

EFFECT OF FAT (25 gm) PLUS PLACEBO ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS

GROUP II-A

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after fat + placebo	Initial	90 minutes after fat + placebo	Initial	90 minutes after fat + placebo
Mean	63.50	66.00	54.25	55.25	54.00	54.00
% Change	-	3.9370	-	1.8433	-	0.00
SD ±	12.099	7.0378	18.0286	18.7374	11.2249	8.9492
SE ±	5.4110	3.1474	8.0628	8.3798	5.020	4.000
p		NS		NS		NS

P = As compared with initial

NS = Not significant

The effect of 25 gm fat plus placebo administration in healthy individuals altered ADP and epinephrine induced platelet aggregation from a mean of 63.50 ± 12.09 to 66.07 ± 7.03 per cent and 54.25 ± 18.02 to 55.25 ± 18.73 per cent respectively, which was statistically not significant. Likewise no significant change was observed in lipoprotein susceptibility (LOS) after fat administration. (Table 3)

TABLE-4
EFFECT OF SINGLE DOSE OF FAT PLUS EUGENOL (10 mg/kg) ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS GROUP II-A

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after fat + eugenol	Initial	90 minutes after fat + eugenol	Initial	90 minutes after fat + eugenol
Mean	53.25	53.75	47.50	48.25	53.00	52.00
% Change	-	0.9389		1.5789	-	1.8867
SD ±	13.1576	11.4222	6.1870	7.4791	9.7467	10.9544
SE ±	5.8844	5.1083	2.7669	3.3448	4.3589	4.8991
p		NS		NS		NS

P = As compared with initial

NS = Not significant

After 90 minutes of administration of fat ± eugenol (10 mg/kg), ADP and epinephrine induced platelet aggregation showed a change of mean from 53.25 ± 13.15 to 53.75 ± 11.42 per cent and 47.50 ± 6.18 to 48.25 ± 7.47 per cent respectively which was not significant statistically. Similarly, lipid peroxidation decreased from a mean of 53 9.74 to 52 ± 10.95 nmol MDA/mg of non HDL-C which is around 1 per cent and was not significant statistically. (Table 4)

TABLE-5
EFFECT OF PLACEBO ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS GROUP I-B

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after placebo	Initial	90 minutes after placebo	Initial	90 minutes after placebo
Mean	58.70	61.45	58.84	62.59	52.00	48.00
% Change	-	4.6848	-	6.3732	-	7.6923
SD ±	10.8489	6.7137	10.9200	13.6243	7.5828	5.7008
SE ±	4.8519	3.0025	4.8837	6.0931	3.3912	2.5495
p		NS		NS		NS

P = As compared with initial

NS = Not significant

Single dose of placebo, increased ADP and epinephrine induced platelet aggregation from a mean of 58.70 ± 10.84 to 61.45 ± 6.71 and 58.84 ± 10.92 to 62.59±13.62 per cent respectively, which was not significant statistically. Similarly placebo reduced lipid peroxidation from a mean of 52.00 ± 7.58 to 48.00 ± 5.70 nmol MDA/mg non-HDL-C which was also not significant statistically (Table 5).

TABLE-6

EFFECT OF SINGLE DOSE OF EUGENOL (15 mg/kg.) ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS
GROUP I-B

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after eugenol	Initial	90 minutes after eugenol	Initial	90 minutes after eugenol
Mean	68.7	66.50	65.95	66.50	54.00	54.60
% change	-	3.2727	-	0.8339	-	1.1111
SD ±	5.1536	7.9744	6.4591	6.5192	6.3835	10.5261
SE ±	2.3048	3.5663	2.8886	2.9155	2.8570	4.7075
p		NS		NS		NS

P = As compared with initial

NS = Not significant

Administration of single dose (15 mg/kg BW) of eugenol in healthy individuals decreased ADP induced platelet aggregation was from a mean of 68.75 ± 5.15 to 66.5 ± 7.97 per cent which is around 3.27 per cent which was statistically not significant. However, epinephrine induced platelet aggregation remained unaffected. Lipid peroxidation was altered by 1.1 per cent from a mean of 54 ± 6.38 to 54.6 ± 10.52 nmol MDA/mg non-HDL-C and was not significant. (Table 6)

TABLE-7

EFFECT OF FAT (25 gm) PLUS PLACEBO ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS
GROUP II-B

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after fat+placebo	Initial	90 minutes after fat+placebo	Initial	90 minutes after fat+placebo
Mean	56.65	58.30	48.65	58.68	53.00	54.00
% Change	-	2.9126	-	20.6166	-	1.8867
SD ±	13.9379	7.6472	13.6959	19.4408	12.0415	19.4935
SE ±	6.2334	3.4200	6.1251	8.6944	5.3852	8.7180
p		NS		NS		NS

P = As compared with initial

NS = Not significant

ADP and epinephrine induced platelet aggregation altered from the mean of 56.65±13.93 to 58.30 ± 7.64 and 48.65 ± 13.6 to 58.68± 19.44 per cent respectively, which was statistically not significant (P = NS). Similarly lipid peroxidation altered from 53 ± 12.04 to 54.00 ± 19.49 which is about 1.88 per cent and was also not significant statistically (Table 7)

TABLE-8
EFFECT OF FAT PLUS EUGENOL (15 mg/kg BW) ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS
GROUP II-B

Case no.	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after fat+eugenol	Initial	90 minutes after fat+eugenol	Initial	90 minutes after fat+eugenol
Mean	68.75	63.90	54.75	52.50	54.00	60.00
% Change	-	7.0545	-	4.1095	-	11.1111
SD ±	8.4731	6.2962	7.9252	6.4346	11.4017	15.8113
SE ±	3.7894	2.8158	3.5443	2.8777	5.0991	7.0712
p		NS		NS		NS

P = As compared with initial

NS = Not significant

Administration of single dose (15 mg/kg) of eugenol fed with 25 gm of fat in 5 healthy individuals. Both concomitantly reduced ADP and epinephrine induced platelet aggregation from a mean of 68.75 ± 8.47 to 63.9 ± 6.29 and 54.75 ±7.92 to 52.5 ± 6.43 per cent respectively. It is evident that ADP induced platelet aggregation was reduced to marked extent about 7.0545 per cent. Same dose of fat plus eugenol altered lipid peroxidation from a mean of 54.00 ± 11.40 to 60.00 ± 15.80 nmol MDA/mg non HDL-C which is around 11.1 per cent and statistically not significant.(Table 8)

TABLE-9
EFFECT OF PLACEBO ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS
GROUP I-C

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after placebo	Initial	90 minutes after placebo	Initial	90 minutes after placebo
Mean	58.95	59.75	55.65	56.75	48.40	47.60
% Change	-	1.3570	-	1.9766	-	1.6528
SD ±	8.5920	11.7614	11.6250	7.5684	8.6775	5.5946
SE ±	3.8425	5.2600	5.1990	3.3847	3.8808	2.5020
p		NS		NS		NS

P = As compared with initial

NS = Not significant

ADP and epinephrine induced platelet aggregation showed 1.35 per cent and 1.97 per cent change which was not significant statistically. On the other hand lipid peroxidation was reduced from 48.40 ± 8.67 to 47.60 ± 5.59 nmol MDA/mg nonHDL-C. However it was not significant. (Table 9)

TABLE-10
EFFECT OF SINGLE DOSE (20 mg/kg) OF EUGENOL ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS
GROUP I-C

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after eugenol	Initial	90 minutes after eugenol	Initial	90 minutes after eugenol
Mean	68.50	59.50	64.50	65.25	55.00	56.00
% Change	-	13.13	-	1.1627	-	1.8181
SD ±	13.8654	13.5092	9.7867	9.3706	10.00	13.8744
SE ±	6.2009	6.0417	4.3768	4.1907	4.4722	6.2050
p		< 0.05		NS		NS

P = As compared with initial

NS = Not significant

Effect of single dose (20 mg/kg BW) of eugenol has been shown in Table 10 on platelet aggregation and lipid peroxidation in healthy individuals. ADP induced platelet aggregation decreased from a mean 68.50 ± 13.86 to 59.50 ± 13.50 per cent which is statistically significant ($p < 0.05$). However, epinephrine induced platelet aggregation remain unaffected. Alteration in lipid peroxidation was statistically not significant.

TABLE-11
EFFECT OF FAT (25 gm) PLUS PLECEBO ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS
GROUP II-C

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after fat+ placebo	Initial	90 minutes after fat+ placebo	Initial	90 minutes after fat+ placebo
Mean	57.55	57.10	58.60	59.25	55.00	54.00
% Change	-	0.7819	-	1.1092	-	1.8181
SD ±	10.2077	3.9314	9.7397	7.6013	14.1421	11.4017
SE ±	4.565	1.7582	4.3558	3.3995	6.3247	5.0991
p		NS		NS		NS

P = As compared with initial

NS = Not significant

ADP and epinephrine induced platelet aggregation varied from 57.55 ± 10.20 to 57.10 ± 3.93 per cent and from 58.6 ± 9.73 to 59.28 ± 7.60 per cent respectively which were not significant. Lipid peroxidation also decreased from a mean of 55±14.14 to 54±11.40 nmol MDA/mg non HDL-C but was also not significant. (Table 11)

TABLE-12
EFFECT OF SINGLE DOSE OF FAT PLUS EUGENOL (20 mg/kg.) ON PLATELET AGGREGATION AND LIPID PEROXIDATION IN HEALTHY INDIVIDUALS
GROUP II-C

	PLATELET AGGREGATION (PERCENT)				Lipid peroxidation susceptibility (nmol MDA/mg of non-HDL-C)	
	ADP		Epinephrine			
	Initial	90 minutes after fat+ eugenol	Initial	90 minutes after fat+ eugenol	Initial	90 minutes after fat+ eugenol
Mean	59.50	53.50	60.25	60.00	58.00	56.00
%change	-	10.0840	-	0.4149	-	3.4482
SD ±	13.5091	12.1642	7.0377	7.0710	8.3666	11.4017
SE ±	6.0416	5.4401	3.1474	3.1623	3.7417	5.0991
p		<0.05		NS		NS

P = As compared with initial

NS = Not significant

After single dose administration of fat (25 gm) plus eugenol (20 mg/kg BW) in 5 healthy individuals lead to reduce ADP induced platelet aggregation up to 10.08 per cent from a mean of 59.5 ± 13.5 to 53.5 ± 12.16 per cent and was statistically significant ($p < 0.05$). However epinephrine induced platelet aggregation remained almost unaffected from a mean of 60.25 ± 7.03 to 60.0 ± 7.07 per cent. Lipid peroxidation also decreased from a mean of 58 ± 8.36 to 56.0 ± 11.4 which is around 3.44 per cent. However the decrease was not statistically significant. (Table 12)

DISCUSSION

The present study was designed to observe the effect of different doses of eugenol on platelet aggregation and lipid peroxidation in healthy individuals.

The study was conducted on 30 healthy individuals divided in two groups. In group I (N = 15), the effect of single dose of eugenol was observed. The group was further divided into three sub-groups of five each and given plain eugenol in 10 mg/kg, 15 mg/kg and 20 mg/kg of body weight respectively. The effect of plain eugenol observed was compared with the effect of placebo given one day prior to eugenol on platelet aggregation and lipoprotein oxidation susceptibility.

Group II (N=15) was similarly subdivided and eugenol was administered in 10, 15 and 20 mg/kg of body weight in single dose. But in this group eugenol was administered with 25 gm of butter and the effect was compared with butter and placebo given one day prior to the fat and eugenol.

In both groups blood samples were collected initially and ninety minutes after ingestion of eugenol or placebo with or without fat and analysed for platelet aggregation and lipid peroxidation.

It has been observed that administration of eugenol in different doses to healthy volunteers variably affect platelet aggregation induced by ADP and epinephrine. The magnitude of responsiveness increases as the dose is increased from 10 mg/kg to 20 mg/kg body weight and at higher dosage (20 mg/kg) it is statistically significant ($P<0.05$). However, lipid peroxidation remains unaltered even in higher doses.

Similarly in group II when fat was administered with eugenol, the response of platelet towards aggregating agents remained same as in plain eugenol group. Here also the response is dose related and statistically significant ($P < 0.05$) at 20 mg/kg body weight dose. Lipoprotein oxidation susceptibility however remains significantly unaltered in all three doses schedules.

In both the study groups, therefore one observation is common, that is eugenol has some potential of lowering platelet aggregation, but the effect is greatly modified by the doses administered. Secondly, as the effect is more or less same in both the groups the absorption of eugenol is practically same and not affected by fat. This observation is similar to those observed in experimental study where eugenol and its metabolites were excreted more than 80 per cent in the urine, after oral ingestion[10]. Except few gastrointestinal side effects such as nausea, epigastric burning and bloating at higher doses, the drug was tolerated well by most of the study subjects.

Eugenol is an important naturally occurring phenolic compound. It has been reported to possess strong antioxidant and platelet aggregation inhibiting property in animal experiments[9].

Eugenol or sodium eugenol acetate inhibited arachidonic acid, collagen, epinephrine and ADP induced platelet aggregation on concentration dependent manner of washed rabbit platelets. The platelet aggregation inhibition effect was resulted by

inhibition of arachidonic acid induced thromboxane B2 and prostaglandin E2 formation[25]. Saed *et al.* Studied eugenol, the active principle of clove oil on human platelet aggregation, arachidonic acid (AA) and platelet- activating factor (PAF) metabolism and in vivo effect on AA and PAF induced shock in rabbits. It was observed that eugenol strongly inhibited PAF induced platelet aggregation with lesser effect against AA and collagen. It inhibited AA metabolism via cyclooxygenase and lipo-oxygenase pathways. Both thronboxane A2 and 12-hydroxy-eicosatetraenoic acid were inhibited by eugenol [26].

Oxidative stress, induced by hydrogen peroxide in islets of Langerhans and its inhibition by eugenol was studied in male mouse. The study revealed that H_2O_2 induced oxidation stress and lipid peroxidation in the isolated islets of Langerhans recovered by administration of eugenol and the recovery was achieved by raising the level of total antioxidant capacity (TAC) and Catalase and reducing malondialdehyde (MDA). It was interesting that low dose administration of eugenol was potent to achieve a therapeutic effect[27]. Antioxidant activity of eugenol was also assessed by *in vitro* and in vivo methods. The study results showed that eugenol inhibited iron and OH radical initiated lipid peroxidation. The inhibitory activity of eugenol was 5 times more than alpha-tocopherol and 10 times less than BHT, the synthetic antioxidant. The mechanism of antioxidant effect was interesting in the sense that eugenol incorporates into mitochondrial membrane and inhibited lipid peroxidation activity as a chain breaking agent[28]. The eugenol abolishes arachidonate, adrenaline and epinephrine induced aggregation *in vitro.* It is mediated by a reduced formation of thromboxane and increased formation of 12-HPETE. This is based on the following experimental evidences: (1) Eugenol inhibits thromboxane B2 formation from added arachidonate to washed platelets, and (2) it inhibits the formation of thromboxane B2 from arachidonic acid labelled platelets after stimulation with calcium ionophore A 23187. The formation of lipoxygenase derived products increased in both the experimental conditions. Some enzymes, the cyclooxygenase of the Arecadonic acid cascade was inhibited by the eugenol[10].

In animal experimental study it was observed that administration of eugenol and acetyl eugenol (100 mg/kg) given orally to rabbits decreases significantly the formation of thromboxane B2 during blood clotting. The effect of eugenol was more pronounced than acetyl eugenol with a biological half life of 72 ± 30 minutes[19]. This probably explains the observation of thepresent work that why the platelets were unresponsive in 10 and 15 mg/kg body weight doses. The eugenol inhibited thromboxane B2 in the dose of 100 mg/kg in animal study while in the present human study the maximum dose administered was only 20 mg/kg and at this dose there is significant inhibition of platelet aggregation induced by ADP but not by epinephrine. Therefore, it might be possible that higher doses are required to block the thromboxane B2 in order to achieve inhibition of platelet aggregation.

Recently, a pharmacodynamic study was performed to determine the level of eugenol in body fluids in human volunteers by HPLC assay. Six volunteers were administered oral dose of 150 mg eugenol once and 24 hours urine, serum and bile samples were collected.

Free eugenol never appeared in measurable concentration in the serum. It was present only as eugenol conjugates with a peak level at 40 minutes. While in the urine less than 0.1 per cent of the drug was excreted as free eugenol. Therefore the study brings a very important conclusion that eugenol undergoes a pronounced first pass effect in the blood. Therefore unconjugated eugenol was not detected after an oral dose of 150 mg and only eugenol conjugate was present. More than 80 per cent of the drug was excreted within 6 hours after oral administration and that too as eugenol conjugates [29].

The work of Fischer and Dengler therefore explains that why the effect of eugenol in the present study is observed at relatively higher doses. Higher dose is probably required to produce inhibition of platelet aggregation as the eugenol in blood is mostly present as conjugates and that conjugates may not have pronounced effect on thromboxane B_2 or aggregation of platelets[28].Same mechanism may explain the failure of lipoprotein oxidation susceptibility to be effected even in higher doses administered in this study.

Antioxidants activity of eugenol has been studied using in-vitro methods where the effect of eugenol on microsomal mixed function oxidase mediated peroxidation using - F^{+3}-ADP-NADPH, carbontetrachloride (CC14) - NADPH and Cumenehydroperoxide (CuMOOH) system was examined. Eugenol clearly inhibited iron or iron chelates induced non-enzymatic peroxidation by scavenging both initiating and propagating radicals. The study shows that eugenol did not inhibit cytochrome P-450 reductase activity but it inhibited P-450-linked mono-oxygenase activities so on the basis of above study eugenol has the potential to be used as therapeutic antioxidant[20].The present study however has failed to demonstrate any observable effect on lipoprotein oxidation susceptibility. This may require a long term administration of eugenol in higher dosage.

CONCLUSION

The present study therefore demonstrates that eugenol, an active ingredient of clove and other essential oils, has some tendency to decrease platelet aggregation in human beings when administered at higher doses (20 mg/kg body weight). Its effect on lipoprotein oxidation susceptibility is not statistically significant. Its use in vivo at present will probably be limited by the quick turnover and relatively short biological half life. However, further studies are needed in high doses to assess its action on platelet aggregation and lipoprotein oxidation susceptibility. Furthermore, pharmacodynamic research is required to keep this molecule active and free (unconjugated) in the blood for a longer time in order to achieve its effective inhibition on platelet aggregation.

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Sildenafil and Human Platelet Aggregation

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ABSTRACT

The effect of the phosphodiesterase-5 inhibitor, sildenafil, on platelet aggregation ex vivo was observed in 30 healthy volunteers. Sildenafil, either 50 or 100 mg, was randomly given to the selected study subjects, and platelet aggregation was observed initially and 2 and 4 hours after sildenafil administration. Adenosine diphosphate (ADP) and collagen were employed as agonists to induce aggregation. Sildenafil, in a single dose of 100 mg, significantly inhibited collagen-induced platelet aggregation at 2 hours ($p<0.05$) and 4 hours ($p<0.001$). Fifty mg sildenafil did inhibit platelet aggregation induced by collagen at 2 and 4 hours after its administration but significantly only at 4 hours ($p<0.05$). ADP induced platelet aggregation, however, it was not significantly inhibited by sildenafil in either dose.

KEYWORDS: Phosphodiesterase, Platelet aggregation, Sildenafil

INTRODUCTION

Sildenafil has been studied and found useful in erectile dysfunction in men with a variety of underlying disorders[1,2] and in women with selective serotonin reuptake inhibitors (SSRIs)-associated sexual dysfunction[3]. Studies in animals and other pharmacodynamic studies have explored its use in esophageal motility dysfunction, [4]lung fibrosis, and pulmonary hypertension[5].

Sildenafil acts by inhibiting cyclic guanosine monophosphate (cGMP), specifically, phosphodiesterase-type-5 (PDE 5) enzyme, which is mainly located in vascular smooth muscle cells and platelets. Physiologically, nitric oxide (NO), released on stimulation, activates the enzyme guanylate cyclase. This, in turn, increases levels of cGMP, which causes smooth muscle relaxation. In the penis, this allows an inflow of blood leading to erection. The cGMP in turn is degraded by PDE 5. Once PDE 5 is inhibited by a phosphodiesterase inhibitor such as sildenafil, the effects of cGMP are enhanced[6,7].

Human platelets have been reported to contain 3 isomers of phosphodiesterases (types I, III, and V).[8] Studies *in vitro* have shown that sildenafil is selective for PDE 5. Its effects are more potent on PDE 5 than on other known phosphodiesterases. It is tenfold more potent than PDE 6. eightyfold more potent than PDE 1, and more than a thousand-fold more potent than PDE 2, 3, and 4. The approximately 4000-fold selectivity for PDE 5 vs. PDE 3 is important because PDE 3 is involved in control of cardiac contractility[9].

The activation of human platelets can be inhibited by 2 intracellular pathways regulated by either cGMP or cyclic adenosive monophosphate (cAMP). However, nitric oxide causes the activation of cGMP-dependent protein kinases, which prevents the agonist-induced activation of

myosinlight chain kinase and protein kinase-C and inhibits the agonist-induced calcium mobilization from intracellular stores without any major effect on the ADP-regulated cation channel. Additionally, cGMP causes an increase of cAMP by inhibition of cAMP phosphodiesterases. Increased cGMP 10 levels inhibit agonist-induced platelet aggregation[10]. Dipyridamole, an ADP uptake and PDE 5 inhibitor, has been extensively used as an antithrombotic agent in clinical application[11]. Moreover, there are reports that PDE 5 inhibitors inhibit platelet aggregation in animal models and that sildenafil exerts antithrombotic effects in combination with a nitric oxide donor in a rat model[12-14]. These findings indicate that inhibition of PDE 5 may influence platelet aggregation. There are few reports that show in-vitro data of human platelet modulation by the PDE 5 inhibitor sildenafil[15-16]. The present study has, therefore, been envisaged to investigate further whether sildenafil in different doses alters human platelet aggregation induced by ADP and collagen.

Methods

The present study was approved by the local ethics committee and conducted on 30 healthy male volunteers between the ages of 30 and 50 years. Underlying diseases like diabetes, hypertension, ischemic heart disease (IHD), hyperlipidemia, kidney diseases, and liver diseases were excluded by relevant investigations. The volunteers were not consuming tobacco in any form and had not taken any type of drug in the previous 15 days. After informed consent, the study subjects were randomly divided into 2 groups of 15 each. Group I (n=15) was administered sildenafil 50 mg and Group II (n=15) was administered sildenafil 100 mg in a single oral dose.

Platelet Aggregation

After an overnight fast 4.5 mL of venous blood was collected without undue pressure and a single dose of sildenafil (50 or 100 mg) was administered. Subsequent blood samples were collected 2 and 4 hours after drug administration. All blood samples collected were mixed to 3.8% of 0.5 ml. of sodium citrate in a 9:1 blood-to-anticoagulant ratio in plastic tubes and subjected to the estimation of platelet aggregation. Platelet-rich plasma (PRP) was prepared by centrifugation of anticoagulant sample at 250 × g for 10 minutes at room temperature. Aliquots of PRP (450 µL) were placed in disposable polystyrene cuvettes.

Platelet-poor plasma (PPP) was obtained by re-centrification of the original blood sample at 1,500 × g for 10 minutes. Platelet aggregation was measured turbidimetrically on ELVI-840 aggregometer and Omniscribe chart recorder. The measurement of aggregation was performed exactly after 30 minutes of sample collection to avoid differences of the aggregation due to altered status of the platelet resulting from ex-vivo conditions. After 10 minutes of equilibrium at 37°C and constant stirring at 1,000 rpm, the aggregation of PRP was induced by ADP (6 µmol/L) or collagen (0.2 µg/mL) (Sigma), the response was recorded for 5 minutes, and the results were expressed as percentage aggregation[17].

Statistical Analysis

All data are expressed as mean $\pm$ SE. The results were analyzed with Student's t test for paired data. A p value of <0.05 was considered statistically significant.

Results

Platelet aggregation was always measured exactly 30 minutes after blood sampling to avoid differences of the aggregation due to altered status of the platelets resulting from ex-vivo conditions. ADP (2 x 10-4mol/L)-induced platelet aggregation in both dose schedules (50 and 100 mg) was not significantly inhibited at 2 and 4 hours after sildenafil administration (Tables 1, II Figure 1).

Table 1: Effect of 50 mg Sildenafil on Platelet Aggregation in Healthy Individuals (n = 15)

	Initial	Platelet Aggregation (%) 2 Hours	4 Hours
ADP	59.73 $\pm$ 3.66	61.16 $\pm$ 2.50	55.390 $\pm$2.34
p Value		NS	NS
Collagen	54.02 $\pm$ 4.58	50.20 $\pm$ 3.94	43.75 $\pm$ 4.34
p Value		NS	< 0.05

p Value as compared to initial, NS = Not significant

Table 2: Effect of 100 mg Sildenafil on Platelet Aggregation in Healthy Individuals (n = 15)

	Initial	Platelet Aggregation (%) 2 Hours	4 Hours
ADP p Value	56.67 + 2.58	56.25 +2.27 NS	55.03+2.84 NS
Collagen p Value	52.92 + 4.05	42.17 + 4.90 < 0.05	29.67 + 5.09 < 0.001

p Value as compared to initial, NS = Not significant

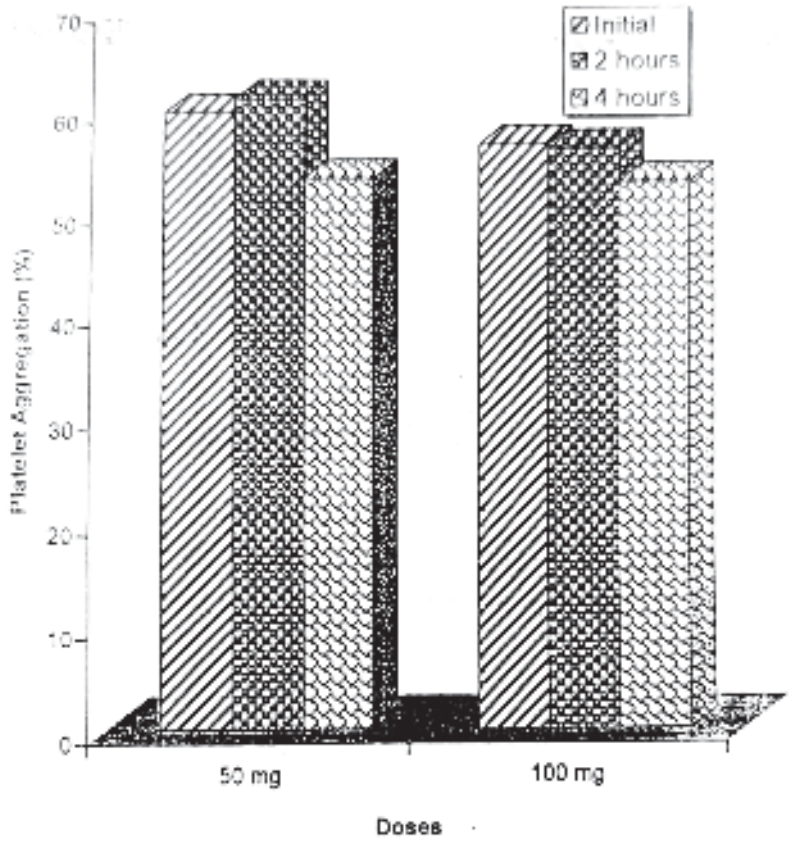
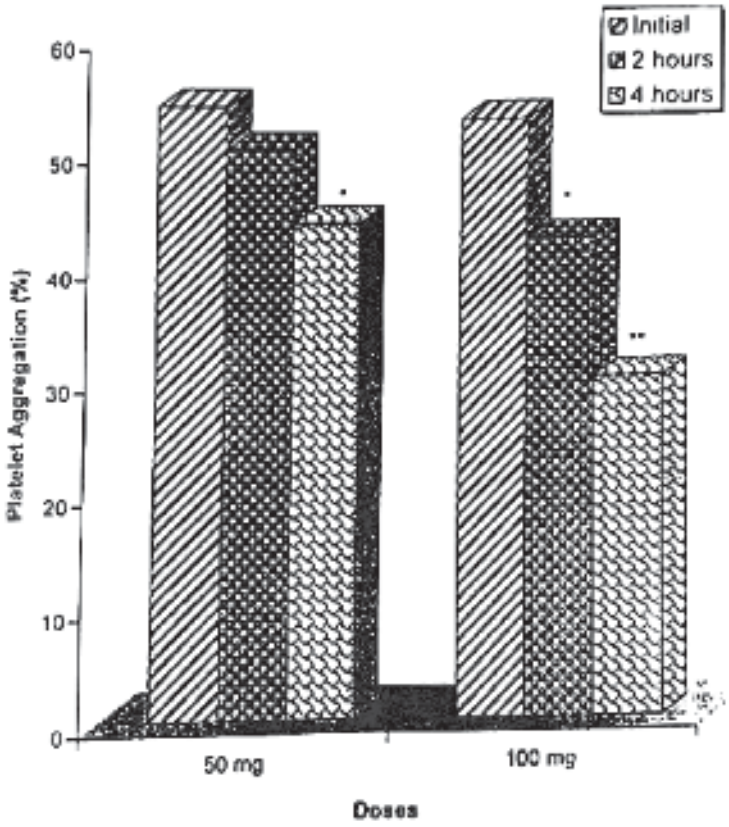


Figure 1: No Significant inhibition of ADP-induced platelet aggregation by Sildenafil

Sildenafil, 100 mg, significantly decreased collagen (0.2 µg/mL)-induced platelet aggregation after 2 ($p<0.05$) and 4 hours ($p<0.001$) (Table II, Figure 2). This effect was, however, overcome when larger concentrations of collagen (>0.2 µg/mL) were used to induce the aggregation (data not shown). This is probably due to the almost maximal aggregation (>70%) achieved with the larger concentration. When 50 mg sildenafil was administered, the collagen-induced platelet aggregation was inhibited at 2 and 4 hours but was statistically significant only at 4 hours (Table 1, Figure 2).



*** p< 0.05**
**** p< 0.001**

Figure 2: Significant inhibition of collagen-induced platelet aggregation by Sildenafil

Side Effects

Sildenafil was well tolerated. Incidences of side effects such as flushing, heaviness of head, stuffiness of nose, and blue vision were higher in those who received 100 mg of sildenafil. The side effects declined, however, after 2 to 3 hours.

DISCUSSION

Time and experience have established the role of sildenafil in the treatment of erectile dysfunction of varied etiologies[2,7]. It is a selective inhibitor of cyclic guanosine monophosphate (cGMP), specifically the phosphodiesterase 5 (PDE 5) enzyme, which is mainly located in vascular smooth muscle cells and platelets. As the cyclic nucleotides are important inhibitors of platelet activation 10 and aggregation, there were investigated in the present study to determine whether medication with sildenafil in healthy volunteers alters platelet aggregation *ex vivo*.

In the present study inhibition of collagen-induced platelet aggregation by sildenafil was clearly evident. The response was dose dependent. Administration of 50 mg sildenafil significantly ($p<0.05$) inhibited collagen-induced platelet aggregation at 4 hours but not at 2 hours. On increasing the dose to 100 mg the platelet aggregation inhibitory response was also significant at 2 hours ($p<0.05$) and was highly significant ($p<0.001$) at 4 hours. Moreover, in 3 volunteers there was complete inhibition of collagen-induced platelet aggregation. As the changes in aggregation are best seen during use of submaximal aggregation stimuli, we used collagen in a concentration of 0.2 µg/ml. to achieve platelet aggregation around 50-55% and not higher concentrations, which can lead to maximal aggregation (>70%), and the effect may be overcome. ADP-induced platelet aggregation, on the other hand, was not significantly inhibited by either dose.

Human platelets have been reported to contain 3 isomers of phosphodiesterase (types I, III, and V)[8]. The activation of human platelets can be inhibited by 2 intracellular pathways

regulated by either cGMP or CAMP. However, nitric oxide causes the activation of cGMP-dependent protein kinases. Additionally, cGMP causes an increase in cAMP by inhibition of cAMP phosphodiesterases[18]. Increased cGMP levels inhibit agonist-induced platelet aggregation.

There are also reports that PDE 5 inhibitor inhibited platelet aggregation and adhesion in animal models[14,15] and that sildenafil exerted antithrombotic effect in combination with a nitric oxide donor in a rat model[12]. There is 1 report that shows in vitro data on human platelets, which were incubated with sildenafil or sodium nitroprusside, or both[15].

ADP and collagen act on platelets via different receptors[19,20], although the underlying signal transduction is not fully understood. However, it is difficult to relate the inhibitory effect of sildenafil on collagen-induced aggregation to a specific pathway in the signal cascade, Unlike ADP, it is possible that only collagen triggers an endogenous nitric oxide release of platelets during activation or aggregation[21,22].

The nitric oxide/cGMP metabolism in platelets is probably activated during aggregation to prevent exceeding aggregation and to act as a negative feedback mechanism. Until now only collagen could be shown to activate this mechanism of generating cGMP, which is degraded by phosphodiesterase type 5[21,22]. This might explain why *in vitro*, without a nitric oxide source such as endothelium, an ADP-induced aggregation is not inhibited by pre-treatment with sildenafil, in contrast to a collagen-induced aggregation.

The above-cited hypothesis is well substantiated by a few of the studies, in which addition of nitric oxide donor before aggregation significantly inhibited ADP and collagen-induced aggregation after administration of the PDE 5 inhibitor sildenafil[16] or zaprinast[23] in doses that otherwise do not inhibit aggregation.

The present observation is important in view of sildenafil administration to subjects receiving other antiplatelet or anticoagulant medications. Sildenafil might further enhance the anti aggregating response and cause bleeding. A recent study[16] has already reported that it enhances bleeding time in volunteers who received sildenafil. However, there are no published data on whether the bleeding time of patients treated with anticoagulants (coumarin) or anti-platelets (aspirin/clopidogrel) is influenced by sildenafil.

Moreover, the positive approach of the present observation is that the drug may prove to be beneficial in those who have incipient endothelial dysfunction, which is now a common denominator in erectile dysfunction, and in the patient with cardiovascular disease. New frontiers about the uses of sildenafil now center around its capability of enhancement of nitric oxide, a signaling molecule that controls a diverse range of physiological processes in many tissues and plays a role in the immune system, nervous system, and in inflammation[24]. The present observation is a further addition to its therapeutic potential.

CONCLUSION

The present study indicates that sildenafil inhibits collagen-induced platelet aggregation *ex vivo*. The effect is greater with a 100 mg dose than with a 50 mg dose of sildenafil; however, ADP-induced platelet aggregation was unaffected by both dose schedules. The exact mechanism is not very clear. However, it is possible that collagen triggers an endogenous nitric oxide release of platelets during activation or aggregation and sildenafil enhances the effect of nitric oxide, a signaling molecule.

CONFLICT OF INTEREST: None

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Research Paper

Assessment of Aspirin Responsiveness by Light Transmittance Aggregometry in Patients with Ischemic Heart Disease – A Study from Southern Rajasthan

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ABSTRACT

The present study was conducted to evaluate the prevalence of aspirin resistance in patients with IHD living in and around Udaipur. Fifty patients of IHD (Group I) either of healed myocardial infarction (>6 months) or angina in whom ischemia can be induced by TMT, and were taking 150 mg of aspirin daily for more than 3 months were selected. Fifty healthy individuals (Group II) were selected as control to know the baseline platelet aggregation in the same age group. Platelet aggregation was assessed employing platelet rich plasma (PRP) on optical aggregometer- ELVI 840.

The study has brought that ten percent of the study population demonstrated aspirin resistance. The incidence of true resistance, that is unresponsive to both, ADP and collagen aggregating agents is 2%, while "Semi responders" constitute 8% of the study population. These patients demonstrated resistance to either of the two aggregating agents. The incidence of clinical resistance in term of recurrence of cardiac event (MI) was observed in 4% of population in whom the true resistance was observed in 2% while 2% were semi responders. Six percent of the study subjects who demonstrated aspirin resistance were semi-responders and all have inducible ischemia and did not develop infarction. Ten percent of the study subjects had recurrent episode of MI in spite of aspirin consumption. In these subjects the true aspirin resistance was observed in 20% of the subjects, twenty percent were semi-responsive, while in 60% aspirin was found to be effective in inhibiting platelet aggregation.

KEYWORDS: Aspirin treatment failure, Aspirin non-responsiveness, PFA-100 device, COX-1inhibition

INTRODUCTION

Cardiovascular diseases account for approximately 12 million deaths annually and are the commonest cause of death globally. Previously considered to be disease of affluent; now it is increasing in developing world too in epidemic proportion. The Asian Indians living in their own country or elsewhere have much higher incidence of coronary artery disease as compared to all other ethnic group. In Indian subcontinent, from 1960's to 1990's the coronary artery disease prevalence increased two folds (from 2% to 4%) in rural and three folds (3.45% to 9.45%) in Urban Indian population[1]. Thus burden of cardiovascular disease in patients and community is enormous.

One way of reducing the burden is to reduce the platelet aggregation in people predisposed to such high risk. Cardiovascular diseases are the result of multiple complex cascade of interaction among the endogenous cells of the arterial wall, the focal haemodynamic environment, blood

components notably monocytes, lipoproteins, inflammatory processes and their mediators and various healing or reparative processes. The role of platelets in thrombosis is central for atherothrombosis.

Platelets are small (2-3 mm in diameter) non nucleated cells containing granules with constituents (e.g. 5-Hydroxytryptamine, catecholamines, and ADP) capable of influencing platelet function. They normally circulate in blood for approximately 10 days before being sequestered in the spleen. Their main function is in primary hemostasis; interacting with injured areas in the vessel wall and form a haemostatic plug that later organizes and incorporates other blood cells and components of the coagulation system[2,3].

Quiescent platelets are discoid in shape and do not adhere to intact endothelium, but a breach of the arterial lining will expose collagen in the deeper tissue layers and this substance activates platelet adhesion, when activated they become irregular and, form pseudopodia. The exposure of sub-endothelial collagen and the release of von Willebrand factor results in binding of these substances to glycoproteins IA/ IIA and IB of the platelet surface[4,5].

For platelets to interact with the endothelium, the platelet glycoproteins IIB-IIIA must first undergo a conformational change. In addition to shape change and adhesion, activated platelets release from their "dense bodies" various agonists, including serotonin and ADP, which intensify aggregation, as does thromboxane A_2 (TXA_2), besides being vasoconstrictive. TXA_2 is derived from arachidonic acid, a substance present in the phospholipids of the platelet membrane. The other inclusion system of platelets, the "alpha granules", contain P-TG (β-thromboglobulin), PF-4 (Platelet factor four), PDGF (Platelet Derived Growth Factor) and t PAI-2 (tissue plasminogen Activator inhibitor); these are also released when thrombocytes are activated and further intensify platelet aggregation.

In opposition to these aggregation promoting factors, the vascular endothelial cells synthesize prostacyclin (PGI_2) which, by stimulating CAMP, inhibit platelet aggregation and release. If endothelial cells are injured, several factors are released e.g. 11 platelet activating factor (PAF), a potent stimulus for platelet aggregation. The endothelial cells also synthesize and release "endothelium derived relaxing factors" (EDRF), one of these is nitric oxide (NO), which like $PG_{1\alpha}$ inhibits platelet aggregation and stimulates vasodilation[5]. The action is mediated by an increase of platelet cAMP and cGMP.

Platelets have been implicated as being pathophysiologically important in hypertension and ischemic heart diseases. They might contribute to coronary artery disease in at least two ways; one by thrombus formation caused by platelet activation in the presence of vascular damage and secondly as a source of mitogenic influence (platelet derived growth factor)[6,7].

In the treatment of CAD aspirin has remained cornerstone of therapy in both primary and secondary prevention of death due to CAD owing to its antiplatelet functions[8, 9]. Aspirin is being used for last 2 centuries. "Willow bark" contains salicin from which salicylic acid is derived. It was used for fever in 18th century as cheap substitute for imported cinchona bark. Its antiplatelet action has recently been recognized[10]. Today nearly all patients of CAD and peripheral vascular disease are receiving this drug.

Aspirin is a cyclo-oxygenase inhibitor thus irreversibly blocks the formation of thromboxane A_2, a potent mediator of platelet aggregation which converts arachidonic acid to prostaglandin G_2. Platelets does not contain nucleus, so once inhibited it can't form cyclo-oxygenase. The enzyme inhibition is permanent and irreversible. Other effects of cyclo-oxygenase inhibition are the block of production of prostacyclin. $PG_{1\alpha}$ that opposes platelet activation. Aspirin inhibits the release of ADP, cationic proteins, PGE_2. $PGE_{2\alpha}$ and phospholipase from the granules of platelets. The use of aspirin as anti thrombotic drug therapy started in 1983 with publication of the Veterans administration cooperative study. This study decreased 1 year event rate by 5% as compared with placebo (10.1%)[11].

A larger number of trials including antiplatelet trialists' collaboration meta analysis, the largest trial ever conducted on aspirin efficacy has proved that aspirin in effective in reducing deaths from myocardial infarction from 25%-68%[12]. However, the antiplatelet effect of aspirin has not been observed to be uniform on all human population and relative risk of recurrent vascular events in patients receiving aspirin therapy remains high (8-18% after 2 years)[13]. Aspirin resistance has been reported to occur in 5% to 45% of general population and its detection is of clinical importance.

The initial evidence that some patients may be resistant to aspirin came from study by Mehta and associates who reported that 30% of patients had minimal inhibition of platelet aggregation after single 150 mg of dose of aspirin[14]. The significant studies by Grundmann and co-workers in ischemic stroke patients with high dose of aspirin proved aspirin resistance to be 34%[15]. Gum and associates conducted studies on 326 patients of IHD and reported a 5% incidence of aspirin resistance by optical platelet aggregation[16]. In a subgroup study of the Heart Outcomes Prevention Evaluation (HOPE) trial sample, Eikelboom and colleagues observed increased adverse events in individuals exhibiting aspirin resistance during a 5-year follow-up. As a measure of *in vivo* thromboxane production, the urinary concentration of 11-dehydrothromboxane B2 was determined. For every quartile that 11-dehydrothromboxane B2 levels increased, the adjusted chances for the composite end-point of myocardial infarction (MI), stroke, or vascular death also increased[17].

Despite consistency of such observation, the prevalence of aspirin resistance has been variable in different populations and there is lack of standardized diagnostic criteria on a single validated method of identifying affected individuals to have aspirin resistance. It has led to wide range of population estimates[18]. Prospective studies have demonstrated that the decrease responsiveness to aspirin therapy is associated with an increased risk of clinical events[19,20].

Aspirin resistance has been observed to affect patients of various categories and healthy controls without vascular disease as well[15,21,22]. Provided more than 12 million deaths caused by CAD annually, even a 5-10% prevalence of aspirin resistance affects more than a million patients[16]. Therefore, it is pertinent to take into account the aspirin resistance while

treating patients with ischemic cardiovascular diseases.

The present study, therefore, has been envisaged to study aspirin responsiveness in patients with ischemic heart disease consuming aspirin 150 mg and living in and around Udaipur.

MATERIAL AND METHOD

The study was conducted on male patients of IHD who were stable in their symptoms, attending OPD or admitted in the wards of Maharana Bhupal Hospital attached to R.N.T. Medical College, Udaipur. After informed consent a total number of 50 subjects were selected for the study in each group:

The study groups included in study are as follows:

Group I : 50 patients of ischemic heart disease (IHD) who are stable in their symptoms and taking 150 mg of aspirin daily from last 3 or more months.

Group II: 50 healthy individuals without any evidence of ischemic heart disease.

Patients with ischemic heart disease (IHD) were selected based on the following investigational criteria:

1. ECG:
   (a) Documentation of old healed myocardial infarction.
   (b) ST depression of ⩾2mm in consecutive leads with or without symptoms.
2. ECHO: Regional wall motion abnormalities (RWMA)
3. Positive TMT:
   (a) Horizontal or down sloping ST segment depression of >1 mm from previous level during TMT with or without symptoms.
   (b) Junctional depression with slowly rising ST slope that remains depressed 1.5 mm or more than 0.80 m seconds after the J point.
   (c) Slowly up sloping ST segment depression with the ST segment being depressed in excess of 2.5 mm, 80 m seconds after the J point.
   (d) Down sloping or flat, ST segment depression in excess of 2.5 mm.
   (e) Horizontal or Down sloping ST segment depression appearing during the first stage of exercise and/or persisting beyond 8 minutes in the recovery phase.
   (f) Complex ventricular, ectopic activity, including multiform ventricular ectopic beats, or runs of ventricular tachycardia or occurrence of ventricular fibrillation.

Exclusion Criteria:

The following subjects were excluded from the study-

1. Those were taking ticlopidine, dipyridamole, clopidogrel, heparin, LMWH (low molecular weight heparins) and corticosteroids and other non steroidal anti-inflammatory drugs.
2. Haemoglobin 8 gm/dL
3. History of myelo - proliferative syndrome & malignant paraproteinemias.
4. Family or personal history of bleeding disorders.
5. Patients with diabetes, hypertension.
6. Patients with peripheral vascular diseases.

Method:

Venous blood samples (9 ml) was collected in the morning in a fasting state without undue pressure of stable cardiac patients of age more than 40 yrs after brief history, physical examination and written consent. Specimens were kept at room temperature and subjected within 1 hour for estimation of platelet aggregation on ELVI-840 aggregometer and Omniscribe chart recorder.

PLATELET AGGREGATION[23]

Most important function of platelets is their role in haemostasis i.e. adhesion to the damaged tissue surfaces and cohesion to one another. This cohesion phenomenon is known as aggregation and may be initiated by a variety of substances including collagen, adenosine 5-diphosphate (ADP), epinephrine, arachidonic acid, serotonin and ristocetin. Aggregation is one of the numerous in vitro tests performed as a measure of platelet function. The described procedure is turbidimetric method of measuring the effect of collagen, ADP and epinephrine on platelets, better termed as light transmittance aggregometry (LTA).

Reagents:

1. 3.8 per cent citric acid (Trisodium salt dehydrate): Prepared by dissolving 3.8 gm citric acid in 100 ml of deionized water.
2. Tris buffer: Tris (hydroxy methyl), methylamine, 1.21 gm. (0.01M), disodium ethylene diamine tetra acetic acid 0.372 gm (0.001 M), sodium chloride 8.76 gm. (0.15 M), dissolved in distilled water adjusted to pH 7.5 with hydrochloric acid and made up to one litre with distilled water.
3. ADP reagent: Adensoine 5-diphosphate lyophilized with buffer salts (supplied by sigma diagnostics). ADP solution was prepared by reconstituting ADP reagent with 1.0 ml deionized water to yield solution containing ADP 2x1 04mol/lit. It was swirled to mix and allowed to stand at room temperature (18-26°) for 15 minutes before use. It should be kept at room temperature only for duration of testing. The reconstituted reagent is stable for one month if stored in refrigerator (2° to 6°C).
4. Collagen Reagent: Collagen (calf skin) acid soluble, approximately 2 mg lyophilized with buffer salts, Collagen solution was prepared by reconstituting a vial of collagen reagent with 1.0 ml deionized water. The, vial was allowed to stand undisturbed for at least 15 minutes at room temperature before use. Warming to 37°C may be necessary for complete dissolution. It was swirled to mix prior to each assay. It should not be vertexed. The solution should be kept at room temperature only for the duration of testing. It is usually stable for at least 2 weeks refrigerated (2° to 6° C). Stability may be extended by freezing.

INSTRUMENTS AND MATERIALS REQUIRED

(1) Instruments:

1. Platelet aggregometer (ELVI 840).
2. Chart recorder (Omniscribe recorder dual pen type L176 2USA)

(2) Materials:

1. Cuvette 250 µl
2. Teflon coated magnetic stirring bars (micro agitators)
3. Pipettes with disposable plastic tips 50 µl and 250 µl.
4. Centrifuge machine.
5. Plastic tubes with caps.
6. Plastic transfer pipettes.

A. Specimen collection:

Blood was collected by avoiding stasis and contamination with tissue fluids into plastic tubes containing 0.1 ml buffer and 3.8 per cent sodium citrate in a ratio of blood to anticoagulant in a ratio of 9: 1.

B. Preparation of platelet rich plasma (PRP):

(i) The anticoagulant sample was centrifuged at 400 rpm, for 10 minutes.

(ii) PRP was removed carefully using a plastic transfer pipette.

(iii) PRP was expelled into a plastic tube and covered and kept at room temperature for duration of the test.

C. Preparation of platelet poor plasma (PPP):

(i) It was prepared by re-centrifuging the PRP at 6000 rpm for 10 minutes.

(ii)Supernatant was transferred to a labelled PPP tube, covered and kept at room temperature for the duration of test.

The platelet count of PRP was adjusted to the range of 4 to 5 lac/cu mm when necessary by addition of autologous PPP to PRP sample. The caution should be taken to assay platelet aggregation within 30 minutes of collection of test samples.

Aggregating agents - ADP and Collagen

ADP - ADP induced aggregation may occur in one or two phases and it may be followed by rapid disaggregation which may be seen in normal man without any hemorrhagic disease.

Collagen - Collagen induced platelet aggregation may occur in an - irreversible single phase curve or a reversible single phase curve depending on the collagen concentration in the PRP.

Procedure:

After preparing PRP and PPP the aggregation was recorded as follows:

1. Cuvette with PRP was introduced into the aggregometer.
2. The electromagnetic agitation was started by means of stiffed control after having introduced a small stiffing bar into the sample.
3. Agitation speed was maintained at 1000 rpm.
4. Baseline of the recorder was adjusted by means of the zero control.
5. The cuvette with PRP was removed and cuvette with PPP was inserted.
6. By means of gain control the maximum excursion of the pen on the recorder was adjusted.
7. Cuvette with PPP was removed and the cuvette with PRP was reinserted and it was readjusted if necessary by means of the zero control.
8. The sliding of the recorder paper was started.
9. The aggregating agents (ADP and Collagen) were added to the PRP by means of micropipette (50 µl).

The aggregation was recorded for a minimum of five minutes and results were expressed as percentage aggregation.

$$\text{Percentage Aggregation} = \frac{90 - CR}{90-10} X100$$

$$= \frac{90 - CR}{80} X100$$

CR is chart reading in terms of number of segments.

Aspirin resistance - Platelet aggregation induced by ADP and Collagen in patient receiving 150 mg of aspirin, more than 60% will be taken as aspirin resistance.

Table 1 shows the profile of 50 male patients of ischemic heart disease, selected for the study. There were 32 patients who had sustained myocardial infarction in the past and were stable in their symptoms. Their mean age was 62.53 years and they were regularly taking 150mg of aspirin daily from last 2 years or more. Eighteen patients were of ischemic heart disease, proved on TMT. Their mean age was 57.7 years and mean duration of aspirin consumption was 4 years and 4 months.

Table 1: Profile of Study Subjects (IHD)

	No. of Patients	Mean age (years)	Mean duration of Aspirin treatment
Old MI	32	62.53	2 years 10 months
IHD – TMT positive	18	57.72	4 years 4 months

MI – Myocardial Infarction, IHD – Ischemic Heart Disease, TMT – Tread Mill Test

Table 2: Platelet Aggregation in Healthy Individuals

	Platelet Aggregation (Percent)	
	ADP	**COLL**
Mean	57.00	52.87
SD ±	9.36	9.88
SE ±	1.32	1.40

Platelet aggregation profile of 50 healthy individuals (Group II), induced by ADP and Collagen have demonstrated that their mean ADP induced platelet aggregation was 57± 9.36 percent, while collagen induced platelet aggregation was 52.87±9.88 percent (Table 2).

Table 3: Platelet Aggregation in Patient with IHD taking Aspirin (150mg)

	Age	**Platelet Aggregation (Percent)**		**Duration of Aspirin Consumption (Months)**
		ADP	**COLL**	
Mean	60.80	32.23	27.43	40.22
SD ±	8.24	16.22	18.88	40.30
SE ±	1.17	2.29	2.67	5.70

Table 3 depicts the platelet aggregation of 50 patients of ischemic heart disease who were taking aspirin (150 mg) daily for more than 3 months. The age varies from 44 to 78 years. The mean duration of aspirin consumption was 40.22±40.30 months. The mean platelet aggregation was 32.23±16.22 percent and 27.43±18.88 percent induced by ADP and Collagen respectively. There are three patients each in ADP and Collagen subsets who demonstrate aggregation of more than 60 percent.

Table 4: Inhibition of Platelet Aggregation by Aspirin (150mg) in Patient with IHD

	Age	**Platelet Aggregation (Percent)**		**Duration of Aspirin Consumption (Months)**
		ADP	**COLL**	
Mean	60.80	66.78	72.58	40.22
SD ±	8.24	17.64	18.88	40.30
SE ±	1.17	2.49	2.67	5.70

The percentage inhibition of platelet aggregation by 150 mg of Aspirin in patients with Ischemic Heart Disease has been shown in table 4. On further analysis of the results, it was observed that except 3 patients (cases 2, 10 & 44) all have demonstrated more than 60 percent inhibition of platelet aggregation induced by ADP. Likewise, 3 patients (cases 18, 26 & 44) also demonstrated platelet aggregation less than 40 percent with Collagen.

Table 5: Profile of Patients Demonstrating Aspirin Resistance

S. No.	ADP ($2x10^{-4}$mol/L)			
	Age	**Diagnosis**	**Duration of Aspirin Treatment**	**Platelet Aggregation (Percent)**
1	55	TMT moderately positive	8 months	65.00
2	68	TMT strongly positive	10 years	68.75
3	58	Recurrent MI 1. Inferior Wall MI 2. Anterior Wall MI	8 years 6 months	75.00

TMT – Tread Mill Test MI – Myocardial Infarction

The profile of three patients who demonstrated aspirin resistance induced by ADP has been given in table 5.Their age was ranging from 55 to 68 years. Two patients were of IHD proved on exercise Test, while one patient was of recurrent myocardial infarction who sustained inferior and anterior myocardial infarction in spite of regular aspirin consumption for last 8 and half year. The aspirin, in this patient, was able to inhibit platelet aggregation only to the extent of 25 percent.

Table 6: Profile of Patients Demonstrating Aspirin Resistance

S. No.	Collagen (0.2µg/ml)			
	Age	**Diagnosis**	**Duration of Aspirin Treatment**	**Platelet Aggregation (Percent)**
1	64	Recurrent MI 1. Anterior Wall MI 2. Inferior MI	12 years	68.75
2	60	TMT Positive	5 years	62.50
3	58	Recurrent MI 1. Inferior Wall MI 2. Anterior Wall MI	8 years 6 months	68.75

TMT – Tread Mill Test MI – Myocardial Infarction

On analysis of profile of patients who manifested aspirin resistance based on Collagen aggregant, it was found that one patient of recurrent myocardial infarction is common in table 5 and 6. Who demonstrates true resistance i.e. both ADP and Collagen induced aggregation more than 60 percent and sustained second myocardial infarction. In remaining two patients one was of recurrent myocardial infarction and other was of inducible myocardial ischemia. In both the patients of recurrent infarction, the duration of aspirin administration was more than 8 years and both were demonstrating platelet aggregation more than 68 percent (Table 6).

Table-7: Profile of Patients with Recurrent Myocardial Infarction

Case No.	Age	Name	Duration of Aspirin Treatment (months)
5	60	Nathulal	60
16	66	Mohan Singh	30
18	64	Kachrulal	144
24	72	Lalu Ram	50
44	58	Dalpat	102

The profile of patients who had recurrent myocardial infarction inspite of aspirin therapy showed that out of 5 patients, one (case number 44) showed true aspirin resistance in whom both ADP and collagen caused more than 60% platelet aggregation. Case number 18, on the other hand, was aspirin semi-responder. Where ADP induced platelet aggregation was 33.75% but collagen induced platelet aggregation was 68.75%. Rest of three cases were aspirin responsive (Table 7).

DISCUSSION

The present study was conducted to observe the prevalence of aspirin resistance among patients of Ischemic Heart Disease (IHD) who are residing in and around Udaipur and taking 150 mg of aspirin for more than 3 months regularly.

Fifty male patients of ischemic heart disease were selected for the study and 50 healthy volunteers were also taken as control for establishment of normal platelet aggregation. All the study subjects were kept overnight fasting and venous blood samples were collected in the morning for platelet aggregation. All the blood samples were subjected for estimation of platelet aggregation using ELVI 840 aggregometer and Omniscribble chart recorder (LTA).

Platelet aggregation measurement in healthy individuals (Table 2) shows that mean aggregation induced by ADP and collagen are 57.00±9.36 and 52.87±9.88 percent respectively. Based on these limits 60% was taken as cut off point. Platelet aggregation more than 60% was taken as aspirin resistance. If both ADP and Collagen induced platelet aggregation is more than 60% than patients were labelled to have "true resistance", while, if one aggregant showed aggregation more than 60% and other less than that they were labelled as "semi responders".

Profile of 50 patient of IHD selected for the study showed that 32 patients were of healed MI and 18 patients were of Angina. All the patients were stable in their symptoms and were taking Aspirin 150 mg daily for than 2 years and 4 years respectively (Table 1).

The mean platelet aggregation induced by ADP and collagen were 32.23±16.22 and 27.43±18.80 percent which reflected good aspirin response. However, on careful analysis of observations (Table 3) 3 patients[2,10,44] showed aspirin resistance (aggregation > 60%) in ADP Induced aggregation and 3 patients[18,26,44] in collagen induced aggregation. In all these patients the percentage of inhibition of platelet aggregation by 150 mg of Aspirin was less than 40% (Table 4).

On further analysis of patients demonstrating aspirin resistance (Table 5, 6); one patient who demonstrated ineffectiveness of aspirin in inhibiting platelet aggregation by both the aggregants had true resistance. He also had recurrent MI in spite of aspirin consumption for last 8.5 years. The other 4 patients were semi-responders who demonstrated failure of aspirin activity with either of aggregants.

The present study therefore brings the fact that aspirin resistance in this area is around 10% out of which 2% is the true resistance and 8% showed semi-responsiveness. Moreover aspirin resistance and duration of aspirin consumption have proportionate relation as the majority of patients demonstrating aspirin resistance were consuming aspirin for more than five years (Figures 1and 2).

The overall prevalence of aspirin resistance in different studies varies from 8% to 45%[12,24,25]. However the dose of aspirin resistance varies in different studies from 75 mg to 325 mg /day as well as methodology used to define aspirin resistance.

Aspirin is the cornerstone of antiplatelet therapy in cardiovascular medicine. Its role in the secondary prevention of vascular events has been proven beyond any doubt. A recently published meta-analysis of 287 randomized trials of antiplatelet therapy by the Antithrombotic Trialists Collaboration has shown a significant reduction in the combined end-point of any serious vascular event in a cohort of high-risk patients with atherothrombotic diseases[26]. However, a substantial proportion of patients manifest "breakthrough" events despite regular intake of aspirin. It is estimated that one in eight high-risk patients suffers from the recurrence of a vascular event within the next 2 years despite regular daily aspirin therapy[27]. Also, by using different methods of measuring platelet activity, several studies have demonstrated marked individual variations in the response to treatment with aspirin[16,24,28,29]. Based on the clinical and laboratory evidence of reduced or absent response to treatment with aspirin in some patients, the concept of "aspirin resistance" has emerged, and has caught the attention of both professionals and the mass media[30].

Unfortunately, aspirin resistance remains a poorly defined term. There are conflicting reports on the incidence and clinical relevance of this phenomenon as this term is being used to describe a number of different phenomena. These include the inability of aspirin to either protect individuals from thrombotic complications; or failure to cause prolongation of bleeding time, or inhibit platelet aggregation ex vivo. or inhibit platelet thromboxane formation[31,32].

Perhaps a clinical definition of aspirin resistance as the failure of the drug to prevent an ischemic event despite regular intake of appropriate doses is the most relevant for practising physicians[31]. It is well known that platelet inhibition is not a uniform process, and considerable inter- and intra-individual variations exist in the antiplatelet effect of aspirin. This mandates functional and biochemical in vitro tests to individualize treatment, and possibly identify the subgroup of patients at risk for future vascular events.

Traditionally, platelet function has been assessed by measuring platelet aggregation in platelet-rich plasma using an optical aggregometer. This test is widely available, and has been used in many investigational studies. Based on this method, 5% and 24% of patients with stable cardiovascular disease on aspirin therapy (325mg/day for at least a week) were defined as "resistant" and "semi responders", respectively[31].

Recently, simpler and more rapid tests of platelet function have been developed. Whole-blood aggregometry is more user

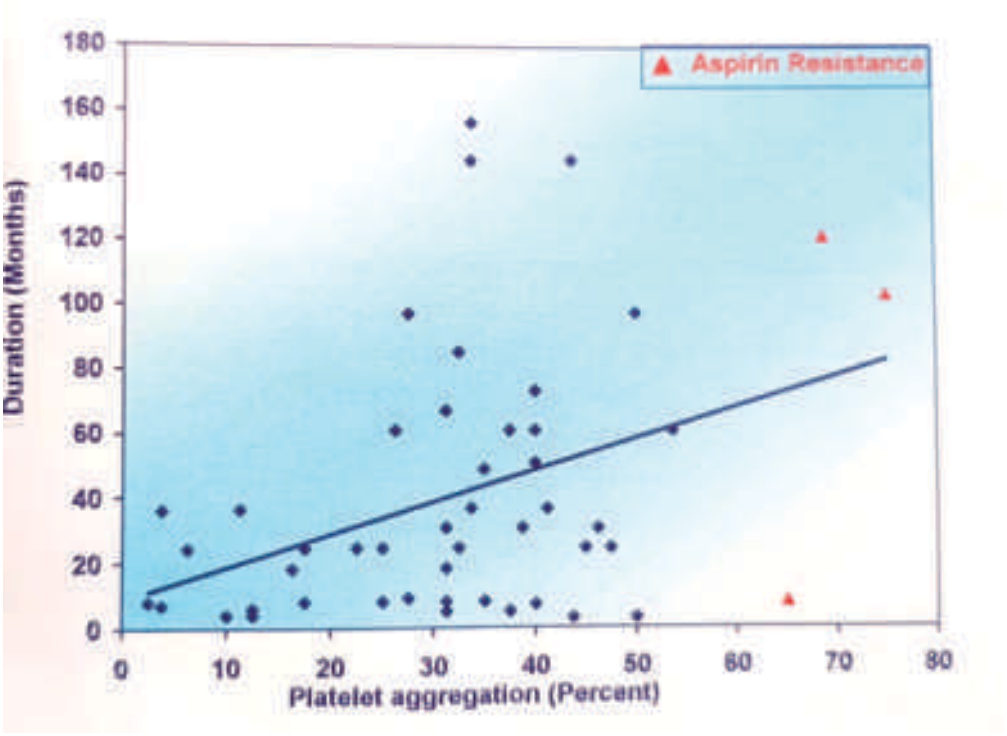

Figure 1: Correlation of platelet aggregation induced by ADP with duration of aspirin therapy

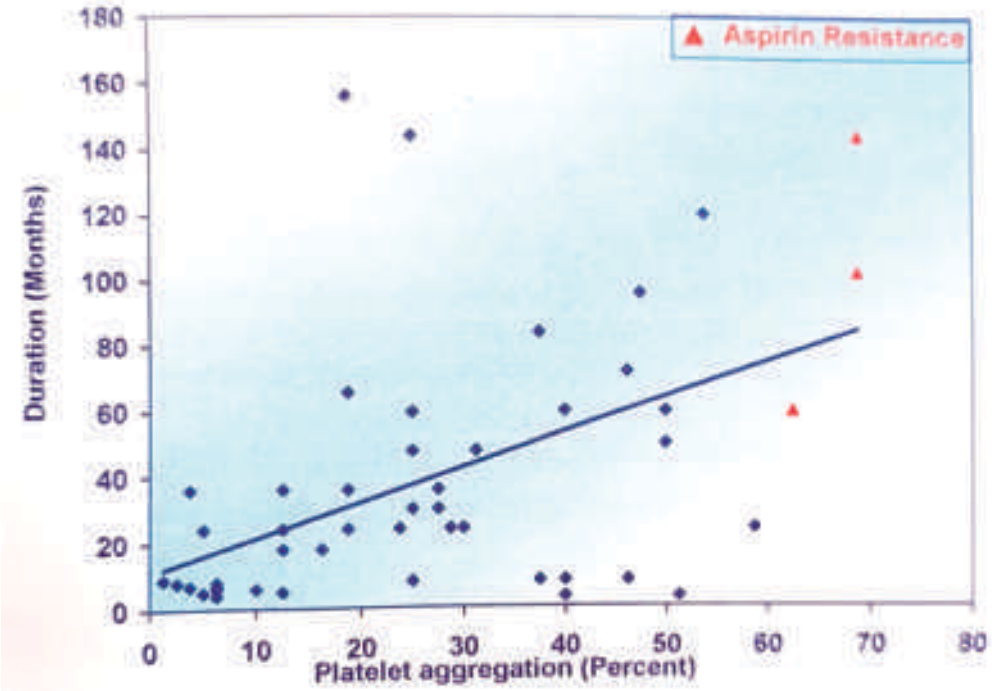

Figure 2: Correlation of platelet aggregation induced by collagen with duration of aspirin therapy

friendly as it eliminates the step of preparing platelet rich plasma. However, the results of this test have not correlated well with those from optical aggregometry[32]. In clinical practice, PFA100 (Dade Behring, Deerfield, Illinois) is the most appealing test at present for the assessment of platelet function. It is a semi-automated analyzer developed to allow the rapid assessment of platelet function using whole blood. The results are easily reproducible and correlate well with the results of optical aggregometry[32,33].

A nonspecific measure of platelet function is the assessment of bleeding time[2, 27]. Other less extensively studied tests include the platelet aggregate ratio, the platelet reactivity index, and the rapid platelet function assay (RPFA)[32,34]. Recently, urinary 11-dehydrothromboxane B_2 levels (a stable metabolite of thromboxane A_2 [TxA_2]) has been used as a marker of suppression of thromboxane formation with aspirin therapy[19]. Since the levels reflect both platelet and non-platelet sources of thromboxane generation, this test lacks specificity. Collectively, these techniques identify an inadequate response to aspirin in 5-60 percent of patients with different vascular atherothrombotic diseases. It is difficult to assess which of these techniques is the most accurate and specific measure of aspirin resistance unless the results are supported by direct comparison with the clinical outcome.

Weber and co-workers classified aspirin resistance into 3 distinct types using simple biochemical tests, and functional *in vitro* studies[31].

TYPE I RESISTANCE (PHARMACOKINETIC TYPE):

When aspirin was taken orally for five days at a dose of 100 mg/day, aspirin responders showed greater than 95% inhibition of thromboxane production and of collagen-induced platelet aggregation as evaluated in vitro. Oral aspirin use for five days did not reduce either thromboxane production or collagen-induced platelet aggregation in patients with "type I resistance" (pharmacokinetic type). However, the addition of 100 μm of aspirin *in vitro* to the platelet-rich plasma significantly changed both of these characteristics. This implies that the pharmacokinetics of low-dose aspirin may vary significantly.

TYPE II RESISTANCE (PHARMACODYNAMIC TYPE):

Neither the oral aspirin consumption nor the in vitro addition of 100 μm of aspirin affected any of the platelet activities. Although the exact mechanism of this kind of resistance is unknown, it may be connected to the enzymatic pathways genetic variation and aspirin sensitivity.

TYPE III RESISTANCE (PSEUDO-RESISTANCE):

Neither the oral aspirin consumption nor the in vitro addition of 100μm of aspirin affected any of the platelet activities. Although the exact mechanism of this kind of resistance is unknown, it may be connected to the enzymatic pathways, genetic variation and aspirin sensitivity. It is possible that certain aspirin-resistant individuals have higher platelet sensitivity to collagen[35]. It's unclear whether this variation has any clinical significance. It is unknown if this change, as assessed in artificial *in vitro* settings, will correspond to a reduced aspirin`s antithrombotic action *in vivo.* 101 It has been suggested that raising the aspirin dosage may help people with type I resistance. Furthermore, additional antiplatelet medications may be beneficial for people with types II and III resistance. The classification and clinical importance has not been investigated yet, though. This problem can only be adequately addressed by prospective follow-up studies in aspirin-resistant patients and their clinical connection.

A few long-term follow-up clinical studies have suggested that aspirin resistance is indeed clinically important[25,36-38].

Grotemeyer and co-workers in a cohort of 180 patients with stroke found that nearly 30% of patients were aspirin non-responders. At a follow-up of 2 years, major clinical vascular end-points were significantly higher in this group as compared to aspirin responders (40% v/s 4.4%, $p<0.0001$). The methodology used by them was platelet reactivity; aggregation induced by blood collection[28].

Mueller and co-workers, in 100 patients undergoing peripheral balloon angioplasty reported an 87% higher risk of re-occlusion on follow-up in patients who failed to show an appropriate response to aspirin[36].

Grundmann and co-workers found that an aspirin non responder status was seen in 34% of patients with recurrent cerebrovascular ischemic events, despite regular use of aspirin for more than 60 months[15].

Buchanen and Pappas, independently conducted aspirin resistance studies on various study groups and healthy controls without vascular diseases, have also shown to have resistance by laboratory testing[21,22].

Chen and associates reported 19.2% incidence of aspirin resistance as defined by Ultra RDFA among 151 patients with coronary disease[20], using ultra rapid platelet function analyser defined aspirin resistance as ARU(ASPIRIN RESPONSE UNITS) >550.

Gum and co-workers reported 5% incidence of aspirin resistant and 23.8% were aspirin semi responders. By PFA-100 (platelet function analysis), 9.5% were aspirin resistant. They found no difference in aspirin sensitivity by race, diabetes, platelet count or liver diseases[19]. They used both optical platelet aggregation using ADP and arachidonic acid as aggregants and PFA (platelet function analyser) for determination of aspirin resistance.

Macchi and co-workers studied 160 stable cardiac patients using PFA-100 (platelet function analyser) and found aspirin resistance in these patients to be 29.2%[39]. Epinephrine closure time less than 186 sec was taken as aspirin resistance by them.

Sibi and co-workers using optical platelet aggregation studied 150 mg dose of aspirin in 75 stable cardiac patients and

reported aspirin resistance to be 26% in studied patients[40]. Methodology used by them was optical platelet aggregation using arachidonic acid and ADP.

Anderson and co-worker studied 129 stable CAD patient using PFA-100 with aspirin resistance define as epinephrine closure time < 196 seconds and reported aspirin resistance of 1.35%[29].

Serum markers such as soluble CD40 ligand and P selection have also been used as markers of platelet activation with variable results[37].

Two recently published studies have highlighted adverse outcomes with aspirin resistance in a larger cohort of patients, and after a longer follow-up period.

In a subgroup analysis from the Heart Outcomes Prevention Evaluation (HOPE) trial population, Eikelboom and Co-workers (2002) found that individuals with aspirin resistance had more bad outcomes during a 5-year follow-up. As a measure of *in vivo* thromboxane production, the urinary concentration of 11-dehydrothromboxane B_2 was determined. With each rising quartile of 11-dehydrothromboxane B_2 levels, the adjusted chances for the composite end-point of myocardial infarction (MI), stroke, or vascular death rose. Individuals with insufficient suppression of TXA_2 and consequent aspirin resistance in the highest quartile were 1.8 times more likely to have composite end-points than patients in the lowest quartile. Likewise, there was a 3.5 times greater risk of cardiovascular mortality and a 2- times higher risk of MI in each group. This substantial and graded correlation between aspirin resistance in the laboratory and unfavourable outcomes was not correlated with traditional risk factors for atherothrombotic vascular diseases[41].

During a mean follow-up period of 679±185 days, Gum and Co workers (2003)[19] emphasised the natural course of aspirin resistance in stable patients with cardiovascular disease. Aspirin resistance was linked to a significantly higher risk of composite end-points such as death, MI, or cerebrovascular accident (CVA) in this prospective, blinded study involving 326 patients when compared to aspirin-responsive patients (24 percent v/s 10 percent, respectively; p=0.03, hazard ratio 3.12)[19].

It is interesting to note that among 50 patients of IHD 32 patients were of healed MI and 5 patients demonstrated recurrent MI in spite of aspirin therapy (Table 7). In these patients of recurrent MI, one patient demonstrated true aspirin resistance, clinically as well as on laboratory study. Aspirin in this patient could not inhibit Platelet aggregation induced by ADP and Collagen. The other patient (case no 18) was aspirin semi-responder while remaining three patients were aspirin responsive. It is clear from the above data that among the patients of IHD who demonstrated recurrence of MI in spite of aspirin therapy aspirin resistance should be seriously thought of Because, as it is evident in the present study. 20% of these patients may have true aspirin resistance and need alternative or combined therapy with other antiplatelet drugs.

Aspirin blocks the formation of TxA_2, a potent vasoconstrictor and platelet agonist by irreversibly inhibiting the enzyme platelet cyclo-oxygenase (COX) (Fig.3). COX has two isoforms of clinical relevance COX-1 isoenzyme is expressed in mature human platelets. The therapeutic efficacy of aspirin in atherothrombotic vascular disease has been clearly attributed to its inhibition of COX-1 activity[38,42]. Importantly, in the low doses necessary to achieve platelet inhibition, aspirin does not inhibit endothelial cell prostaglandin synthesis, particularly prostacyclin, which is a potent vasodilator[33,34]. COX-2 isoenzyme plays a dominant role in the processes of inflammation andcancers[38]. Aspirin acts as an anti-inflammatory agent due to the inhibition of COX-2 activity at higher doses. Although much is currently known about effect of aspirin on platelets, the mechanism by which some patients are resistant to this effect has not been clearly established.

A number of extrinsic variables can alter aspirin`s capacity to deactivate platelets. Aspirin`s antiplatelet impact has been demonstrated to be influenced by smoking elevated cholesterol levels, and circumstances linked to an accelerated platelet turnover[43,44]. While full inhibition of COX-1 is anticipated with low-dose aspirin, greater doses may be needed for certain people to have the desired antiplatelet effect.

Helgason and co-workers in patients with stroke reported the effect of dose escalation of aspirin in non-responders as judged by aggregation studies. An initial 25% incidence of aspirin resistance (daily dose 325 mg) fell to 8% with dose escalation up to 1300 mg. However, a recently published meta-analysis does not support this contention, and it may not be practical in many patients due to gastrointestinal side-effects[24].

Secondary aspirin resistance may be influenced by certain medication interactions, particularly those involving non-steroidal anti-inflammatory medicines (NSAIDs). Since aspirin and NSAIDs are both frequently given medications, it is possible that many people are taking both on a long-term basis. Given that both of these medications function by suppressing the COX enzyme, there is a chance that they will interact competitively. NSAIDs, on the other hand, are reversible inhibitors of this enzyme, unlike aspirin. Aspirin`s long-lasting antiplatelet activity has been demonstrated to be blocked by NSAIDs (such as ibuprofen), which modifies the drug`s cardioprotective effects. In individuals who initially react to aspirin, this can potentially result in secondary aspirin resistance[43]. This is because an NSAID competitively inhibits the active site inside the COX-1 channel, preventing aspirin from reaching its target[44]. Furthermore, there is currently proof that this medication combination has negative long-term clinical outcomes[21,45].

MacDonald and Wein[46] reported a cohort of patients' secondary prophylaxis with aspirin, and highlighted that on concomitant administration of ibuprofen was associated with a significant increase in the all cause mortality as well as cardiovascular mortality on long-term follow-up. The absence of COX-2 in mature human platelets explains why selective COX-2 inhibitors (coxibs) do not inhibit the effects of low-dose aspirin on platelet function in comparison with ibuprofen[44,47]. These drugs would logically seem preferable to ibuprofen when

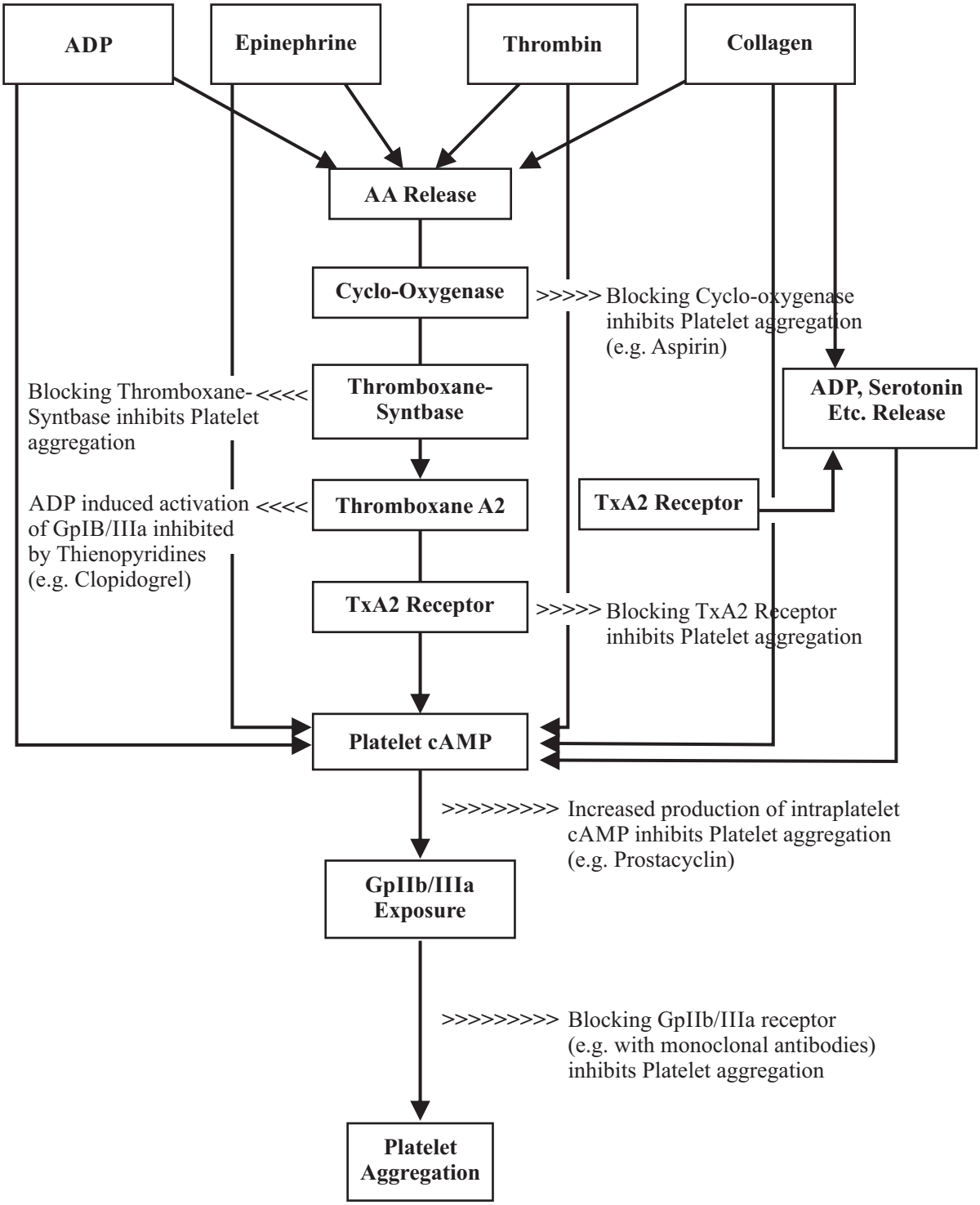

Figure 3: Platelet activation mechanism leading to platelet aggregation

patients taking aspirin for cardio protectiveness require chronic treatment with NSAIDs.

A number of intrinsic mechanisms have been postulated to cause aspirin resistance. The inability of treatment to sufficiently reduce TXA_2 Synthesis is a critical factor in the pathophysiology of aspirin resistance [19, 48]. The emergence of aspirin resistance has been linked to COX-2, despite aspirin`s almost 170-fold greater potency in inhibiting COX-1. It has long been assumed that mature platelets are only COX-1 isoenzyme-containing. 99 Contemporary data, however still subject to debate, has demonstrated that COX-2 mRNA is present in platelets[48,49]. Patients differ in the extent of their COX-2 expression, and some may express COX-2 at higher levels than others, particularly under stress. Due to low dosage Aspirin`s inability to block the COX-2 enzyme, individuals on aspirin treatment may have an alternative pathway for platelet-mediated thromboxane synthesis, which might lead to aspirin resistance[16,47].

Nucleated cells, such as monocytes and macrophages, have also been linked to the processes behind aspirin resistance in addition to platelets. In terms of their capacity for synthesis, these cells are second only to platelets in terms of TxA_2 availability[50]. These cells can renew the enzyme, nevertheless, in contrast to anucleate platelets. Prostaglandins are produced by this regenerated, unrestrained COX-1 in nucleated cells, which is then transferred to the platelets to make aspirin-insensitive/resistant thromboxane, avoiding platelet COX-1. These nucleated cells have the ability to produce their own TxA_2 in addition to that which is mediated by COX-1 through COX-2, which is not blocked by aspirin at low concentrations[19,30]. In contrast to constitutively expressed COX-1, inflammatory stimuli increase COX-2 expression in nucleated cells by a factor of 10–20[19,32]. These nucleated cells may activate platelets with the help of the TxA_2 they manufacture, starting a chain reaction50. There is evidence that atherosclerotic tissue has an upregulated level of COX-2. Aspirin resistance and acute coronary syndromes may result from the macrophages in the atherosclerotic plaque contributing considerably to the pool of TXA_2 that is not inhibited by modest dosages of aspirin[48]. Studies have demonstrated that erythrocytes can increase platelet reactivity and be prothrombotic. Not all individuals experience a consistent blocking of this cell-to-cell contact by aspirin, which might offer a different route for the development of thrombus [51].

The varying effects of aspirin in different persons may also be due to genetic variances. Firstly, a genetic foundation for aspirin resistance may be provided by polymorphisms or mutations of the COX-1 gene, which renders it relatively resistant to the action of aspirin. Single nucleotide polymorphisms (SNPs) of COX-1 may occur and influence an individual`s susceptibility to aspirin's inhibitory effect.117 SNPs are thought to act as mediators of phenotypic variation and provide the genetic foundation for a drug's variable response. Second, the variable effects of aspirin in different people may potentially be due to genetic variations in the glycoprotein IIb/IIIa receptor complex. The last common route for platelet activation is the glycoprotein IIb/IIIa receptor. The PIA_1 and PIA_2 alleles are defined by a common polymorphism involving the replacement of Leu33 for Pro, respectively. The majority of research show that PIA_3 carriers are less sensitive to aspirin`s antithrombotic actions and exhibit increased platelet activation by agonists, despite contradictory data. The amount to which the glycoprotein IIb/IIIa polymorphism influences aspirin`s functions contributes to both the drugs clinical effectiveness and resistance to its effects is still unknown, though.

Despite consistency of such observation, the prevalence of aspirin resistance has been variable in different populations and there is lack of standardized diagnostic criteria on a single validated method of identifying affected individuals to have aspirin resistance. It has led to wide range of population estimates[18]. Even though, aspirin resistance should be seriously considered in patients of IHD or stroke who are taking the prescribed dose regularly but getting recurrent coronary events or stroke. These patients need supplementation or supplant of other antiplatelet drugs. Unfortunately there are reports showing that clopidogrel, a thienopyridine derivative, commonly used as an antiplatelet agent, also demonstrates resistance in many patients.

CONCLUSION

The present study demonstrated that aspirin non-responsiveness in IHD patients living in and around Udaipur is 10%, of which 2% are having true resistance and 8% are semi-responders. The real prevalence in population around the study area is thought to be much more than reflected by the present study because the present study has excluded patients with diabetes, hypertension, history of smoking and female patients in whom incidence of aspirin resistance is reported to be high. Aspirin resistance or non-responsiveness is clinically important particularly in patients of recurrent infarction in whom chances of aspirin resistance are 40%. Aspirin resistance and duration of aspirin consumption have proportionate relation as majority of patients demonstrating aspirin resistance were consuming aspirin for more than 5 years. Further large scale study is warranted including different population with different disease using other parameters of assessment of platelet function.

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Research Paper

Effect of Physical Stress on Platelet Aggregation in Patients with Ischemic Heart Disease

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ABSTRACT

Effect of physical stress on platelet aggregation was evaluated in thirty patients with ischemic heart disease (IHD) - After collecting fasting blood samples, they were subjected to treadmill test up to stage III, and blood samples were again collected. All the blood samples were subjected for the study of platelet aggregation using ADP and collagen as aggregating agents. The study demonstrated that ADP induced platelet aggregation is variably affected by physical stress while collagen induced platelet aggregation was significantly ($p< 0.05$) and consistently increased after physical exercise, in patients with IHD.

KEYWORDS: Stress, ADP, Collagen, Aggreganometer, IHD

INTRODUCTION

Experimental and clinical data suggest that platelets may contribute to the adverse clinical events associated with atherosclerotic coronary disease[1]. Platelet aggregation in coronary vessels occurring during stress has been implicated as a mechanism of imbalance between myocardial blood supply and demand[2]. The evidence of platelet involvement in myocardial ischemia is derived from several animal and human studies[3,4].

Platelet upon activation generate a potent pro-aggregant and a vasoconstrictor prostaglandin, thromboxane-A2, which has been shown to be increased in patients with coronary artery disease during spontaneous or pacing induced myocardial ischemia[5,6]. Several studies relating platelet aggregation function to exercise have been reported[7-9]. Moderate and strenuous exercise is known to enhance the platelet aggregation in patients with coronary artery disease[10-12]. Not only has this, but the effect of low-grade exercise also demonstrated enhanced platelet aggregability in-patient with obstructive coronary artery disease. In a recent study, shear induced platelet aggregability before and after mild exercise in 27 patients with documented coronary artery disease was assessed. *ex vivo* platelet aggregability was assessed in flowing whole blood as the time to occlude collagen and ADP coated ring. Patient with coronary artery disease showed a significant increase in aggregation at peak compared with base line, whereas no significant change occurred in controls. This effect is independent of myocardial ischemia and has been

observed despite aspirin consumption[13]. The failure of aspirin to attenuate the platelet response to exercise has also been reported by others[11,14,15].

It is interesting to note that even low grade exercise also enhances platelet aggregation which is probably dependent on shear stress. The mechanism by which shear stress induces platelet aggregation is not clear however it involves the increase in intracytoplasmic ionized calcium, von Willibrand Factor and functional platelet receptor complex Gp Ib/IX/V and Gp III b/II a. vWF and Gp l b a interaction causes associated increase in intracytoplasmic (Ca2+) and platelet aggregation. Both of these are potentiated by vWF binding to activated platelet GPIII b/II a complex in presence of released ADP. Fibrinogen, Gp l b a and extra cellular Ca2+ are absolutely required for these above reactions. If the effect of released ADP is blocked, shear induced platelet aggregation is inhibited without affecting shear induced increase in calcium. It is also observed that neither calcium nor aggregation response to shear stress is inhibited by blocking platelet cyclo-oxygenase with acetyl salicyclic acid[16,17].

The present study has been envisaged to evaluate the effect of physical stress on platelet aggregation in patients of coronary artery disease.

MATERIAL AND METHODS

Patients' Selection:

The study included 30 male patients of ischemic heart disease (IHD) between the ages of 40 to 60 years. Patients of ischemic heart disease were either of old healed myocardial infarction (> 6 months) or stable angina pectoris with or without positive Treadmill Test (TMT). The criteria for diagnosis of IHD were same as that of WHO[18].

Exclusion Criteria:

The following patients were not included in this study:

1. Patients with valvular heart disease;
2. Bleeding tendency;
3. Uncontrolled diabetes;
4. Patients with peripheral arterial disease;
5. Those patients who were consuming tobacco in any form or taking treatments with corticosteroid, anticoagulant and antiplatelet drugs.

Protocol :

After informed consent of the selected study subjects, initial fasting blood samples were collected. All selected individuals were subjected to stress testing on a computerized 12 lead TMT machine for less than or equal to stage three of Bruce protocol. Blood samples were again collected at the end of the test. All the blood samples were subjected for measurement of platelet aggregation.

Exercise Testing[19]:

Measurements of cardiovascular function during rest are poor predictors of circulatory performance. Exercise is currently the most convenient way of stimulating the myocardium to demand maximal blood flow so that even a moderate impairment of coronary blood flow capacity becomes detectable.

Modes:

1) Dynamic lower extremity testing:

1. Treadmill: It is most widely used method for exercise testing.
2. Bicycle ergometer: It is used when a patient is unable to do treadmill exercise, i.e., during radionuclide ventriculography and for dynamic testing during cardiac catheterisation.
3. Master's step test: Seldom used

2) Dynamic upper extremity testing:

1. Arm ergo meter: sometimes used in patients with PVD, orthopaedic abnormality and other limitation to lower extremity effort.

Exercise Test Protocols:

1) Heart rate limited or sub maximal exercise

2) Symptom limited testing

3) Treadmill protocols: There are several protocols available, the choice of which depends upon expected effort tolerance of the patient:

a) Bruce protocol: This is preferred for evaluating patients with little or no symptomatic limitation. This has a relatively higher initial workload with greater subsequent work increments. The subjects start out at 1.7 mph on a 10% inclined & progress to their maximal capacity at 3 minutes intervals.

b) Naughton protocol: This has low initial workload and small work increment with subsequent stages, and is used after myocardial infarction or coronary artery bypass graft surgery.

The workload achieved in all protocols is expressed in terms of MET (metabolic equivalent). This term is used to describe the energy cost of physical activity. One MET is approximately equal to an oxygen consumption of 3.5 ml/ Kg/min.

Preparation of Patient:

A detailed clinical examination and evaluation resting 12 lead ECG is done before treadmill test is ordered. The patient should be fasting for at least 2 hours before the test; whether any medication being taken should be discontinued depends on purpose of testing. Leads monitored are 12 lead EGG. The patient is continuously monitored as he goes through various stages of exercise. Reassurance & encouragement of the patient during test enable one to obtain a truly symptom limited

exercise test.

In the post exercise period, the sitting position is mostly frequently employed. Cardiac auscultation is carried out to detect out any gallop sound, mitral regurgitation or pulmonary rales. Observation after exercise is continued for 6 minutes or longer till all exercise-induced abnormalities have disappeared.

Exercise Test Response

Symptomatic end points:

In symptom limited exercise test, the following symptoms are used as end points:

1) Angina pectoris

2) Dysponea and Fatigue

3) Leg fatigue, Claudication, Joint pain

ECG Patterns and their Significance

Normal Exercise Electrocardiogram

When the heart rate increases with exercise, a number of predictable changes occur in the ECG tracing. PR interval shortens, P wave becomes taller & atrial repolarisation rate (Ta wave) becomes prominent causing depression of the PQ segment. This results in J point depression, which is usually of short duration (0.04) sec. The normal ST segment with exercise is up sloping and slightly convex in form and returns to base line within 0.04 to 0.06 sec after J point.

ST segment changes:

1) Up sloping segment: These are abnormal when the degree of depression at 0.08 sec from the J point is 1.5 mm or more below base line.

2)Horizontal segment: When ST segment depression is horizontal or down sloping, 1.0 mm depression at 0.08 sec from the J point is considered a positive response and correlates well with the actual presence of CAD.

3) Rounded segment depression: A rounded configuration of ST segment depression represents a positive response - patients with this pattern are at higher risk of future coronary events

4) ST segment depression late in recovery period: In few patients depression is absent during and immediately after exercise but occurs three to eight minutes into the recovery period. This is almost associated with normal coronaries.

5) ST Elevation: When ST segment elevation occurs with exercise it usually indicates a ventricular kinetic or dyskinetic segment or presence of high-grade lesion in the proximal left anterior descending coronary artery.

T wave changes:

1) Tall T wave in lateral precordial leads after exercise are normal and are due to increased stroke volume.

2) T wave inversion during exercise is a non-specific finding and not considered in the evaluation of ischaemia .On the other hand, the evolution of a downsloping T wave after exercise is often associated with ischaemia. Normalisation of T wave with exercise such as a flat or inverted T wave at rest becoming upright with exercise has been considered as a sign of ischaemia.

U wave:

Occurrence of inverted or diphasic U waves with exercise is often associated with CAD and in particular with high grade proximal left anterior descending artery stenosis.

R wave amplitude:

The R wave amplitude in lateral precordial leads normally decreases with exercise. In patients with severe CAD, R wave amplitude increases with exercise.

Predictive Implications

Two of the important factors in the analysis of patients undergoing stress test are:

1. **Pre-test disease prevalence:** Patients with typical anginal pain have 95 % chance of having the disease; in those with atypical chest pain it is 60% and in patients with nonanginal pain it is 10%. It is in the group of a typical chest pain that treadmill test is of considerable value in masking CAD.
2. **Sensitivity & specificity of the test:** Sensitivity provides an index of the capability of the test to detect an abnormality. Specificity indicates the ability of the test to recognize a normal subject.

Prognostic Significance

Stress test can be a useful tool in patients with CAD to predict the prognosis of the disease and assess the result of developing future cardiac event.

Platelet Aggregation[20]

Most important function of platelets is their role in hemostasis i.e. adhesion to damaged tissue surface and cohesion to one another. This cohesion phenomenon is known as aggregation and may be initiated by a variety of substances including collagen, adenosine 5-diphosphate (ADP), epinephrine, serotonin and ristocetin. Aggregation is one of the numerous *in vitro* test performed as a measure of platelet function. The described procedure is a turbidimetric method of measuring the effect of collagen, ADP and epinephrine on platelets.

I. Reagents

1) 3.8 per cent citric acid (Trisodium salt dehydrate): prepared by dissolving 3.8 gm citric acid in100 ml of deionised water.

2) Tris buffer: Tris (hydroxyl methyl), methylamine,1.21 gm (0.01 M), disodium ethylene diamine tetra acetic acid 0.372 gm (0.001 M), sodium chloride 8.76 gm (0.15 M), dissolved in distilled water adjusted to pH 7.5 with hydrochloric acid and made upto one litre with distilled water.

3) ADP reagent --- Adenosine 5-diphosphate lyophilized with buffer salts (supplied by sigma diagnostics). ADP solution was prepared by reconstituting ADP reagent with 1.0 ml deionized water to yield solution containing ADP 2 x 10^{-4} mol/lit. It was swirled to mix and allowed to stand at room temperature (18-26°C) for 15 minutes before use. It should be kept at room temperature only for duration of testing. The reconstituted reagent is stable for one month if stored in refrigerator (2° to 6°C).

4) Epinephrine reagent : Epinephrine bitartrate lyophilized with buffer salts. Epinephrine solution was prepared by reconstituting epinephrine reagent with 1.0ml deionized water to yield solution containing epinephrine 1 x 10^{-4} mol / lit. It was allowed to stand at room temperature for 15 minutes before use. The reconstituted reagent is stable for one month, if stored in refrigerator (2° to 6°C).

5) Collagen reagent: Collagen (calf skin) acid soluble, approximately 2 mg, lyophilized with buffer salts. Collagen solution was prepared by reconstituting vial of collagen with 1.0 ml deionised water. The vial was allowed to stand undisturbed for at least 15 minutes at room temperature before use. Warming to 37°C may be necessary for complete dissolution. It was swirled to mix prior to each assay. It should not be vertexed. The solution should be kept at room temperature only for the duration of testing. It is usually stable for at least 2 weeks refrigerated (2-8°C). Stability may be extended by freezing.

II. INSTRUMENTS & MATERIALS

1. Instruments (Fig. 1)

1. Platelet aggreganometer (Elvi 840)
2. Chart recorder (Omniscribe recorder dual pen type <176 USA)

2. Materials

1. Cuvette 250µl
2. Teflon coated magnetic stirring bars (micro agitators)
3. Pipettes with disposable plastic tips 50µ1 and 250µl
4. Centrifuge machine
5. Plastic tubes with caps
6. Plastic transfer pipettes

III. SPECIMEN COLLECTION

Blood was collected by avoiding stasis and contamination with tissue fluids into plastic tubes containing 0.1 ml buffer and 3.8 per cent sodium citrate in a ratio of blood to anticoagulant in a ratio of 9:1.

IV. PREPARATION OF PLATELET RICH PLASMA (PRP)

1. The anticoagulant sample was centrifuged at 400 RPM for 10 minutes.
2. PRP was removed carefully using a plastic transfer pipette.
3. PRP was expelled into a plastic tube covered and kept at room temperature for duration of the test.

V. PREPARATION OF PLATELET POOR PLASMA (PPP)

1. It was prepared by re-centrifuging the PRP at 6000 RPM for 10 minutes.
2. Supernatant was transferred to a labelled PPP tube, covered and kept at room temperature for the duration of the test.

The platelet count of PRP was adjusted to the range of 4 to 5 lac / cu mm when necessary by addition of autologus PPP to PRP samples. The caution should be taken to assay platelet aggregation within 30 minutes of collection of test samples.

VI. AGGREGATING AGENTS

ADP - ADP induced aggregation may occur in one or two phases and it may be followed by rapid disaggregation, which may be seen in normal man without any hemorrhagic disease.

Epinephrine - Epinephrine induced platelet aggregation may occur in one or two phases and is largely irreversible and epinephrine induced platelet aggregation sometimes absent in patients who appear to be totally normal and may be related to temporary saturation of catecholamine binding sites on platelets.

Collagen - Collagen induced platelet aggregation may occur in an irreversible single-phase curve or a reversible single-phase curve depending on the collagen concentration in the PRP.

VII. PROCEDURE

After preparing PRP and PPP the aggregation was recorded as follows:

1.Cuvette with PRP was introduced into the aggregometer.

2.The electromagnetic agitation was started by means of stirred control after having introduced a small stirring bar into the sample.

3.Agitation speed was maintained at 1000 RPM.

4.Baseline of the recorder was adjusted by means of the zero control.

5.The cuvette with PRP was removed and cuvette with PPP was inserted.

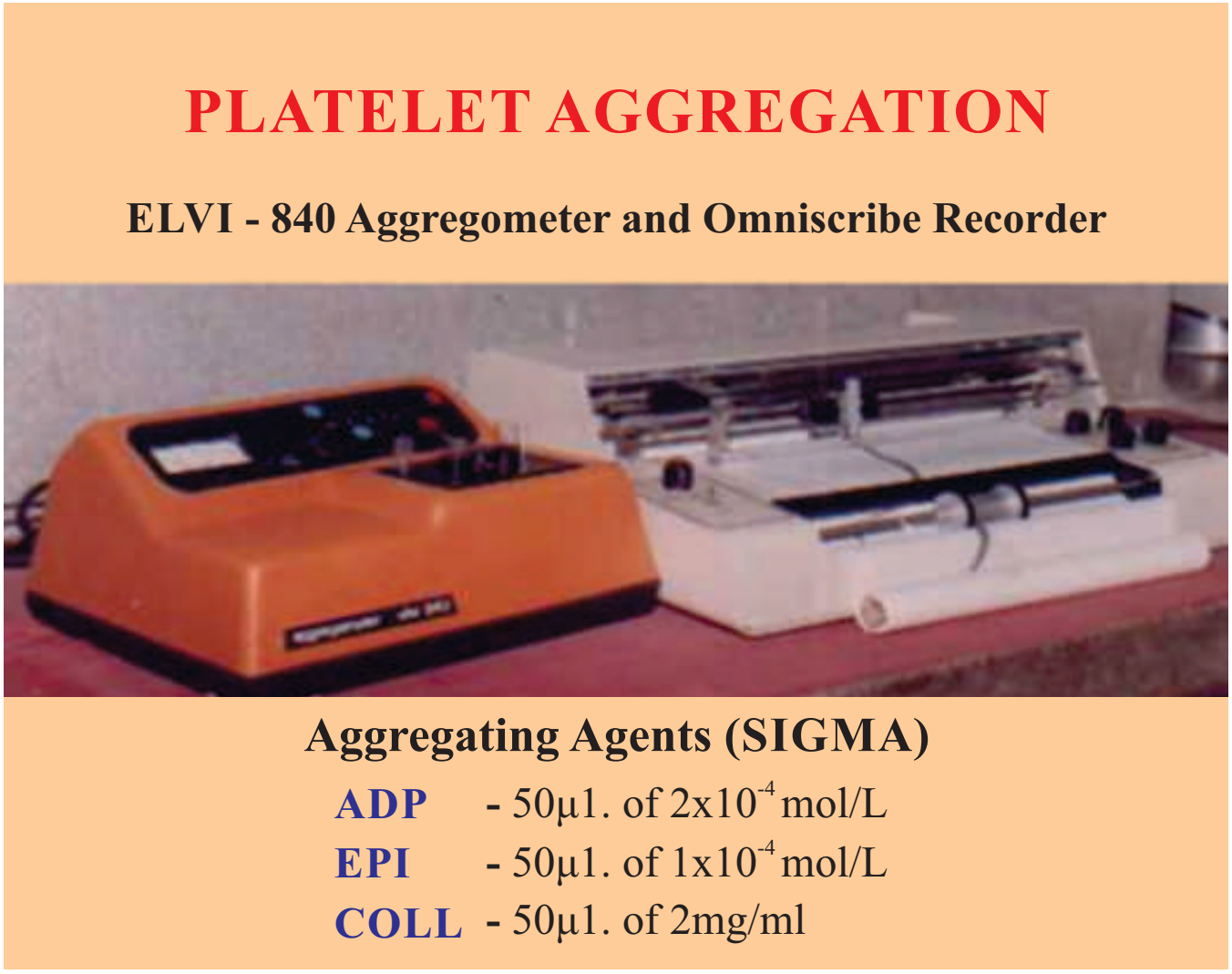

Figure 1: Platelet aggregometer (Elvi 840) with chart recorder (Omniscribe recorder dual pen type 176 USA)

6.By means of gain control the maximum excursion of the pen to the recorder was adjusted.

7.Cuvette with PPP was removed and the cuvette with PRP was reinserted and it was readjusted if necessary by means of the zero control.

8.The sliding of the recorder paper was started.

9.The aggregating agents (ADP, and collagen) were added to the PRP by means of micropipette (50µ1)

The aggregation was recorded for a minimum of 5 minutes and results were expressed as percentage aggregation.

$$\text{Percentage Aggregation} = \frac{90 - CR}{90 - 10} \times 100$$

$$= \frac{90 - CR}{80} \times 100$$

CR is chart reading in terms of number of segments.

VIII. EXPECTED VALUES

Platelet aggregation studies were performed on 20 healthy adults using the three aggregation reagents. The results were as follows -

ADP = 80-100

Epinephrine= 67-97 % (subjects with secondary aggregation phase)

Collagen = 26-59 % (subjects without secondary aggregation phase)

Collagen = 80-100%

However due to differences in instrumentation and technique, each laboratory should establish its own normal ranges for each reagent.

IX. PERFORMANCE CHARACTERISTICS

Duplicate aggregation determinations on platelet rich plasma from normal individuals yielded an average difference of ±3% aggregation for collagen, ADP and epinephrine aggregations determinations.

STATISTICAL ANALYSIS[21]

Results were statistically analysed with Student`s t-test and a 'p' value of less than 0.05 was considered as significant difference in analysis.

OBSERVATIONS AND RESULTS

The following observations were made on 30 male patients of known ischemic heart disease. The mean age of subjects was 57 years, they were subjected to treadmill stress (TMT) test. On an average, they exercised to stage III ± I of Bruce protocol. 17 patients had positive test and 13 had negative stress test.

Collagen induced platelet aggregation was increased from mean value of 49.75 to 54.90 %. This had led to increase in post exercise aggregation to the extent of 10 percent and that is statistically significant ($p<0.05$). However, ADP induced platelet aggregation was increased to the extent of 5 percent but it was not significant (p NS) statistically (Table 1).

DISCUSSION

The present study was conducted on 30 male patients of known ischemic heart disease between the ages of 40 - 60 years. They were subjected to treadmill exercise to the stage III. Blood samples were collected before and after exercise test for platelet aggregation.

The mean age of the patient selected was 57 years, 17 patients had positive ischemic response to exercise while 13 had negative test. Exercise had significantly ($P < 0.05$) increased collagen induced platelet aggregation to the extent of 10% while ADP induced platelet aggregation was not significantly altered (Table 1).

The present study therefore suggests that treadmill exercise test which is basically a physical stress test does not significantly increase ADP induced platelet aggregation in patients with documented IHD. Collagen induced platelet aggregation on the other hand has been observed to increase significantly (<0.05) after exercise (Table 1) (Fig. 2, 3, 4 and 5).

Table 1: Effect of Physical stress on platelet aggregation (percent) in Patients with IHD

	ADP (2 X 10^{-4})		Collagen (0.2 µg/ micro lit.)	
	Pre TMT	**Post TMT**	**Pre TMT**	**Post TMT**
Mean	53.63	56.50	49.75	54.90
% Change		5.30		10.35
S.D.±	10.56	12.84	10.70	12.75
S.E.±	3.34	4.06	3.38	4.03
P value		NS		< 0.05

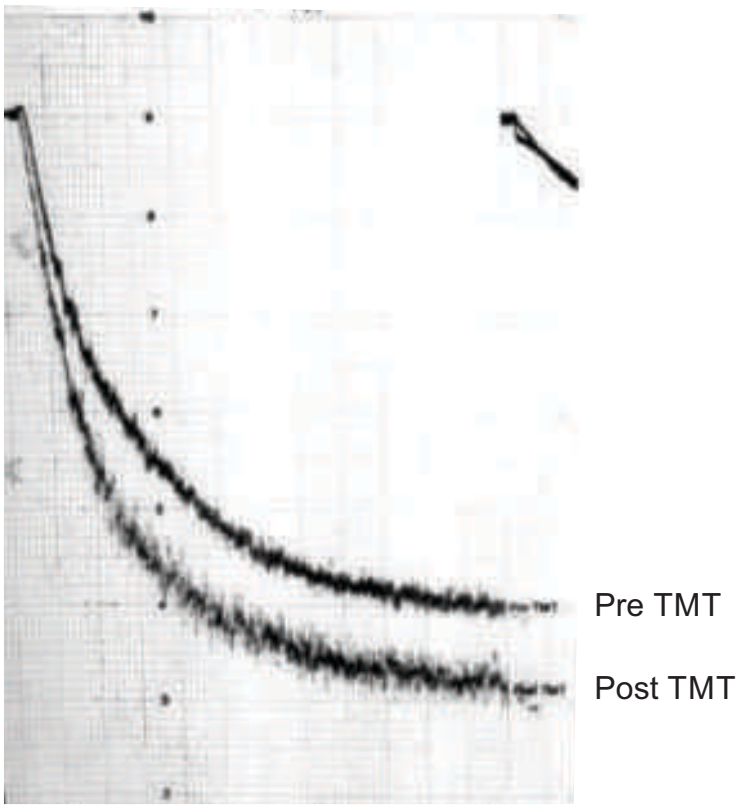

Figure 2: Effect of exercise on ADP induced platelet aggregation

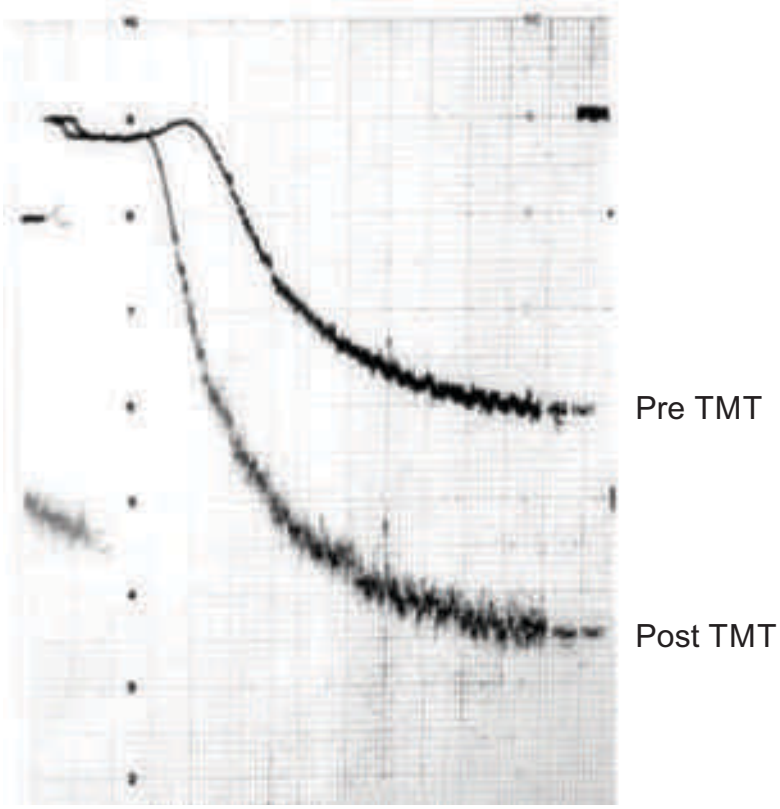

Figure 3: Effect of exercise on Collagen induced platelet aggregation

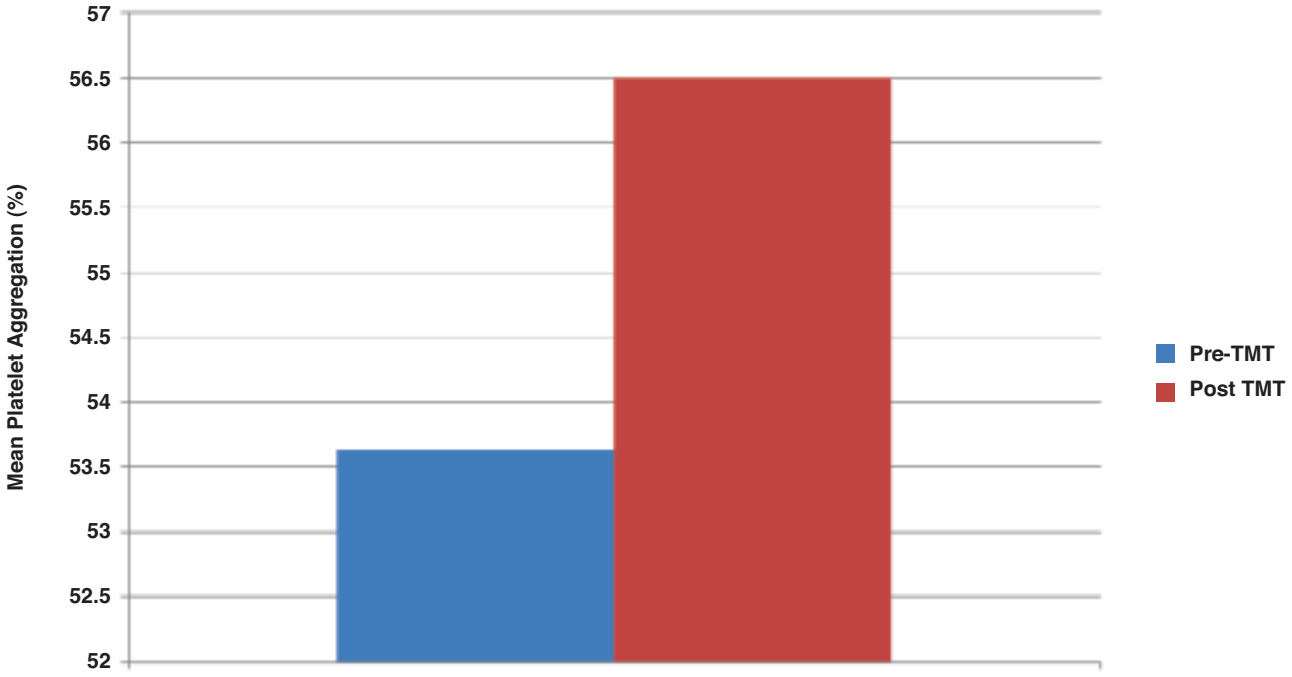

Figure 4: Effect of physical stress on ADP induced platelet aggregation

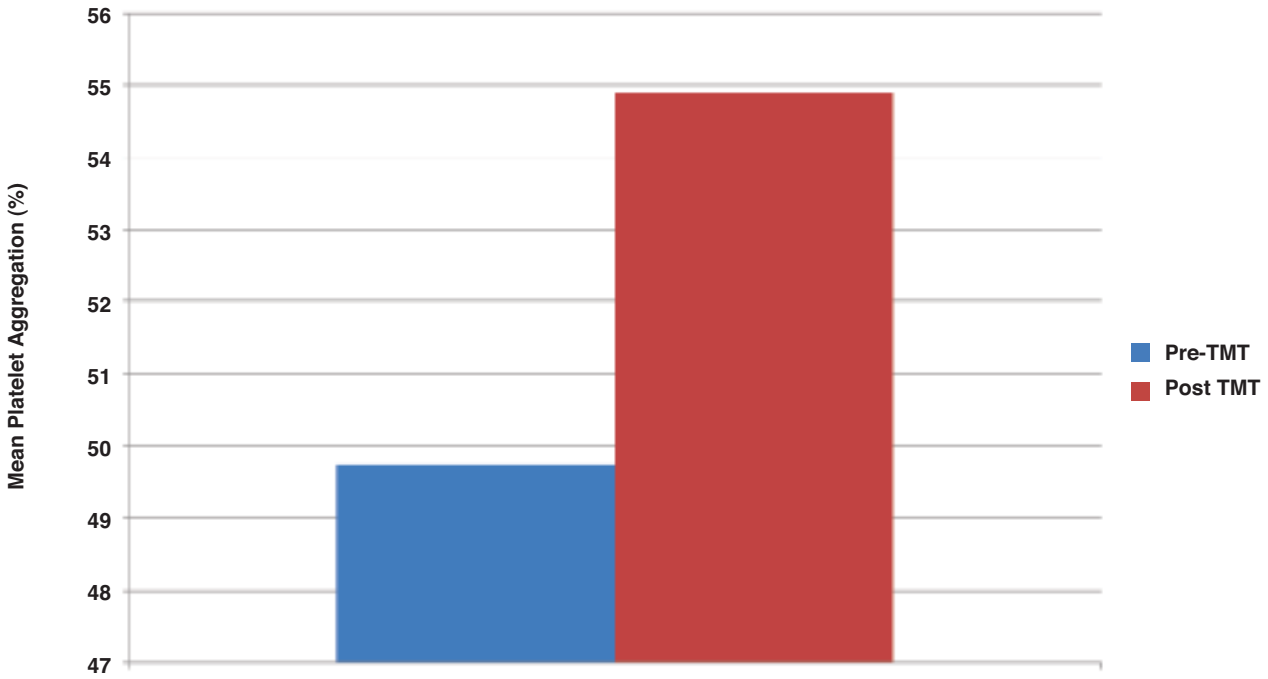

Figure 5: Effect of physical stress on Collagen induced platelet aggregation

There are controversial reports on the effect of exercise on platelet aggregation not only on healthy individuals but also in patients of CAD. Some of the inconsistencies regarding the effect of exercise on platelet aggregation can perhaps be explained by differences in physical conditions, i.e., healthy young individuals who do not engage in regular exercise increase their aggregability during exercise whereas those who are regularly participating in an exercise show the opposite effect[22,23].

In elderly, the failure of exercise to reduce platelet aggregability can be explained by their generally low level of physical activity. Another factor could be that young people release more platelet inhibitors such as prostacycline and NO (nitrous oxide) from the vessel wall than do the elderly. In mild hypertensive, the response to maximal exercise is an increased *in vivo* platelet activity and a similar reaction has been noted in patients with IHD[24].

In the present study also, the response to exercise on platelet aggregation is variably observed in patients of IHD with ADP induced aggregation. However, the response of collagen induced aggregation was consistently observed to be increased and was statistically significant. The possible cause of inconsistent observation of exercise induced aggregation may be because of hurmoral factors which may be stimulated and released by exercise such as catecholamines, vWF, leucocytes, thrombocytes, release of PF-4 which causes platelet leukocyte interaction and alteration in fibrinolytic system. The response to collagen-induced aggregation, which has consistently increased after stress, may be more sensitive indicator of underlying atherosclerosis involving the endothelial functions. This has already been reported that platelet collagen interaction *in vitro* may be comparable to platelet damage to vessel walls interaction *in vivo*[30].

Andreottii & Associates[13] have demonstrated that even low grades of exertion transiently enhance platelet aggregability in patients with CAD and not in subjects without apparent CAD. The increase in aggregability is independent of myocardial ischaemia. These findings suggest that platelet aggregability is enhanced by exercise in the presence of coronary atherosclerosis *per se*, as a result of hemodynamic factors interacting with arterial obstruction or more likely with endothelial dysfunction[26].

Another study has demonstrated increase platelet aggregability response to shear stress in patients with acute MI[14]. They observed that shear induced platelet aggregation was significantly higher in patients with acute MI than in patients with stable CAD and normal subjects. This variability also explains the observation in present study, which shows that response to stress is variable in patients with stable CAD.

Effect of physical and mental stress on platelet aggregation was also evaluated in 113 patients with stable angina and 50 healthy individuals by Wallen and associates[27]. They observed that platelet functions were more or less same at rest but physical exercise increased the platelet aggregability in both the groups. Platelet responses to mental stress were highly variable but more pronounced in angina patients than healthy controls.

Platelet activation, aggregation and adhesion is a complex phenomenon responsible for arterial thrombosis. It does involve the coagulation system with fibrin formation. The event starts with response of platelet against tissue or endothelial injury. The platelets come in rescue to produce platelet plug to stop bleeding (primary hemostasis). Platelets, as has been described, have multiple surface receptors. These receptors, when stimulated, produce change in shape of platelets. The major receptors involved are glycoprotein Ib (Gp Ib) receptor, which binds to von Willebrand factor (vWF). Besides these, there are receptors for adenosine diphosphate (ADP), thrombin and thromboxane A2.

With the shape change, there is change in the surface of the platelet that leads to expression of a second binding site, the glycoprotein IIb/IIIa (Gp IIb/IIIa) receptor. These Gp IIb/IIIa receptors bind fibrinogen to bridge between adjacent platelets. Furthermore, the surface of the platelet also expresses binding sites for factor V, which is an essential cofactor in the generation of thrombin[28,29].

The exact underlying mechanism of exercise-induced alteration in platelet aggregability remains unclear. However, there are certain evidences pointing towards the possible mechanism.

It has been observed that intense physical exercise increases plasma level of von Willebrand factor (vWF). Not only this but platelet aggregation is also associated with enhanced expression of adhesion molecules on platelets such as P-selectin (CD 625P) and glycoprotein (Gp) IIb/IIIa[30].

Exhaustive exercise leads to activation of several `stress hormones`. Epinephrine and vasopressin are the key regulators of the stress response. The magnitudes of responses are modulated by the relative intensities and duration of physical exercise. Epinephrine and vasopressin trigger the activation of endothelial cells, which may result in the release of ultra large vWF multimers (ULvWFM), which in turn induce platelet activation and thrombus formation under the state of high shear stress[31].

The biological activity of the platelet activating ULvWFM is regulated by a specific plasma metallo-protease ADAMTS-13 (A Disintegrin and Metallo-protease with Thrombo Spondin-1Repeats)[32]. Moreover, there is a compelling body of evidence that support the concept of a reciprocal behavior of the proteolytic activity of ADAMTS-13 and plasma vWF[33].

A similar association could be shown for various (patho-) physiological conditions, including systemic inflammation following the endotoxin challenge and stimulation of endothelial vWF release by desmopressin[34,35].

CONCLUSION

The present study clearly demonstrated that physical stress in terms of exercise increases platelet aggregation in patients with IHD. The response to collagen challenge is more specific and predictable, while ADP induced platelet aggregation shows variable response. The exact mechanism of exercise induced platelet aggregability is not clearly understood. However, there is a complex mechanism involving von Willebrand Factor, P-selectin, glycoprotein receptors and a specific plasma metallo-protease ADAMTS-13.

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Research Paper

Effect of Long - Acting Phosphodiesterase Type-5 Inhibitor - Tadalafil on Human Platelet Aggregation

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ABSTRACT

Introduction: *Erectile dysfunction is predominantly a vascular disease and may even be a marker for occult-cardiovascular disease.There are reports that PDE5 inhibitors inhibit platelet aggregation in animal models and only few studies presenting in vitro data of human platelet modulation by PDE type-5 inhibitor Sildenafil. The present study therefore, was planned to evaluate effect of long-acting phosphodiesterase type-5 inhibitor, tadalafil on human platelet aggregation.*

Methods: *The study was conducted on 30 healthy male volunteers between the age of 30 to 50 years. Tadalafil 10 mg and 20 mg was given to 15 patients in each group. Blood samples were collected after four and twenty-four hours of drug administration. All blood samples were subjected for the estimation of platelet aggregation on ELVI-840 aggregometer and Omni scribe chart recorded.*

Results: *Administration of Tadalafil has decreased platelet aggregation after 4 hrs and 24 hrs; which in both the cases was statistically significant. However, the decrease in platelet aggregation at the end of 24 hrs as compared to 4 hrs was not significant.*

Conclusion: *Tadalafil is effective inhibitor of platelet aggregation induced by ADP and collagen. Collagen induced aggregation is more significantly blocked by Tadalafil. The dose of 20 mg is more effective in inhibition of platelet aggregation at 24 hours as compared to 10 mg.*

KEYWORDS: Phosphodiesterases; cGMP; cAMP; NO; Erectile dysfunction

INTRODUCTION

Erectile dysfunction (ED) is a common condition and studies predict that it will become even more common in future. There is increasing evidence to suggest that it is predominantly a vascular disease and may even be a marker for occult-cardiovascular disease. The common pathological process is at the level of endothelium. The Massachusetts Male Aging Study (MMAS), a large population based random sample, confirmed that ED is highly correlated with vascular disease such as hypertension, heart disease and diabetes[1].

Several families of phosphodiesterase (PDE) enzyme have been identified and characterized[2]. Since selective pharmacological inhibitors of isoforms-5 (cGMP-specific PDE), such as sildenafil, vardenafil, tadalafil, have become available, the physiological function and interaction of different PDE isoforms, their tissue distribution and therapeutic potential of PDE 5 inhibition have attracted increasing interest[3]. The differential distribution of PDE isoforms in various tissues as well as selectivity of pharmacological agents is the basis for potential tissue specific effects of PDE inhibitors[4]. PDE5 is found in high concentration in smooth muscle cells of corpora cavernosa. Physiologically, nitric oxide (NO), which is released during sexual stimulation in the corpora cavernosum, increases concentration of cGMP by activating soluble guanylate cyclase, this in turn mediates vasorelaxation and subsequent filling of corpora cavernosa with blood. The cGMP is degraded by PDE5 and once PDE5 is inhibited by a phosphodiesterase inhibitor such as sildenafil, vardenafil or tadalafil, effects of cGMP are enhanced and erection is supported (Figure 1). PDE5 is also expressed in various other tissues, such as arterial vasculature, including pulmonary and coronary arteries, venous vasculature, skeletal muscles, visceral and tracheobronchial muscles and platelets.

Human platelets reported to contain three isomers of phosphodiesterase's (type 1,3,5)[5]. The activation of human platelets can be inhibited by two intracellular pathways, regulated by either cGMP or cAMP. However, nitric oxide causes the activation of cGMP dependent protein kinases, which prevents the agonist induced myosin light chain kinase and protein kinase C and inhibits the agonist induced calcium mobilization from intracellular stores without any major effect on ADP regulated cation channel [6]. Additionally, cGMP causes an increase of cAMP by inhibition of cAMP phosphodiesterase. Increased cGMP level inhibits agonist induced platelet aggregation [7].

There are reports that PDE5 inhibitors inhibit platelet aggregation in animal models and only few studies presenting *in vitro* data of human platelet modulation by PDE type-5 inhibitor Sildenafil[8-10]. The present study therefore, was planned to evaluate effect of long-acting phosphodiesterase type-5 inhibitor, tadalafil on human platelet aggregation.

MATERIAL AND METHODS

The present study was conducted on apparently 30 healthy male volunteers between the ages of 30 to 50 years. After obtaining institutional ethical approval and informed consent, the study subjects were randomly divided into two groups. Group I (n = 15) was administered Tadalafil 10 mg and Group II (n = 15) was administered Tadalafil 20 mg.

Exclusion criteria

- Diabetes, hypertension, hyperlipidaemia, ischemic heart disease
- Hepatic and liver dysfunction
- Smokers or consuming tobacco in any form.
- Use of drugs – NSAIDs, Antiplatelet, Nitrates, CYP inhibitors (erythromycin, ketoconazole, Cimetidine).

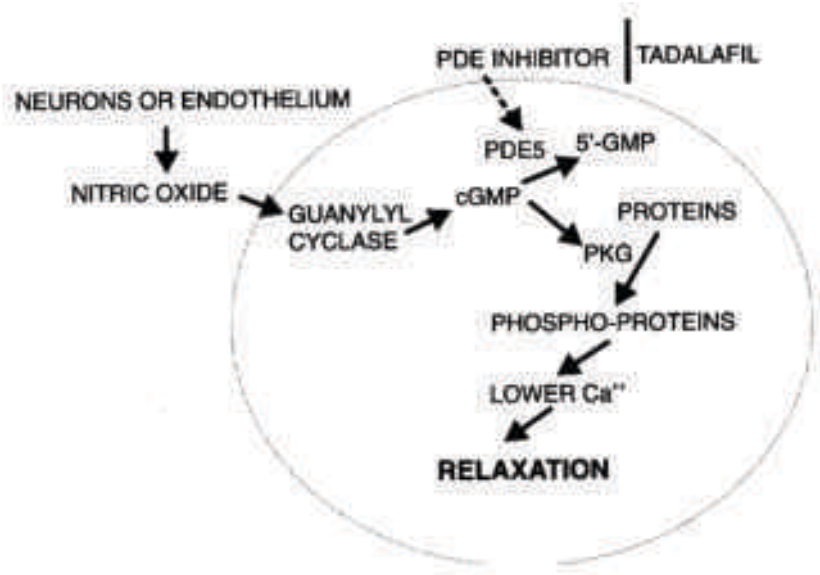

Figure 1: Regulation of penile corpus cavernosum smooth muscle relaxation and effect of PDE 5 inhibitor. (PDE, phosphodiesterases)

Study protocol

After an overnight fast, 4.5 ml of venous blood was collected

without undue pressure and a single dose of Tadalafil (10/20 mg) was administered. Subsequent blood samples were collected after four and twenty-four hours of drug administration. All blood samples were subjected for the estimation of platelet aggregation on ELVI-840 aggregometer and Omni scribe chart recorded.

Platelet aggregation

Most important function of platelets is their role in haemostasis i.e. adhesion to the damaged tissue surfaces and cohesion to one another. This cohesion phenomenon is known as aggregation and may be initiated by variety of substances including collagen, adenosine 5' diphosphate (ADP), epinephrine, serotonin and ristocetin. Aggregation is one of the numerous in vitro tests performed as a measure of platelet function. The described procedure is a turbidimetric method of measuring the effect of collagen, ADP and epinephrine on platelets.

Reagents

- 3.8 per cent citric acid (Trisodium salt dehydrate): Prepared by dissolving 3.8 gm citric acid in 100 ml of deionized water.
- Tris buffer: Tris (hydroxyl methyl), methylamine, 1.21 gm (0.01 M), disodium ethylene diamine tetra acetic acid 0.372 gm (0.001 M), sodium chloride 8.76 gm (0.15 M), dissolved in distilled water adjusted to pH 7.5 with hydrochloric acid and made up to one litre distilled water.
- ADP reagent: Adenosine 5'-diphosphate lyophilized with buffer salts (supplied by Sigma Diagnostics). ADP solution was prepared by reconstituting ADP reagent with 1.0 ml deionized water to yield solution containing ADP 2x10-4 mol/l. It was swirled to mix and allow to stand at room temperature (19-26°C) for 15 minutes before use. It was kept at room temperature only for duration of testing. The reconstituted reagent is stable for one month if stored in refrigerator (2 to 6°C).
- Collagen reagent: Collagen (calfskin) acid soluble, approximately 2 mg lyophilized with buffer salts. Collagen solution was prepared by reconstituting a vial of collagen reagent with 1.0 ml deionized water.

The vial was allowed to stand undisturbed for at least 15 minutes at room temperature before use. Warming to 37°C may be necessary to complete dissolution. It was swirled to mix prior to use and kept at room temperature only for the duration of testing. It is usually stable for at least 2 weeks, refrigerated (2-6°C) stability may be extended by freezing.

Specimen collection

Blood was collected by avoiding stasis and contamination with tissue fluids into plastic tubes containing 0.1 ml buffer and 3.8 per cent sodium citrate in a ratio of blood to anticoagulant in a ratio of 9:1.

Preparation of platelets rich plasma (PRP)

The anticoagulant sample was centrifuged at 400 rpm for 10 minutes. PRP was removed carefully using a plastic transfer pipette and was expelled into a plastic tube and covered it and kept at room temperature for duration of the test.

Preparation of platelets poor plasma (PPP)

It was prepared by again centrifuging the PRP at 6000 rpm for 10 minutes. Supernatant was transferred to a labelled PPP tube, covered and kept at room temperature for the duration of test.

The platelet count of PRP was adjusted to the range of 4 to 5 lac/mm3 by addition of autologous PPP to PRP sample. The caution should be taken to assay platelet aggregation within 30 minutes of collection of test samples.

Aggregating agents

ADP induced aggregation may occur in one or two phases and it may be followed by rapid disaggregation. Collagen induced platelet aggregation may occur in an irreversible single-phase curve or a reversible single-phase curve depending on the collagen concentration in the PRP.

Procedure

Cuvette with PRP was introduced into the aggregometer. The electromagnetic agitation was started by means of stirred control after having introduced a small stirring bar into the sample. Agitation speed was maintained at 1000 rpm. Baseline of the recorder was adjusted by means of the zero control. The cuvette with PRP was removed and cuvette with PPP was inserted. By means of gain control the maximum excursion of the pen on the recorded was adjusted. Cuvette with PPP was removed and cuvette with PRP was reinserted and it was readjusted, if necessary, by means of the zero control. The sliding of the recorded paper was started. The aggregating agents (ADP and collagen) were added to the PRP by means of micropipette. The aggregation was recorded for a minimum of five minutes and results were expressed as percentage aggregation.

Statistical Methods

Mean, percentage changes, standard deviation and standard errors of the mean were obtained. P values were calculated to determine the statistical significance of effect of Tadalafil administration to healthy humans (n=30) in doses (10 and 20 mg) on mean value of platelet aggregation induced by ADP and collagen. A $P < 0.05$ was considered significant.

RESULTS

The effect of Tadalafil on ADP induced platelet aggregation (%) in healthy volunteers has been shown in table 1. Administration of 10 mg of Tadalafil has decreased platelet aggregation by approx. 17 per cent and 13 per cent at 4 hrs and 24 hrs respectively which in both the cases was significant. However, the decrease in platelet aggregation at the end of 24 hrs as compared to 4 hrs was not significant (Figure 2).

Table 1: Effect of Tadalafil on ADP (2 × 10-4 mol/L) induced Platelet Aggregation (%) in Healthy Volunteers (n = 15)

Dose	Initial	After 4 hours	After 24 hours
10 mg	44.08 ± 3.13	36.58 ± 2.83 *P < 0.01	38.50 ± 2.77 *P < 0.02 **P = NS
20 mg	40.08 ± 3.66	36.08 ± 2.89 *P < 0.01	36.33 ± 3.56 *P < 0.05 **P = NS

All values are expressed as Mean ± Standard Error of Mean (SE)
P values:
*As compared to Initial
**As compared to 4 hr
NS = Not significant

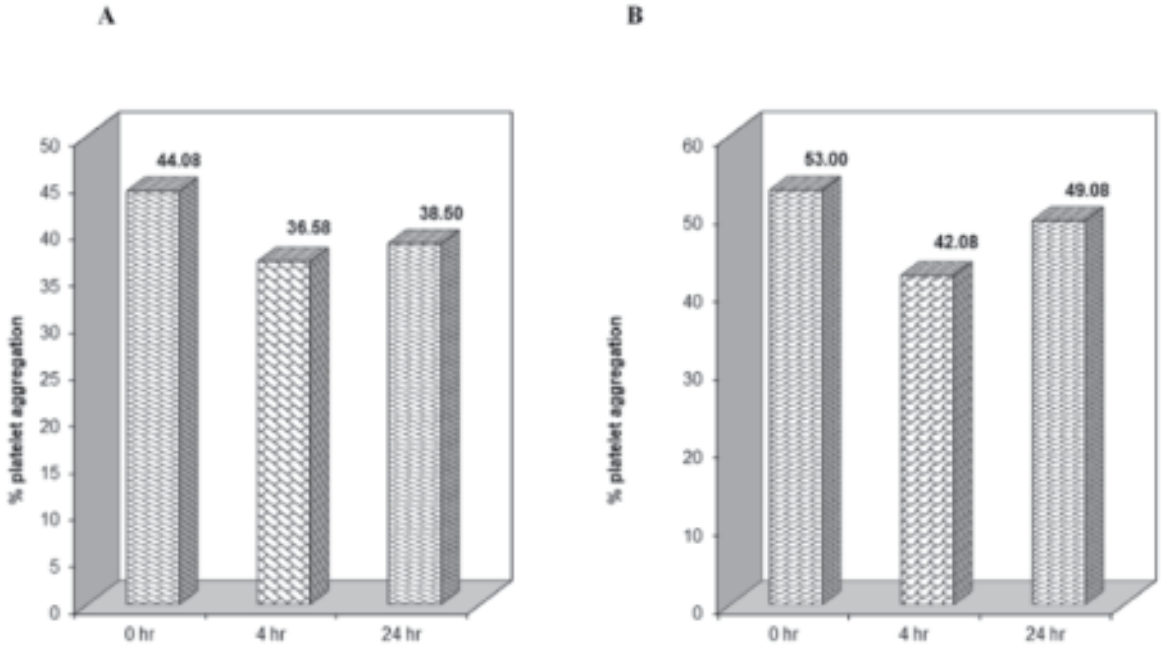

Figure 2: Effect of 10 mg Tadalafil on ADP (Panel A) and Collagen (Panel B) induced Platelet Aggregation in Healthy Volunteers

The effect of Tadalafil (20 mg) on ADP induced platelet aggregation in healthy volunteers is shown in table. Administration of 20 mg of Tadalafil has decreased platelet aggregation by about 10 per cent at the end of 4 hrs and about 9 per cent at the end of 24 hrs. This effect was seen in almost all individuals. The mean value of platelet aggregation has decreased from 40.08 ± 3.66 to 36.08 ± 2.89 at the end of 4 hrs and 36.33 ± 3.56 at the end of 24 hrs, which is statistically significant in both cases. However, difference in platelet aggregation at the end of 24 hrs compared to aggregation at the end of 4 hrs was not significant (Figure 3).

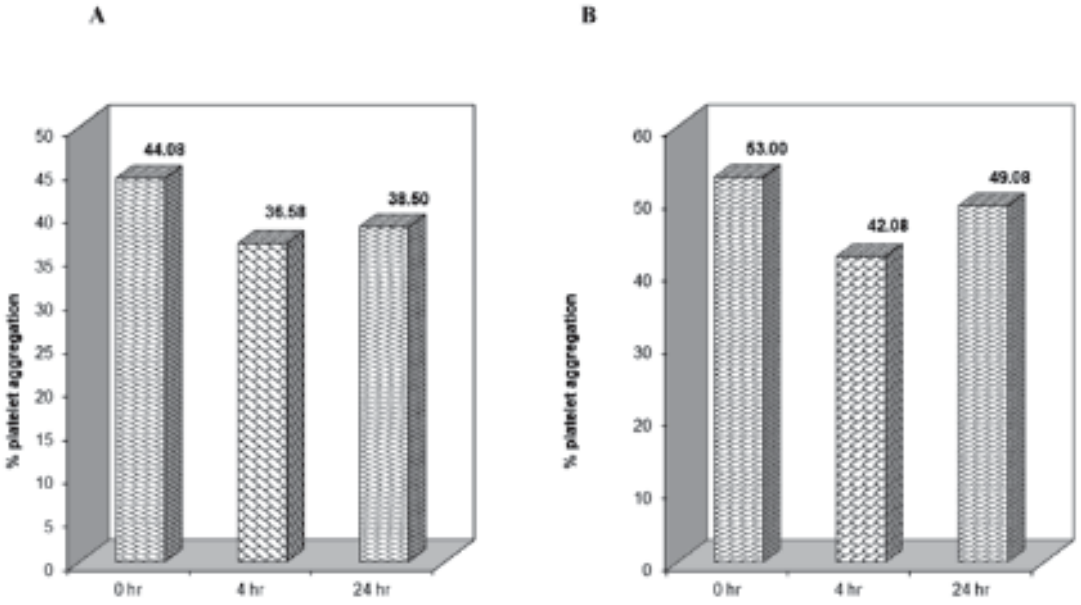

Figure 3: Effect of 20 mg Tadalafil on ADP (Panel A) and Collagen (Panel B) induced Platelet Aggregation in Healthy Volunteers

Table 2 shows the effect of Tadalafil on collagen induced platelet aggregation in healthy volunteers. Administration of 10 mg Tadalafil has decreased platelet aggregation by about 21% and 7% at the end of 4 hrs and 24 hrs. At the end of 4 hrs, the mean value of platelet aggregation has decreased from 53.00 ± 1.37 to 42.08 ± 2.17, which is statistically highly significant (P < 0.001). Similarly, at the end of 24 hrs, the mean value of platelet aggregation has decreased to 49.08 ± 1.14, which again is statistically highly significant (P < 0.001) (Figure 2). However, when platelet aggregation at the end of 24 hrs was compared to aggregation at the end of 4 hrs, the aggregation inhibition effect of tadalafil was no more seen and there was a significant (P < 0.001) rise in platelet aggregation.

Table 2: Effect of Tadalafil on Collagen (0.2 µg/ml) induced Platelet Aggregation (%) in Healthy Volunteers (n = 15)

Dose	Initial	After 4 hours	After 24 hours
10 mg	53.00 ± 1.37	42.08 ± 2.17 *P < 0.001	49.08 ± 1.14 *P < 0.001 **P < 0.001
20 mg	53.91 ± 1.83	46.66 ± 1.96 *P < 0.001	49.75 ± 2.06 *P < 0.002 **P = NS

All values are expressed as Mean ± Standard Error of Mean (SE)
P values:
*As compared to Initial
**As compared to 4 hr
NS = Not significant.

Administration of 20 mg Tadalafil has decreased platelet aggregation by 13 per cent and 8 per cent at the end of 4 hrs and 24 hrs respectively. The mean of platelet aggregation has decreased from 53.91 ± 1.83 to 46.66 ± 1.96 and 49.75 ± 2.06 at the end of 4 hrs and 24 hrs respectively (Figure 3). This effect was statistically significant at both the levels. When platelet aggregation at the end of 24 hrs was compared to 4 hrs, the difference was not significant.

DISCUSSION

It has long been accepted that elevation of cGMP has an inhibitory effect on platelet aggregation and PDE-5 inhibitor inhibits platelet aggregation and adhesion in animal models. There are only few reports of the effect of PDE 5 inhibitors on human platelet aggregation. Human platelets have been reported to contain 3 isomers of phosphodiesterase (Type I, II and V). The activation of human platelets can be inhibited by the intracellular pathways, regulated by either cGMP or cAMP. However, nitric oxide causes the activation of cGMP-dependent protein kinase. Additionally, cGMP causes an increase in cAMP by inhibition of cAMP phosphodiesterase. Increased cGMP levels inhibit agonist induced platelet aggregation. Tadalafil, is an orally administered phospho-diesterase-5 inhibitor, used for the treatment of erectile dysfunction. It acts by producing, elevation of cAMP in the presence of nitric oxide, and activation of guanyl cyclase that is released during sexual stimulation.

The present study was envisaged to observe the effect of longacting phosphodiesterase type-5 inhibitor, Tadalafil, on human platelet aggregation ex vivo. Administration of 10 mg Tadalafil significantly inhibited platelet aggregation induced by ADP at 4 hrs ($P < 0.01$) and 24 hrs ($P < 0.02$). The platelet aggregation inhibition was maximum at 4 hours and thereafter the inhibition was less. At 24 hrs, there was still significant ($P < 0.02$) inhibition of platelet aggregation as compared to baseline values, however, it was not significant as compared to 4 hours value. Collagen induced platelet aggregation inhibition, on the other hand, was more significant at 4 hours ($P < 0.001$) and 24 hours ($P < 0.001$) when compared to initial value. The effect did last for 24 hours, but when compared to 4 hours level, the aggregation was significantly ($P < 0.001$) more, suggesting thereby that the inhibition is rapidly reverting back. Increasing the dose of Tadalafil to 20 mg, the platelet aggregation induced by ADP was again inhibited significantly at 4 hours ($P < 0.01$) and 24 hours ($P < 0.05$), but importantly, both 4 hours and 24 hours values were essentially same, meaning thereby that the inhibition of platelet aggregation which was maximum at 4 hours was persistent to 24 hours. Likewise, the collagen induced platelet aggregation was very significantly decreased at 4 hours ($P < 0.001$) and 24 hours ($P < 0.002$) and the difference between 4- and 24-hours level was statistically not significant.

Wallis and colleagues3 have published their work showing *in vitro* data of human platelets that have been incubated with sildenafil or sodium nitroprusside or both. Berkels and associates[10] have demonstrated modulation of human platelet aggregation by sildenafil. The study revealed that sildenafil (50 mg and 100 mg) did not inhibit ADP induced platelet aggregation, whereas the collagen-induced aggregation was markedly reduced after 1 hr and significantly inhibited after 4 hrs of 100 mg of sildenafil administration. Verma and Jain observed the effect of PDE 5 inhibitor sildenafil on platelet aggregation ex-vivo in 30 healthy volunteers[11]. Sildenafil, in a single dose of 100 mg, significantly inhibited

collagen-induced platelet aggregation at 2 hrs ($P < 0.05$) and 4 hrs ($P < 0.001$). 50 mg sildenafil did inhibit platelet aggregation induced by collagen at 2 hrs and 4 hrs after its administration, but significantly only at 4 hrs ($P < 0.05$). ADP induced platelet aggregation, however, was not significantly inhibited by sildenafil in either dose. The effect of another PDE 5 inhibitor Zaprinast, which is 10 times less potent, has been reported[12]. Addition of Zaprinast to a nitric oxide solution resulted in potent increase of the inhibitory effect of nitric oxide. Li and associate studied the effect of Sildenafil on human platelet aggregation induced by restocetin or thombin[13]. They suggested that Sildenafil may have a biphasic effect on platelets, initially potentiating platelet aggregation and then inhibiting the response. They also observed that sildenafil sensitizes human platelets to the pro-aggregatory effects of ristocetin in a concentration dependent fashion (0.05- 1.1 μM). Similar observations were made with thrombin, wherein the pro-aggregatory effect was blocked by a protein kinase G (PKG) inhibition. The point to be seriously considered is that the effects of cGMP on platelet sensitization was biphasic; at relatively low concentration/short incubation time (<5 min), cGMP promoted platelet aggregation while at higher concentration and longer incubation times (5-10 min) the more customary inhibitory effects were observed. From a clinical perspective, the author speculate that sildenafil may potentiate platelet aggregation in patients with pro-thrombotic conditions and that this may explain the thrombotic complications in a small number of patients taking sildenafil. However, their clinical extrapolations are not supported by clinical data for Tadalafil, nor for the PDE 5 inhibitor class generally.

The present study conclusively demonstrates that Tadalafil in both dosage schedules inhibit platelet aggregation induced by ADP and collagen and the effect lasts up to 24 hours. However, increasing the dose from 10mg to 20 mg, the effectivity increases at 24 hours. The significant ($P < 0.001$) rise in aggregation induced by collagen observed at 24 hours with 10 mg Tadalafil group was checked when 20 mg was administered. Not only this, the ADP induced platelet aggregation was also significantly blocked by Tadalafil, the effect not observed by sildenafil. No adverse effects were observed in both dosage schedule in any of the volunteers.

The present observation is important in view of Tadalafil administration to subjects receiving other antiplatelet or anticoagulant medications. It might further enhance the antiaggregatory response and cause bleeding. However, no published data are available suggesting that bleeding time of patients treated with anticoagulants and antiplatelets is

increased by Tadalafil or any other PDE 5 inhibitors.

Erectile dysfunction is now been considered as vascular endothelial dysfunction, usually associated with other conditions such as hypertension and coronary artery disease. Administration of PDE-5 inhibitor Tadalafil will not only improve the erectile function for 48-72 hours but also check the thrombotic predisposition by favourably affecting platelet aggregation. This preposition however, needs further evaluation.

CONCLUSION

Tadalafil is an effective inhibitor of platelet aggregation induced by ADP and collagen. Collagen induced aggregation is more significantly blocked by Tadalafil. Twenty mg dose of Tadalafil is more effective in inhibition of platelet aggregation at 24 hours as compared to 10 mg dose. The possibility of interaction with other antiplatelet and anticoagulant medication needs further studies.

CONFLICT OF INTEREST: None

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Review

Platelet Physiology and Mechanics of Aggregation: A Review

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ABSTRACT

Platelets are anucleate blood cells that play a crucial role in haemostasis and thrombosis. They also participate in various inflammatory and immune processes, as well as in the pathogenesis of cardiovascular diseases. Platelet aggregation is the result of a complex interaction between platelet surface receptors, soluble agonists, and subendothelial components. Platelet aggregation can be modulated by antiplatelet drugs, which target different pathways of platelet activation and inhibit the formation of thrombi. Antiplatelet therapy is widely used for the prevention and treatment of arterial thromboembolic events, such as myocardial infarction, stroke, and peripheral vascular disease. However, antiplatelet therapy is not without limitations, such as bleeding risk, interindividual variability, and drug resistance. This review summarizes the current knowledge on platelet aggregation and antiplatelet drugs, with a focus on their mechanisms of action, clinical indications, and challenges.

KEYWORDS: Platelets, Thrombocytes, Aggregation, Haemostasis, Thrombolytic

INTRODUCTION - The Anucleate Cell

Giulio Bizzozero made the discovery of platelets in 1882[1], but for many years afterward, only biologists were interested in the dynamic and multifunctional properties of platelets. The tiniest blood particles are anucleate, discoid platelets, whose shape reveals their dynamic nature. They are mostly linked to the process of haemostasis, which starts blood coagulation. Despite their high level of activity, they often prefer to be dormant and only become active when a blood artery is compromised. However, platelets have several multifunctional purposes that monitor the body's homeostasis in addition to their primary role in haemostasis, or blood coagulation. One of the most accessible markers was finally determined by its great sensitivity to various illness states. It returns to its previous state as a significant inflammatory marker while maintaining connections with leukocytes and endothelial cells[2]. Certain physiologically active indicators, such as CD36, CD41, CD42a, CD42b, and CD61, are highly dependent on platelet reactivity for various disease aetiology. Several platelet secretory products and active surface receptors are among them. A vast area for studying illness development is made possible by platelets, which have a tendency to change the production and signalling of these markers in various disease diagnostic and prognostic situations.

Platelet activity is mostly linked to the start of coagulation cascades. When a blood artery is damaged, platelets primarily target the subendothelial surface, which maintains haemostasis. The activity of platelet adhesion to subendothelial surfaces is promoted by a variety of proaggregatory stimuli, commonly referred to as platelet agonists. A platelet undergoes a morphological change during this process, releases its granule contents, and

progressively adheres to other platelets to create aggregates[3]. Thus, reducing blood loss continues to be its major objective. But as was previously said, platelets are essential to the pathophysiology of many diseases and are not just involved in controlling thrombosis and haemostasis. For many years, the relationship between platelet interaction and the development of cardiovascular disease remains unclear[4]. Another intriguing topic to investigate is platelet hyper aggregation in diabetic individuals with CVD. In certain circumstances, platelet hyperactivity in a variety of conditions can have negative consequences, particularly in coronary artery disease, where hyper aggregation obstructs blood flow.

The linked processes of thrombosis and haemostasis are mediated by interactions between blood components and vascular walls. The physiological halt of bleeding at the site of vascular damage is known as haemostasis. The coordinated action of platelets, the vessel wall, and coagulation factors orchestrates it. The abnormal production of blood clots brought on by improper activation of haemostatic systems is referred to as thrombosis. The essential blood components in these processes are platelets, which do not interact with blood arteries in a physiological state. Nevertheless, they quickly attach themselves to the broken artery wall at the site of injury in reaction to vascular damage, which sets off a series of processes that include the recruitment of more platelets (aggregation), leukocytes, and the activation of blood coagulation[5]. While these processes facilitate the halting of bleeding and the healing of wounds during "physiologic" haemostasis, they also play a role in "pathologic" thrombosis, which is a complication of several human diseases, including atherosclerosis. While the outcomes of haemostasis and thrombosis are different, there is a significant commonality in the molecular processes behind both reactions.

STRUCTURE AND PHYSIOLOGY

Mammalian platelets are anucleated cells arising from cytoplasmic fragmentation of megakaryocytes in the bone marrow, and have a typical diameter of ~2–3 μm. Platelets circulate in a discoid form (***Figure 1***) and their average lifespan in humans is ~10 days[6]. However, following activation, they undergo dramatic changes in shape and ultrastructure; the membranes become ruffled with cytoplasmic projections and the granules are centralized and discharged[7,8]. Normal human platelet count is ~150,000–400,000/μl, though spontaneous bleeding resulting from reduced (but functionally normal) platelets is unusual at levels >10,000/μl[9].

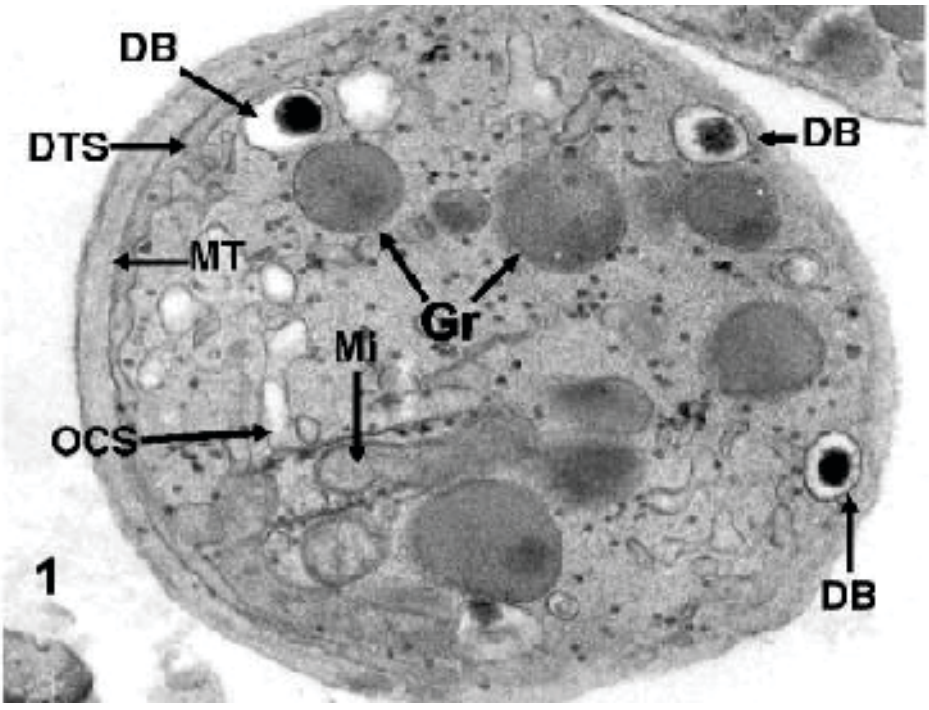

Figure 1: TEM image of Human Platelet, Granules (Gr), Dense Granules (DB), Open Canalicular System (OCS), and the Dense Tubular System (DTS), Glycogen (Gly), Mitochondria (Mi), Circumferential band of Microtubules (MT). (Re-distributed with permission from Reference 8)

Platelets participate actively in a wide range of physiological and pathologic processes despite not having a nucleus. Numerous mediators found in platelets control thrombosis and haemostasis in addition to performing a wide range of other tasks including chemotaxis, which is the attraction of other cells, vasomotor activity, cell proliferation, and inflammation. Both the cytoplasm and the cell membrane include components that are relevant to thrombosis, mostly in the form of platelet granules. The typical bilayer of phospholipids that makes up the platelet membrane also contains membrane glycoproteins that interact with different types of ligands, such as fixed ligands found on other cells or within the vessel wall that the platelets adhere to, or soluble ligands that activate the platelets. The network of many invaginations into the platelet interior, which is connected to the exterior via tiny holes known as the open canalicular system, is one of the platelet's distinctive characteristics (OCS)[10,11].

Platelet Markers

The phospholipid bilayer that makes up the platelet plasma membrane is where a variety of surface receptors and lipid rafts are expressed, aiding in intracellular trafficking and signalling. CD36, CD63, CD9, GPCR, IIbIIIa, and GLUT-3 are some of these markers. Additionally, these surface receptors cause the release of α granules, which are involved in coagulation, inflammation, atherosclerosis, host defence against microbes, angiogenesis, wound healing, and carcinogenesis[12]. GPCR is one of these surface receptors that has been shown to be essential for the secretion of ADP, the main secretory product, from dense granules. The inner layer of the plasma membrane contains asymmetrically ordered phospholipids (phosphatidylserine and phosphatidylinositol, for example) that stabilise the membrane's surface when it is not procoagulant[13].

Activation and Storage

In order to start coagulation cascades, the platelet surface progressively exposes amino phospholipids during activation (***Figure 2***) through ATP-dependent floppies and scramblases[14]. The "tunnel" system that runs the length of a platelet cell and is still attached to the plasma membrane is called the open canalicular system (OCS)[15]. The primary function of OCS is to allow external substances to enter platelets and to expel the granules inside. It not only serves as a significant location for the storage of glycoproteins in the plasma membrane but also aids in the development of filopodia during platelet activation[16]. The main function of the dense tubular system of platelets, which is a closed-channel network of remnant endoplasmic reticulum, is calcium sequestration through a series of events that activate the G protein-coupled receptor PAR-1[17,18]. Platelets' highly specialised cytoskeleton keeps the cell from shearing in the circulation and preserves its discoid features. The actin cytoskeleton, the marginal microtubule coil, and the Spectrin-based membrane skeleton make up its major parts.

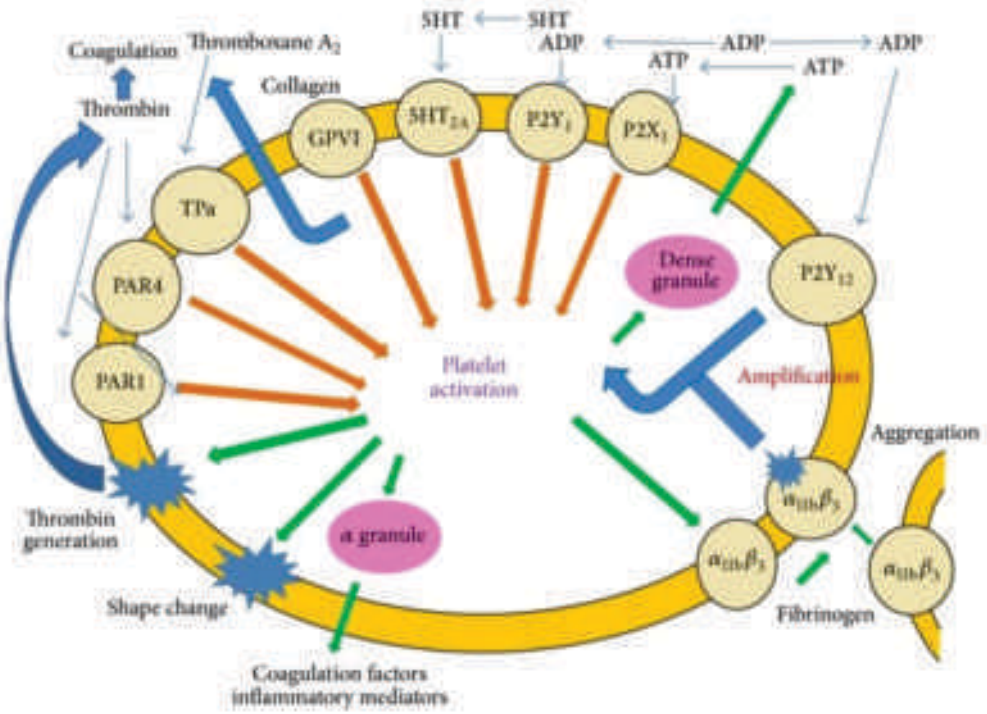

Figure 2: Following platelet activation, ADP is secreted in dense granule form, activating P2Y12 and amplifying procoagulant, proinflammatory, and aggregation responses. (Copyright Ghoshal K., Bhattacharyya M[19])

The two main storage granules found in platelets are called Dense and α granules, and they are responsible for storing physiologically active chemicals that are specifically engaged in the onset of coagulation and the recruitment of additional cells during inflammation[20]. The more common α granule has proteins (including fibrinogen, vWf, and GPIIbIIIa) that start the coagulation cascades. The α granule also contains a number of membrane proteins necessary for platelet function, including as CD36, P-selectin (CD62P), and GPIIbIIIa. The majority of cellular P-selectin is also present in the membrane of α granules. It has been revealed that P-selectin recruit's neutrophils through P-selectin glycoprotein ligand (PSGL1)[19,21]. Numerous hemostatically active substances, including as catecholamines, serotonin, calcium, adenosine 5′-diphosphate (ADP), and adenosine 5′-triphosphate, are released during platelet activation and are stored in dense granules (ATP). ADP is a mild platelet agonist that causes aggregation, granule release, and a change in platelet morphology[22].

Dense Granules

Because of their high calcium and phosphate content, platelet dense granules—the smallest granules—appear as dense masses under electron microscopy (***Figure 1***)[23]. Each platelet has three to eight thick granules. They also have elevated levels of serotonin and adenine nucleotides. Little GTP-binding proteins and related adhesion molecules, including as GPIb, GPIIb/IIIa, and P-selectin, which are typically found on other platelet compartments, have also been observed to be present in dense granules. Dense granule membrane proteins combine with the platelet plasma membrane during platelet activation, releasing the contents of the granules into the extracellular space. Both local vasoconstriction and the recruitment of more platelets (aggregation) are facilitated by the released components (e.g., serotonin). Dense granules' ADP is considered to come from the storage pool since it is mostly used in haemostasis and does not equilibrate with the metabolic pool of ADP. While the processes for releasing dense granules are similar to those found for α-granules, there have been reports of differences in the roles of certain SNARE proteins between the two granule populations[24]. Patients with a lack of these granules have bleeding disorders, demonstrating the critical role that dense granules play in proper haemostasis. Hermansky-Pudlak syndrome (HPS) and Chediak-Higashi syndrome are two uncommon human disorders linked to a susceptibility to bleeding. These illnesses have been linked to platelet dense granule deficiency[25].

α Granules

The most common, heterogeneous, and biggest platelet granules, measuring between 200 and 400 nm, are called alpha (α) granules[12]. Each platelet contains around 50–60 granules, which are what give the cytoplasm in peripheral blood smears its granular look (stained with Romanowsky stains). Most of the platelet factors involved in thrombosis and haemostasis are found in these granules. These comprise a number of coagulation-related factors as well as big polypeptides such thrombospondin, P-selectin, platelet factor 4, and beta thromboglobulins (Factors V, XI, XIII, fibrinogen, von Willebrand factor and high molecular weight kininogens). Numerous adhesion molecules, including vitronectin and fibronectin, that are involved in the contact between platelets and artery walls are also present in α-granules. Some proteins found on the membrane of α-granules are also expressed on the membrane of platelets, including P-selectin, GPVI, GP IIb/IIIa, and GPIb complex. Furthermore, proteins implicated in inflammation and wound healing, as well as a variety of chemokines and mitogenic growth factors such as transforming growth factor-β, vascular endothelial growth factor, and platelet-derived growth factor, are found in α-granules. Platelet α-granule components are being increasingly understood; for instance, a recent proteomic investigation of α-granules identified 284 non-redundant proteins, 44 of which had not been previously reported in these granules[26]. Moreover, the composition of α-granules varies, and different agonists may cause the release of distinct subpopulations of these granules[27]. Research is now being done to better understand the mechanisms underlying the differential release of α-granules and the wide spectrum of consequences that result from the release of their many elements.

The intricate process of α-granule secretion during platelet activation includes coalescence inside the platelet centre, granule fusion with the OCS and with one another, as well as fusion with the plasma membrane[12,20,28]. The intricate machinery required for granule release is present in platelets and includes membrane lipids, related proteins, and soluble NSF [N-ethylmaleimide-sensitive factor] attachment protein receptors (SNAREs)[28,29]. Certain components of α-granules, including P-selectin, perform their primary physiological function after being integrated into the platelet membrane[30]. After being released from granules, additional granule contents function and take part in platelet aggregation, thrombosis, platelet sticky contacts with leukocytes and other substrates, and the release of several growth factors that regulate cell proliferation. A rare genetic condition called Gray Platelet syndrome (GPS) is characterised by a deficiency of platelet α-granules, which leads to both quantitative and qualitative platelet dysfunction as well as a susceptibility to bleeding[31]. In GPS, aberrant α-granule development during megakaryocytic differentiation prevents proteins endogenously produced by megakaryocytes or endocytosed by platelets from entering the α-granules of platelets. Fibrosis ensues from the continuous release of α-granule contents, including cytokines and growth factors, into the bone marrow (myelofibrosis). In peripheral smears, the platelets have a grey morphology[31].

PLATELET RECEPTORS AND ADHESION MOLECULES

Selectin

The biggest adhesion molecule in the selectin family is P-selectin (CD62P), which has a molecular weight of 130 kDa. It is mostly seen on platelet α-granules, but it has also been reported on dense granules[32]. It is also found in endothelial

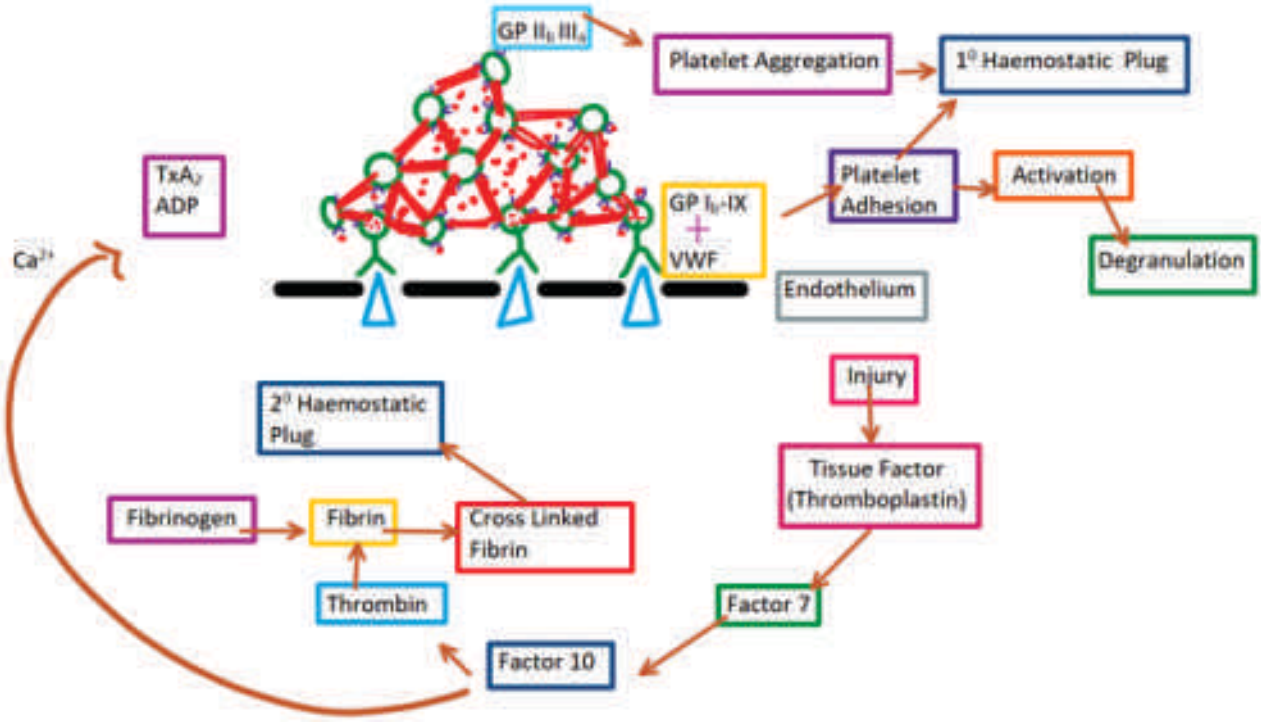

Figure 3: Platelet adhesion and Haemostatic Plug formation

cells' Weibel-Palade structures. There is not much P-selectin visible on the surface of platelets when they are at rest and not activated. On the other hand, P-selectin is rapidly expressed on the surface of activated platelets (or endothelial cells) due to the fusing of granule membranes with the cell membrane. It has been shown that the peak surface expression of P-selectin ranges from around 30 seconds to 10 minutes after stimulation, however the kinetics of this response depend on the agonist and dosage[33,34]. One of the most widely utilised indicators of platelet activation is P-selectin surface expression. 30 P-selectin glycoprotein ligand-1 (PSGL-1), expressed mostly on leukocytes[35], von Willebrand factor[36], glycoprotein Ibα[37], and sulfatides[38] are some of the ligands for P-selectin. In addition to its role in thrombosis and haemostasis, platelet P-selectin also plays a role in the interactions of endothelial cells, leukocytes, and platelets during inflammation[39]. Furthermore, thrombosis may be facilitated by a soluble version of P-selectin that is found in plasma[40].

GP (Glycoprotein) Ib/IV/IX

The primary platelet receptor for von Willebrand factor (vWF) is this big glycoprotein receptor complex, which is made up of four different components. These include the primary binding site for vWF, GPIbα (around 145 kDa), GPIbβ (around 20 kDa), GPIX (around 16 kDa), and GPV (around 82 kDa). Two GPIbβ subunits and GPIbα are connected by membrane-proximal disulfide linkages. GPIb, sometimes referred to as the α/β2 complex, has a non-covalent association with GPIX, whereas GVP has a more tenuous association with two GPIb-IX complexes[41]. When vWF binds to GPIb, signal transduction pathways are triggered, activating platelet integrin GPIIb/IIIa (αIIb/β3). This platelet integrin can then bind fibrinogen or vWF to drive platelet aggregation. Not only is GPIbα the primary vWF receptor on platelets, but it has also been shown to bind a wide range of ligands, such as kininogen[48], integrin Mac-1 on leukocytes[43], integrin Mac-1[42], thrombin[44], coagulation factors XI, XI, and VIIa[45-47], and thrombospondin[42]. On the other hand, GPIbα's interaction with vWF is more understood than its binding to these other ligands. GPIb's cytoplasmic C-terminal tails interact with filamin, calmodulin, 14-3-3, and the regulatory p85 subunit of phosphoinositide 3-kinase in addition to having many serine phosphorylation sites[41]. GPIb-IX-V is connected to the membrane cytoskeleton by interaction with filamin; effective production of the GPIb-IX complex on the transfected cell's plasma membrane requires the presence of all three subunits. The Bernard-Soulier Syndrome is a bleeding illness caused by deficiency or malfunction of the GPIb complex[49] and several mutations causing the disease have been linked to the genes encoding GPIbα, GPIbβ, and GPIX[50]. Although vWF is typically available in plasma and this receptor is constitutively present on the platelet plasma membrane, interaction of the receptor with its ligand requires a conformational change in one or both of these components. These modifications are brought about by variations in blood flow and the ensuing shear stress. In vitro evaluation of this interaction is conducted using the antibiotic Ristocetin, which causes these alterations in the absence of shear stress[51].

GP IIb/IIIa

Because platelet GP IIb/IIIa (αIIbβ3) is crucial for platelet aggregation, it has been the subject of the most research. It is a heterodimer, just like other integrins, with an alpha subunit (αIIb, about 136 kDa) and a beta subunit (β3, ~92 kDa). Unstimulated human platelets have around 80,000 copies of GP IIb/IIIa on their surface, and during platelet secretion, extra molecules from the membranes of platelet granules are translocated to the platelet surface[52]. When platelets are at rest, this molecule is constitutively produced on the plasma membrane in an inactive state; nevertheless, upon activation, it changes shape. This integrin is made up of flexible stalks that carry its transmembrane (TM) and cytoplasmic domains, as well as a large extracellular nodular head that contains its ligand-binding site[53,54]. The alpha subunit's nodular head is folded into a β-propeller shape, and the extracellular part of the αIIb stalk is made up of two "calf" and one "thigh" domain. The β3 head is composed of a hybrid domain with a fold like that of I-set immunoglobulin domains and a βA-domain with a metal ion-dependent adhesion site (MIDAS) motif. A PSI (plexin, semaphorin, integrin) domain, four tandem epidermal growth factor (EGF) repeats, and a distinct carboxyterminal βTD domain make up the β3 stalk. The head area is notably hunched over in a small "V" form while at rest . A structural alteration of the headpiece is brought about by activation, which causes the αIIb and β3 domains to move from their closed conformation with adjacent stalks to their open conformation with separated stalks. This reveals the ligand binding site, which is made up of a β3βA-domain and forms a “cap” with four loops on the upper surface of the αIIbβ-propeller domain. In addition to fibrinogen, platelet GPIIb/IIIa can bind to additional ligands such vWF, fibronectin, and vitronectin[55]. For individuals suffering from thrombotic diseases, the tTis molecule is a prominent target for targeted treatment[56].

Collagen Receptors

Primary haemostasis requires platelets to interact with subendothelial collagen. The two main collagen receptors, α2β1 integrin and glycoprotein VI (GP VI, approximately 65 kDa), are important for haemostasis. These receptors have varying degrees of affinity when binding to particular collagenous sequences[57]. Sequences containing the GER triplet (glycine-glutamic acid-arginine) are bound by α2β1, whereas sequences containing two or more GPO triplets (glycine-proline-hydroxyproline) are bound by GPVI[58,59]. Through the stimulation of the small GTPase Rap1b, which is dependent on phospholipase C (PLC), platelet adhesion facilitated by integrin α2β1 leads to the activation of platelet GPIIb/IIIa[60]. Platelet GPVI is expressed constitutively on both α-granules and the platelet plasma membrane[61]. After platelet activation, GPVI surface expression rises and intracellular expression falls, which is in line with their release from α-granules and plasma membrane integration. Comprising two C2 immunoglobulin-like domains and an arginine residue in the transmembrane region that forms a salt bridge with the aspartic acid residue of the Fc receptor γ (FcRγ)-chain, GPVI is a member of the immunoglobulin superfamily[62]. When collagen activates its immunoreceptor, its tyrosine-based activation motif (ITAM) is phosphorylated. This sets off a series of events involving many adaptor proteins, culminating in the phosphorylation and activation of PLCγ2[63,64]. Collagen type III, for example, is one of the collagen types to which GPVI primarily binds. A propensity for bleeding is linked to the lack of GPVI in humans, a crucial adhesion molecule involved in haemostasis and thrombosis[65].

Thrombin Molecules

Thrombin is a powerful platelet stimulant and an essential part of the blood coagulation cascade. Protease-activated receptors are responsible for mediating platelet responses to thrombin (PAR). Among G protein-coupled receptors, PAR are distinct in that thrombin's proteolytic cleavage of the receptor results in the unmasking of a particular ligand[66]. When thrombin attaches itself to the extracellular domain of PAR-1 and PAR-4, the receptor is activated and signalling is induced. The cleaved amino terminus has a tethered ligand attached to it. Activating the thrombin receptor potently without requiring receptor cleavage, synthetic peptides known as thrombin-receptor agonist peptides (TRAPs) imitate the new amino terminus and cause platelet activation, secretion, and aggregation. To stop platelet activation once thrombin binds to platelets, both PAR-1 and -4 activation must be blocked. In mouse platelets, the reaction to thrombin is mediated by PAR-3 and PAR-4, not PAR-1 and PAR-4[67,68]. Although PAR3 is expressed in human platelets as well, it does not seem to have a role in the platelet responses to thrombin, in contrast to mice[67,69]. Platelet activation, shape change, and granule release are all caused by thrombin signalling through either PAR1 or PAR4; PAR1-dependent reactions are seen at lower thrombin concentrations than those caused by PAR4[67]. Though both PAR-1 and PAR-4 bind to Gq and G12/13 G-proteins, activating phospholipase C, calcium mobilisation, and protein kinase C, the signalling pathways downstream of PAR-1 and PAR-4 in human platelets are not well understood[70]. Large-scale clinical studies are being conducted on thrombin receptor antagonists, which may offer a potential target for therapeutic antithrombotic treatment[71].

ATP and ADP

Although the reaction to ADP differs from that to thrombin, it has long been known to stimulate platelet adhesion and aggregation. Human platelets undergo an initial, reversible aggregation without granule release when exposed to modest amounts of ADP. Increased ADP concentrations cause prostaglandin production and granule release, which results in a distinctive biphasic response with irreversible aggregation.

The G-protein coupled P2Y receptor family of G protein-coupled, seven transmembrane domain receptors mediates the human platelets' response to ADP (P2Y1 and P2Y12). The P2Y1 receptor mediates platelet shape change and aggregation by coupling to Gq and releasing intracellular calcium ions. Antithrombotic drugs including ticlopidine, clopidogrel, and prasugrel target the P2Y12 receptor, which is linked to the suppression of adenylyl cyclase via Gi[72]. A lifelong bleeding disease is linked to mutations in the P2Y12 receptor[73].

Although ATP is not as strong as ADP, it nevertheless causes shape changes in platelets and increases their reactivity to other agonists like collagen. ATP-induced signalling is carried out by a ligand-gated ion channel (P2X1)[74].

Thromboxane A_2

A consequence of the metabolism of arachidonic acid is thromboxane A2 (TXA2). Two isoforms of thromboxane receptors, TPα and TPβ, have been discovered; TPα is expressed more often in human platelets than TPβ[74,75]. Moreover, TP is a member of the G protein-coupled receptor (GPCR) family. Studies have suggested that it might interact with Gq and G13 to activate phospholipase C and RhoGEF, respectively[76]. TXA2 is released by many cells, including platelets, and it exerts paracrine and autocrine actions. Through altering platelet shape, aggregation, degranulation, and amplifying reactivity to additional agonists, TXA2 raises platelet activation. Aspirin, a commonly used antiplatelet medication, works by inhibiting cyclooxygenase-1 to reduce the production of TXA2, and direct inhibitors of TP-α are being researched for potential therapeutic use[77,78]. Platelets express receptors for other prostanoids, including prostaglandin E2 and prostacyclin, which prevent platelet aggregation (which has a biphasic effect on platelets)[79].

PLATELET AGGREGATION

Physiology and Mechanics

After platelets first adhere to the site of damage, aggregation—which involves platelet-to-platelet adhesion—is required for efficient haemostasis. A variety of agonists, including collagen and adenosine diphosphate (ADP), which are present at the sites of vascular damage, activate platelets after adhesion. By attaching to certain receptors on the previously mentioned platelet surface, these agonists stimulate platelets. When these receptors are occupied, a chain of subsequent processes occurs that raise the intracytoplasmic concentration of calcium ions. Calcium influx via the plasma membrane[80] and release from intracellular reserves cause a rise in intracellular calcium in platelets. Phospholipase Cβ (PLCβ) is activated by receptors linked to G-proteins, such as those to thromboxane A2 (TXA2), thrombin, and ADP. On the other hand, phospholipase Cγ (PLCγ)[81] is preferentially activated by receptors acting through non-receptor tyrosine kinase pathways, such as collagen receptor GpVI. Two second messengers, inositol triphosphate and diacylglycerol (DAG), are produced when PLCβ or PLCγ is activated (IP3). While IP3 releases calcium from intracellular reserves, DAG facilitates calcium influx. Furthermore, certain agonists, such as ATP binding to the ligand-gated ion channel receptor P2X1[74], can directly trigger calcium influx.

Numerous structural and functional alterations in the platelet are brought about by an increase in the concentration of platelet-free calcium. The platelet undergoes a significant morphological transition from a disc to a spiny sphere (a process called shape change). The platelet's granules are centralised, and their contents are released into the open canalicular system's lumen before exiting the system altogether (the release reaction). Arachidonic acid is released from membrane phospholipids by membrane phospholipase A2, which is activated by an increase in platelet calcium. The enzyme cyclooxygenase 1 transforms arachidonic acid into prostaglandin H2 (PGH2), an intermediate product (COX-1). Thrombin synthase[82] proceeds to further convert PGH2 into TXA2. TXA2 is a powerful platelet activator. The shape-change response produces lengthy membrane projections that enable platelets to connect and form aggregates. The platelet cytoskeleton, which is made up of an ordered web of actin filaments, microtubules, and many related proteins connected to a range of platelet signalling molecules, is responsible for mediating shape change in platelets[83]. Actin polymerization, myosin light chain phosphorylation, and extensive cytoskeleton network remodelling are the outcomes of platelet shape change; these reactions vary depending on the time and stimuli[83,84].

Platelet aggregation is facilitated by the membrane protein GPIIb/IIIa complex, which is a major adhesion molecule. Platelets have a high density of GPIIb/IIIa integrin receptors on both the plasma membrane and α-granules[52]. In platelets that are at rest, it exists in an inactive state. Almost all agonists that activate platelets cause GPIIb/IIIa to undergo conformational changes, or "inside-out signalling," which makes them capable of binding soluble plasma fibrinogen. Thus, GPIIb/IIIa undergoes conformational changes that are directed toward the cytoplasm upon ligand attachment (a process known as "outside-in signalling"). It is still unclear exactly which set of circumstances led to these signalling events[53,86]. It is unclear how exactly receptor clustering, phosphorylation, and interactions with cytoskeletal and other cytoplasmic molecules cause conformational changes in GPIIb/IIIa. However, on neighbouring platelets[80], the receptor-bound fibrinogen serves as a link between two GPIIb/IIIa molecules. This completes the general process by which platelet chemical agonists cause platelet aggregation. When high shear circumstances are present, platelet aggregation is mediated by fibrinogen binding to GPIIb/IIIa[87]; however, in the case of platelet aggregation produced by low shear, vWF acts as a bridge molecule in place of fibrinogen.

Some molecules have lately been postulated as potential mediators of these reactions, even though GPIIb/IIIa is the most extensively investigated mediator of stabilising thrombi and connecting platelets to each other. These consist of CD40 ligand[88,89], SLAM (signalling lymphocyte activation molecule) family proteins, and junctional adhesion molecules (JAMs). It is yet unclear how these processes relate to one another in platelet aggregation.

Through many feedback amplification loops, activated platelets attract more platelets to the expanding haemostatic plug. They do this by releasing platelet agonists such ADP and serotonin that is stored in α-granules and by synthesising de novo proaggregatory TXA2. By encouraging the inclusion of more platelets in the haemostatic plug established at sites of

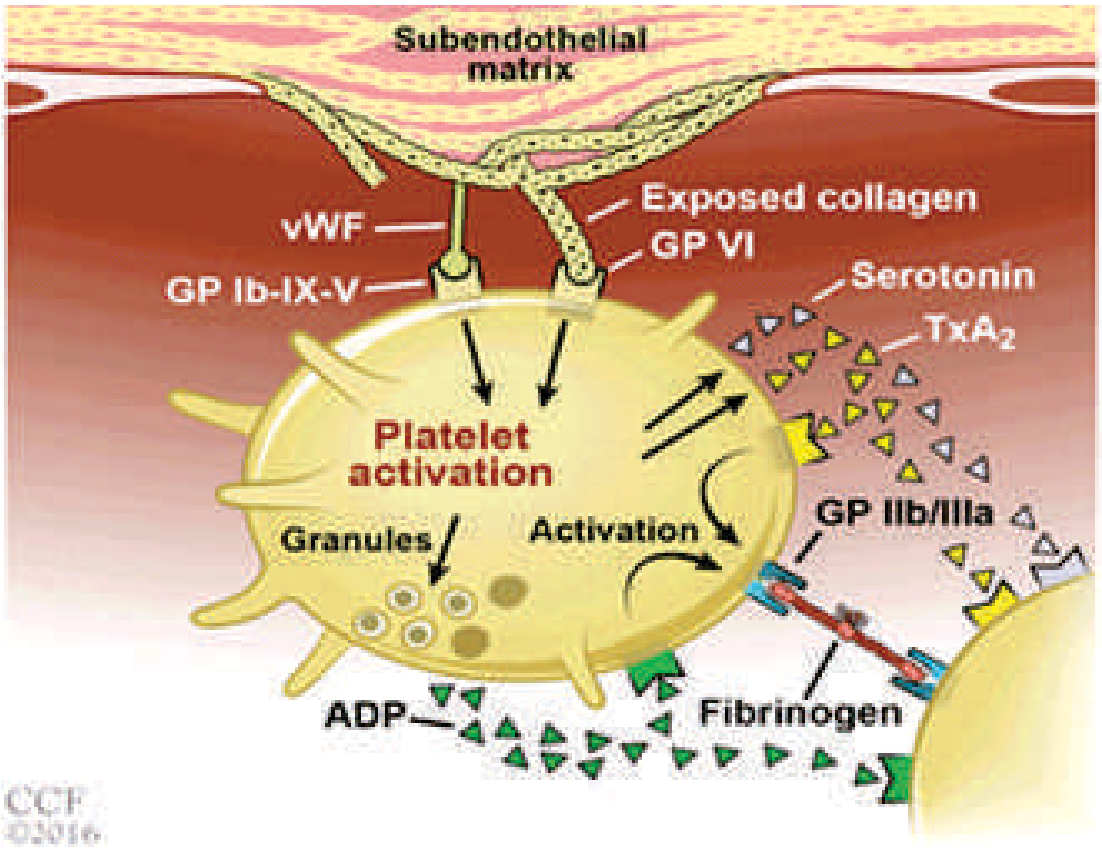

Figure 4: Activation of Platelet Aggregation Cascade

vascular damage, the release of ADP and TXA2 synthesis consolidates the original haemostatic plug. Last but not least, platelets also contribute significantly to secondary haemostasis by acting as a very potent catalytic surface to initiate the coagulation cascade. Negatively charged phospholipids go from the membrane bilayer's inner to outer leaflets when platelets are activated. Procoagulant vesicles rich in anionic phospholipids are known to bleb and release in response to the trans bilayer migration of these molecules. Microvesicles and active platelets both serve as binding sites for coagulation system cofactors and enzymes, which effectively produce thrombin, a powerful platelet agonist.

Pseudopods begin to grow on platelets when intracellular Ca2+ concentration rises over a certain threshold. Platelet-platelet aggregation is triggered by the exposure and activation of platelet fibrinogen receptors (GPIIb/IIIa) during shape change. This reversible process is often referred to as primary aggregation. Nevertheless, fibrinogen cannot be bound by platelets that are at rest. An essential route for platelet activation is the arachidonic acid thromboxane pathway. Acetylsalicylic acid, or aspirin, is a medication often used to treat cardiovascular disease (CVD). It works by permanently acetylating and inactivating COX, which stops TxA2 from being produced. This decreases platelet aggregation. Since anucleate platelets are unable to generate enzymes from scratch, mature normal human platelets exclusively express COX-1. Consequently, the effects of aspirin on them are cumulative and long-lasting. As a result, aspirin's cardioprotective action is achieved by permanently and irreversibly impairing thromboxane A2-dependent platelet activity, which lowers the risk of acute arterial thrombosis[90,91].

Another significant platelet activator is ADP. On the platelet membrane, P2Y12, an ADP-specific receptor, is linked to inhibitory G-proteins and mediates the release of Ca2+ produced by ADP. This process inhibits adenylate cyclase and activates the GPIIb/IIIa receptor, which causes platelet aggregation. By blocking the P2Y12 receptor, ticlopidine, clopidogrel, and other thienopyridines decrease platelet activation[92]. The active platelet releases chemicals including serotonin and thromboxane A2, which strengthen the platelet-rich clot and initiate irreversible secondary aggregation (Figure 5)[93]. These compounds also give crucial positive feedback.

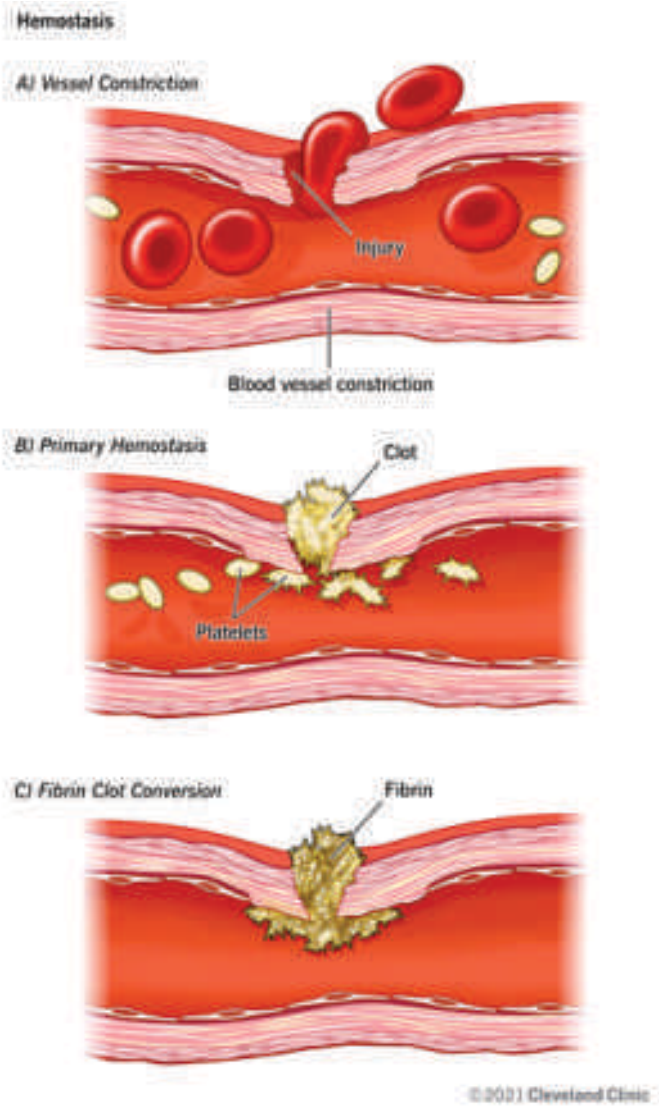

Figure 5: Haemostasis

Substances produced by platelet granules that attract other platelets and blood cells enhance the platelet response. In primary haemostasis, the platelet plug that first forms is rather fragile. The production of thrombin and fibrin, as well as the coagulation cascade, prolong secondary haemostasis. Platelet membrane phospholipids become negatively charged during platelet activation, which helps to activate coagulation (e.g., FV, FVIIIa, FIXa, and FX). This stage involves the prothrombinase complex (FXa, FVa, Ca2+, and prothrombin) binding to the platelet membrane. Thrombin production initiates further platelet activation. The blood clot is strengthened by the "red thrombus" that forms as a result of these cascades[94].

Assessment

The assessment of bleeding time (BT) using the Duke procedure95 marked the beginning of the history of platelet function testing (PFT). This was the initial test to evaluate platelets' ability to form a plug. It has long been the only screening test that can detect acquired or congenital platelet disorders[96]. Born's research suggest that the primary technique for diagnosing platelet function in the 1960s was light transmission aggregometry (LTA), a ground-breaking platelet aggregation test in platelet-rich plasma (PRP)[97]. This test measures the platelets' capacity to clump together in response to exogenous aggregating agents, or agonists, such collagen, adenosine-diphosphate (ADP), arachidonic acid (AA), and epinephrine (EPI)[98]. Other PFT techniques have become accessible since the late 1980s, including platelet aggregometry in whole blood (WB), activated platelets studied ex vivo by flow cytometry, the determination of certain chemicals generated by platelets, and the evaluation of platelet nucleotides[99,100].

But the growing number of antiplatelet medication users, who are more likely to bleed, particularly after trauma and surgery, has also made PFT a valuable tool for presurgical and perioperative settings, where it can be used to predict bleeding and track the effectiveness of various prohemostatic treatments. In this case, there are now greater opportunities to use PFT in general laboratories, various clinical settings, and specialised clinical or research laboratories due to the introduction of new, easier-to-use tools for evaluating platelet function at the point-of-care (POC) or bedside.[101]

1. Prostanoids Modalities

The primary by product of platelet metabolism of arachidonic acid is TXA2. At the location of vascular damage, it is produced and released from platelets, intensifying platelet activation[102]. Phospholipase A2 facilitates the extraction of arachidonic acid from membrane phospholipids (PLA2). It is converted to prostaglandin G2 and H2 by cyclooxigenase-1 (COX-1), and subsequently to TXA2, a strong vasoconstrictor, by thromboxane synthase. By hydrolysing TXA2, thromboxane B2 (TXB2)[103], a physiologically inactive and stable compound, is produced. TXB2 is converted into two main metabolites: 11-dehydro-TXB2 by dehydration and 2,3-dinor-TXB2 through β-oxidation. Urine contains 11-dehydro-TXB2, and the amount expelled indicates the total amount of platelets that may make TXA2[104]. The aspirin action on COX-1 lowers the urine 11-dehydro-TXB2[105,106] levels. These days, enzyme-linked immunoassays (ELISA)[107] are used instead of immunoradiometric assays (IRMA)[108] or radioimmunoassay (RIA).

2. Platelet Function Analysis

Siemens, Munich, Germany, manufactures the PFA-100 and PFA-200 platelet function analysers, which quantify in vitro the stopping of high-shear blood flow caused by the platelet plug. This point-of-care whole blood technique is straightforward, quick, and needs no sample preparation or small sample quantities. Its drawbacks include the need for pipetting and its reliance on haematocrit and von Willebrand factor levels. In the PFA-100 method, two distinct cartridges are applied: collagen plus ADP (CAPD) and collagen plus epinephrine (CEPI). Within the cartridges, citrated whole

blood runs via a capillary at a high shear rate, ending in a collagen-coated membrane with an opening of 147 μm that is filled with either EPI or ADP. Closure time is the amount of time until clot formation obstructs the opening (CT).[109]

The technique has been employed to track the results of acetylsalicylic acid treatment. In individuals on aspirin treatment, a brief CEPI CT scan may be suggestive of elevated residual platelet reactivity[110,111]. It is not advised to use PFA-100 to track the effects of thienopyridines[111]. Yet, the recently released INNOVANCE PFA P2Y cartridge demonstrated sensitivity to P2Y12 inhibition and was on par with other platelet function tests that are now accessible[113].

3. ROTEM System

One additional module that may be added to the ROTEM is the ROTEM Platelet System. The technique uses whole blood impedance aggregometry as its foundation. It offers details on the aggregation and function of platelets as well as how medications are impacted by platelet function. Unfortunately, there aren't much data on this relatively new method's applicability in clinical practice[114].

4. Thrombo-Elasto Graphy (TEG)

The viscoelastic alterations of the whole clotting process are measured by Thromboelastography (TEG) and thromboelastometry. There are several tests available for both the intrinsic and extrinsic pathways. The function of platelets in clot formation may be assessed by selectively activating the extrinsic route. More precisely, the platelet contribution to clot strength is measured by the thromboelastometry platelet test and the thromboelastogram platelet mapping system. The worldwide functional tasks of platelets in haemostasis-thrombin production, clotting, clot retraction, and fibrinolytic activation—are tested in both procedures[115].

Based on these principles, the most popular techniques include Sonoclot analysis, Thromboelastography (using the TEG Platelet Mapping System; Haemoscope, Braintree, MA, USA), and thromboelastometry (using the ROTEM; TEM Int, Munich, Germany) (Sonoclot Signature; Sienco, Arvada, CO, USA). A revolving mechanism with a pin held aloft by a torsion wire is a feature of TEG and ROTEM. A pin that is inserted into the Sonoclot device is pushed up and down at an ultrasonic pace. Whole blood samples are first subjected to the proper reagents to begin the test. All phases of the clot's development and resolution involve measurements and displays of changes in elasticity[116].

The original TEG has been modified to become the TEG platelet mapping system. It is a point-of-care technique suitable for keeping an eye on all kinds of antiplatelet treatments. Through four distinct whole blood tests, it offers information regarding platelets. A sample that has been activated with kaolin exhibits the potential for maximal clot strength by producing a robust thrombin response and cleaving all of the fibrinogen that is present. The strength of the clot resulting from fibrin is demonstrated by one aliquot that only contains Activator F, which blocks all thrombin. Additionally, all thrombin is blocked in the third and fourth experiments, which additionally activate platelets at the thromboxane A2 or ADP receptors. The patient's whole haemostatic potential is used as the baseline to determine the degree of inhibition[117].

5. Plateletworks

The foundation of Plateletworks (Helena Laboratories, Beaumont, TX, USA) is platelet aggregation that is dependent on GP IIb/IIIa. Samples with and without agonists are compared in terms of platelet count (ADP or AA). The procedure uses whole blood and needs very little sample preparation. The fact that samples must be measured shortly after blood is drawn is a drawback. This aspect restricts its use, and only a small number of studies have linked the approach to clinical outcomes[109].

6. IMPACT-R

Cone-and-plate technology is used by the Impact R analyser (DiaMed, Cressier, Switzerland) to measure shear-induced platelet aggregation. Under arterial flow conditions, the apparatus evaluates platelet adhesion and aggregation in anticoagulated whole blood. Platelet adhesion and aggregation occur as soon as plasma proteins from a blood sample are applied to the surface of a polystyrene well. Adherent platelets are quantified by an image analyser. The proportion of the well surface covered by aggregates is used as an index of adhesion, and the average size of the aggregates is used as an index of aggregation to represent the results. There is a clinical version of the device as well as a research version with an adjustable shear rate. The method's simplicity, lack of sample preparation, and small sample sizes are its advantages. Nevertheless, because pipetting is necessary, it is not a true point-of-care technique[109].

7. Global Thrombosis Test (GTT)

Using native, non-anticoagulated whole blood, the global thrombosis test (GTT) (Montrose Diagnostics Ltd., London, UK) is a new technique based on platelet activation owing to severe shear stress. This quick point-of-care test provides information on the patient's thrombotic condition. Its clinical utility is being assessed[118].

8. Verify Now

A point-of-care tool called VerifyNow (Accriva Diagnostics, San Diego, CA, USA) uses turbidimetric-based optical detection to assess platelet aggregation in anticoagulated whole blood. Beads coated with fibrinogen promote platelet aggregation; the amount of active GP IIB/IIIA receptors on the surface of the beads determines how many platelets aggregate there. The aspirin effect may be studied with the Aspirin Test, which uses AA as an agonist. The Platelet Reactivity Unit (PRU) Test assesses the clopidogrel impact by using PGE1 as a suppressor of intracellular free calcium and ADP as an agonist. As a baseline, a second channel studies platelet aggregation triggered by thrombin receptor activating peptide (TRAP-). The technique requires little sample volume and is quick and easy to use. Pipetting is not necessary[109].

9. Impedance Method (Aggregometry)

When an agonist causes platelet aggregation, impedance aggregometry analyses the change in electrical impedance between two electrodes. The method's premise is comparable to optical aggregometry, except it may be performed on whole blood, negating the requirement to prepare a platelet suspension. An increase in electrical impedance results from platelets aggregating to platelets that are adhered to the electrodes. The measurement of the increase in electrical impedance expressed in Ohms is used to evaluate platelet aggregation[99]. Patients with thrombocytopenic syndrome may also benefit from impedance aggregometry[119]. Since impedance aggregometry is done on whole blood, other blood components can affect platelet aggregation, allowing for a more physiological assessment of platelet function. It also occurs on a solid surface, mimicking the platelet adhesion and aggregation process in physiology.

Despite being based on impedance aggregometry, the Multiple Electrode Aggregometry (MEA) is a point-of-care tool[120]. Its five channels allow for the simultaneous monitoring of several agonists or substances. Two pairs of sensor electrodes are inserted into each cuvette as a means of integrated quality control. Each sensor unit is used independently to assess platelet aggregation concurrently in triplicate. The process of pipetting is mechanised. MEA can be used to identify increased platelet reactivity during therapy and to monitor antiplatelet treatments[121,122]. Finding patients with a bleeding diathesis is another appropriate use for it.

10. Flow Cytometric Methods

Numerous assays are used in flow cytometry (FC) study of platelets for a number of objectives, such as thrombopoiesis investigation, detection of illnesses related to platelet function, and antiplatelet medication monitoring[123].

Using antibodies coupled to fluorescent dyes, which may attach to certain proteins on cell membranes or inside cells and show their presence, flow cytometry quantifies the amount of these proteins. The fluorescent molecules of dyes linked to platelets are excited to a higher energy state by a light source. When the dyes go back to rest, they release light at various wavelengths. Double labelling is the process of coupling a particular secondary antibody to a fluorochrome that identifies the primary antibody[104].

The sorts of activation-dependent monoclonal antibodies that target granule membrane proteins or conformational changes of GPIIb/IIIa have been researched the most. The fibrinogen binding site revealed by a conformational shift in GPIIb/IIIa brought on by platelet activation is the target of the monoclonal antibody PAC-1. PAC-1 exclusively attaches to activated platelets as a result. A different surface marker that is frequently employed in relation to platelet activation targets platelet surface P selectin (CD62P). Monoclonal antibodies specific to P selectin exclusively bind to degranulated platelets because P selectin is expressed on the platelet surface membrane only after α-degranulation. Nevertheless, it was shown that circulating degranulated platelets quickly lose their P-selectin in vivo, which may restrict the use of this technique[109].

Activation-dependent platelet signalling is measured by the phosphorylation of the vasodilator-stimulated phosphoprotein (VASP). Its benefits include minimal sample quantities needed, the use of whole blood, stability (enabling samples to be sent to a distant lab), and reliance on the P2Y12 receptor, which is where clopidogrel acts. Its drawbacks include the need for skilled personnel and intricate sample preparation[124].

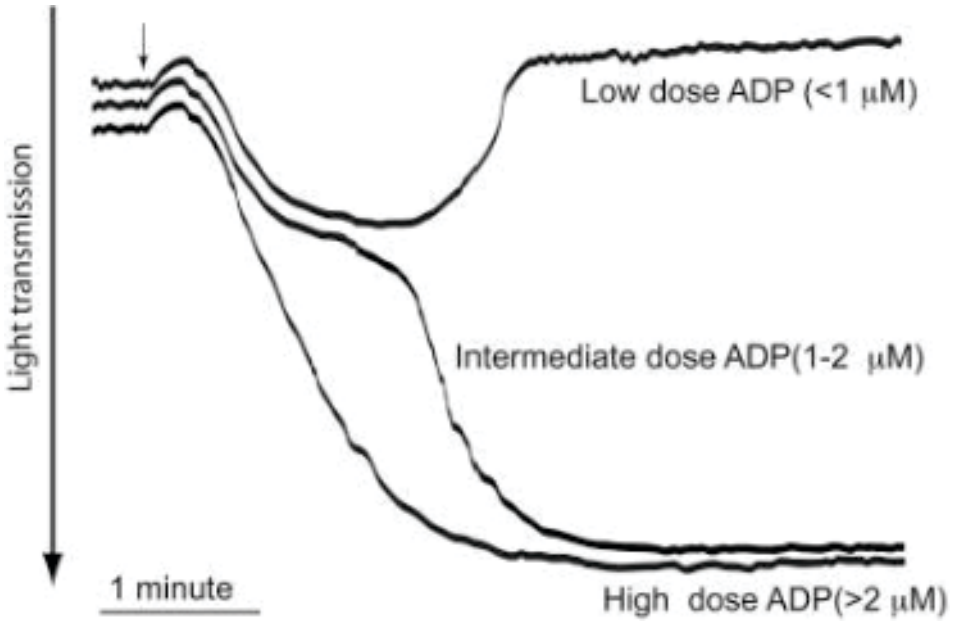

Figure 6: Human platelets responding to ADP are traced using platelet aggregometry.

11. *in vivo* Tests

Platelet aggregometry may not accurately capture the intricacies of platelet aggregation *in vivo*, even while it enables evaluation of platelet aggregation kinetics under meticulously regulated experimental circumstances (agonist dosage, platelet numbers, temperature, etc.). Numerous intravital video microscopy techniques have been employed to observe platelet aggregation in vivo during thrombus development; they are covered in more depth in another source[125].

These techniques typically involve an injury to microvascular walls, for example by micropuncture, electrical stimulation, laser, chemical, or photochemical injury[125,126]. The nature of vascular injury, as well as the vessel type may determine the molecular mechanisms responsible for platelet recruitment in the individual models. In some models (e.g., micropuncture, chemical stimulation) platelet adhesion occurs at sites of endothelial denudation and reflects the mechanisms of platelet adhesion to the subendothelial matrix outlined earlier. In others, platelet adhesion is evident without overt endothelial denudation[127]. Vascular injury results in platelet adhesion, which may progress to formation of an occlusive thrombus, as illustrated in ***Figure 7***. The kinetics of platelet adhesion and aggregation may be monitored in real-time with these techniques, and they have provided important novel observations of the mechanisms mediating platelet recruitment in vivo. These approaches also illustrate the redundancy of many mechanisms responsible for platelet recruitment. For example, mice lacking both vWF and fibrinogen are able to form occlusive thrombi following injury, albeit with marked delay in responses[128]. While intravital microscopy to visualize thrombus formation is not a new technique (e.g., Bizzozero used it in the late 19th century[129]), advances in image acquisition and processing techniques, molecular biology and genetic models of disease have expanded the recent interest in this approach to study interactions of platelets with vascular walls.

ANTI PLATELET DRUGS

When an atherosclerotic plaque suddenly ruptures or fissures, platelet adhesion, activation, and aggregation can be understood as a physiological repair response. However, if this process continues unchecked through a series of self-sustaining amplification loops, it can result in intraluminal thrombus formation, vascular occlusion, and subsequent ischemia or infarction. Antiplatelet medications already on the market disrupt one or more phases of platelet release and aggregation[130], resulting in a substantial decrease in thrombosis risk that is inextricably linked to an elevated risk of bleeding[131].

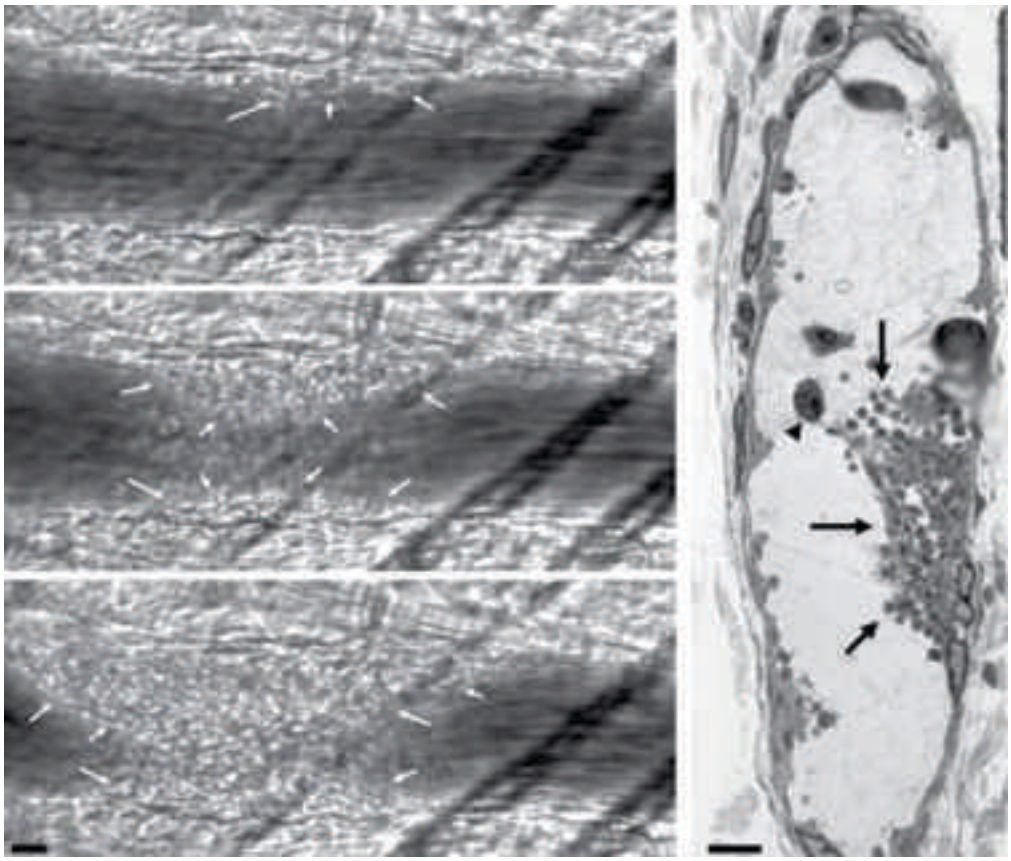

Figure 7: *in vivo* aggregation assessment in mouse model

ASPIRIN

The most researched antiplatelet medication is aspirin. Based on more than 100 randomised trials with high-risk patients, aspirin lowers the risk of vascular mortality by around 15% and nonfatal vascular events by approximately 30%[132].

Pharmacology and Mechanism of Action

The ability of aspirin to permanently inhibit prostaglandin H-synthase-1 and prostaglandin H-synthase-2 (also known as COX-1 and COX-2, respectively)[133] is the most well-characterized mechanism of action of the drug. The conversion of arachidonic acid to prostaglandin H2, the first committed step in the production of prostanoids, is catalysed by COX isozymes (PGH2). The direct antecedent of TXA2 and PGI2 is PGH2.

The acetylation of a strategically placed serine residue (Ser529 in COX-1, Ser516 in COX-2), which blocks substrate access to the catalytic region of the enzyme[134], is the molecular mechanism behind aspirin's persistent suppression of COX activity.

Low dosages of aspirin (75–150 mg) administered once day can result in total or almost complete suppression of platelet COX-1. However, because nucleated cells quickly resynthesize the enzyme, inhibiting COX-2-dependent pathophysiologic processes (such as inflammation and hyperalgesia) necessitates higher aspirin dosages and shorter treatment intervals.

Aspirin is rapidly absorbed in the stomach and upper intestine. Plasma levels peak 30 to 40 min after aspirin ingestion, and inhibition of platelet function is evident within 1 h. In contrast, it can take 3 to 4 h to reach peak plasma levels after administration of enteric-coated aspirin. Therefore, if a rapid effect is required and only enteric-coated tablets are available, the tablets should be chewed instead of swallowed intact. The oral bioavailability of regular aspirin tablets is ~40% to 50% over a wide range of doses. A considerably lower bioavailability has been reported for enteric-coated tablets and for sustained-release, microencapsulated preparations[135].

Aspirin is a useful antithrombotic drug when used long-term at levels between 50 and 100 mg/d; findings suggest that it may even be useful at doses as low as 30 mg/dl[132]. Placebo-controlled randomised trials have demonstrated this. A 75 mg/d dose of aspirin was demonstrated to (1) lower the risk of stroke or death in patients with transient cerebral ischemia[138], (2) lower the risk of acute myocardial infarction (MI) or death in patients with unstable angina [136] or chronic stable angina[137], and (3) lower the risk of stroke following carotid endarterectomy[139].

A meta-analysis of 14 studies on Aspirin efficacy was done. It shown that taking aspirin, as opposed to taking a placebo or receiving no therapy, was linked to a decreased risk of myocardial infarction (risk ratio [RR], 0.83, 95 percent confidence interval [CI]: 0.73–0.95, P = 0.005). Furthermore, aspirin usage was not linked to a decreased risk of cardiovascular or all-cause death when compared to the control groups. Regarding safety, using aspirin was linked to an increased risk of haemorrhagic stroke (RR, 1.30, 95 percent CI: 1.06–1.60, P = 0.011), severe bleeding (RR, 1.40, 95 percent CI: 1.25–1.57, P = 0.000), and gastrointestinal bleeding (RR, 1.58, 95 percent CI: 1.25–1.99, P = 0.000). Furthermore, the clinical features of the patients did not significantly alter the therapeutic outcome[140].

DIPYRIDAMOLE

A pyridopyrimidine derivative having antiplatelet and vasodilator characteristics is dipyridamole. There is debate over dipyridamole's antiplatelet agent's mode of action. It has been suggested that cyclic AMP, an inhibitor of platelet aggregation, can be produced by blocking cyclic nucleotide phosphodiesterase, which is the enzyme that breaks down cyclic adenosine monophosphate [AMP] to 5′-AMP. Another option is to block the uptake of adenosine, which binds to A2 receptors, stimulates platelet adenyl cyclase, and increases cyclic AMP. Furthermore, there have been reports of direct PGI2 synthesis stimulation and protection against its degradation; however, these actions need dipyridamole concentrations significantly higher than the low-micromolar plasma levels attained with oral administration of normal dosages (100-400 mg/d)[142].

Pharmacology

Conventional formulations of dipyridamole have very varied absorption rates, which might lead to a low level of systemic bioavailability. A combination tablet containing low-dose aspirin and a modified-release formulation of dipyridamole with enhanced bioavailability has been created. As a glucuronide conjugate, dipyridamole is excreted mostly via biliary excretion and is heavily protein bound to albumin. It is also prone to enterohepatic recirculation. Reports indicate a terminal half-life of ten hours. This aligns with the bid regimen that has been employed in the latest clinical research[143].

According to a meta-analysis of six randomised trials with 7,648 patients who had a history of TIA or stroke and for which stroke was reported as an outcome, aspirin plus dipyridamole (dose range: 50–1,300 mg/d) reduced stroke by 23% (RR: 0.77; 95 percent CI: 0.67–0.89) when compared to aspirin alone (dose range: 50–1,300 mg/d), with no statistically significant evidence of heterogeneity[144]. Trials using dipyridamole's immediate-release preparation (four trials) and those using its extended-release preparation yielded consistent estimates. The combination of aspirin plus dipyridamole is superior to aspirin alone for the prevention of vascular events in patients with a history of TIA or stroke, according to a Cochrane review of 29 randomised trials involving 23,019 patients. However, no evidence of a benefit of the combination was found in studies involving patients with a history of coronary or peripheral arterial disease, or in other high-risk patients[145].

CILOSTAZOL

A 2-oxoquinolone derivative called clostazol is said to have antiproliferative, antiplatelet, and vasodilatory qualities that lessen the proliferation of smooth muscle cells and the development of neointimal hyperplasia following endothelial

damage. Within the first two weeks of initiating therapy, headaches occur in up to 25% of patients, and GI side effects are frequently caused by ciprofloxacin. Because of the risk of inducing ventricular tachycardia in patients with heart failure, cilostazol should not be used. This effect has been linked to an increase in intracellular cyclic AMP, which is also probably responsible for the medication's vasodilatory effects[141].

Pharmacology

Significant variation exists in the absorption of cilostazol taken orally. The rate and amount of medication absorption are increased when food is administered concurrently. Due to its strong albumin binding and extensive metabolism by cytochrome P450 (CYP450) enzymes, Cilastazole is excreted in urine together with its metabolites. Its half-life is 11 hours, and in individuals with severe renal impairment, the half-life is extended[141].

Cilostazol (50 mg bid or 100 mg once daily) increases the maximal and pain-free walking distance in patients with intermittent claudication[146], prevents thrombotic events in patients with peripheral arterial disease, and prevents restenosis and target vessel revascularization in patients undergoing coronary or peripheral artery stenting, according to meta-analyses of mostly small, open-label, placebo- and active-controlled trials[147,148].

THIENOPYRIDINES GROUP

CLOPIDOGREL

When platelets transit through the liver, clopidogrel is quickly absorbed and converted into a highly labile active metabolite[149] that binds to the platelet P2Y12 receptor irreversibly[150.] With a half-life of about 8 hours, SR 26334, an inactive carboxylic acid derivative, is the primary systemic metabolite of clopidogrel.

The pharmacological pattern of clopidogrel's active metabolite is comparable to that of aspirin; modest dosages administered repeatedly daily result in cumulative inhibition of platelet activity. It takes seven to ten days following the final clopidogrel dosage for platelet function to recover to normal. Permanent inhibition of COX-1 and the P2Y12 receptor by the active moieties of aspirin and clopidogrel is consistent with both the cumulative nature of the inhibitory effects and the delayed rate of recovery of platelet thromboxane synthesis (aspirin) or ADP-induced platelet aggregation (clopidogrel) (active metabolite). This also explains why people with typical platelet turnover rates need take aspirin and clopidogrel once daily, even though their half-lives in the bloodstream are brief[141].

Because there were few phase 2 trials and only one big phase 3 study that compared the effectiveness and safety of clopidogrel (75 mg/d) with aspirin (325 mg/d), the clinical development of clopidogrel was unique.

In the Clopidogrel vs. Aspirin in Patients at risk for Ischemic Events (CAPRIE) trial, 6,400 patients were divided into three groups based on their increased risk of recurrent ischemic events: those who had recently suffered a stroke, those who had recently had a MI, and those who had symptomatic peripheral arterial disease. Clopidogrel decreased the absolute risk by 0.51 percent and the relative risk of MI, ischemic stroke, or vascular death by 8.7 percent (95 percent confidence interval [CI], 3 percent to 65 percent) in the whole CAPRIE trial group of 19,185 high-risk patients when compared to aspirin[151].

TICLOPIDINE

A single oral dosage of ticlopidine can be quickly absorbed up to 90% of the time[152]. One to three hours after a single oral dosage of 250 mg, plasma concentrations peak. Over 98% of the ticlopidine that is absorbed is reversibly linked to albumin and other plasma proteins. Ticlopidine is quickly and thoroughly metabolised. In humans, 13 distinct metabolites have been found. Only the ticlopidine 2-keto derivative has the ability to suppress ADP-induced platelet aggregation more effectively than the original molecule[152]. Ticlopidine's apparent elimination half-life is 24 to 36 hours following a single oral dosage and up to 96 hours following 14 days of consecutive administration[152]. Ticlopidine's recommended dosage is 250 mg bid.

Due to its documented toxicity, ticlopidine's place in the current treatment arsenal is questionable. In the majority of jurisdictions, clopidogrel has essentially taken its place.

PRASUGREL

Following oral consumption, prasugrel is quickly absorbed and transformed into its active metabolite, which reaches peak concentrations within 30 minutes of dosage. Food has little effect on absorption. The primary method of metabolite elimination is renal excretion, with the active metabolite having a half-life of approximately 4 hours[153]. S-methylation and cysteine conjugation transform the active metabolite of prasugrel into inactive metabolites.

According to preliminary pharmacological tests, prasugrel inhibits ADP-induced platelet aggregation more consistently and completely than clopidogrel, and its effects are seen more quickly in healthy persons[154], as well as in patients with stable coronary artery disease[154,155]. In comparison to clopidogrel, which goes through a two-step hepatic conversion process, prasugrel's more fast beginning of action may be partially explained by the CYP450 enzymes' single-step hepatic conversion to its active metabolite[156].

Prasugrel was approved by the FDA with a boxed warning about the possibility of severe or deadly bleeding. Patients who have a history of stroke or who are bleeding actively should not use this medication. The goal of the Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage Acute Coronary Syndromes (TRILOGY ACS) experiment is to see whether lowering the dosage of prasugrel[157] can result in a better benefit/risk ratio.

GP IIb / IIIa GROUP

ABCIXIMAB

A humanised form of a mouse antibody's Fab fragment that targets GpIIb-IIIa is called abciximab. When GpIIb-IIIa is blocked by abciximab, a phenotype resembling Glanzmann thrombasthenia[158] is produced. Each platelet has around 40,000 antibody molecules attached to its surface; however, since they interact in a bivalent manner, each platelet most likely has 80,000 GpIIb-IIIa receptors[159]. Antibody concentrations that reduce the number of accessible receptors to less than 50% of normal dramatically suppress platelet aggregation. About 80 percent receptor inhibition virtually eliminates platelet aggregation; nevertheless, bleeding time is only slightly impacted at this level of blocking. The bleeding period only noticeably prolongs when there is more than 90% receptor blockade[159].

About 1–2% of people receiving abciximab experience thrombocytopenia. Re-administration of abciximab appears to enhance the risk of thrombocytopenia[160,161]. The platelet count usually starts to decline within 24 hours after starting therapy, although it can start to decline as soon as 2 hours in. As a result, the package insert for abciximab stipulates that a platelet count must be taken two to four hours after starting treatment. It is thought that antibodies are the cause of the thrombocytopenia[162]. When the medication is stopped, the thrombocytopenia usually goes away. Platelet transfusions can be administered if need[163].

TIROFIBAN

Tirofiban is a derivative of nonpeptide tyrosine that binds specifically to GpIIb-IIIa[164,165]. Tirofiban has a 1.5–2 hour plasma half-life, and its excretion by the kidneys and liver helps to remove it; urine and faeces include unaltered tirofiban[166]. Patients with renal insufficiency need to have their doses adjusted, whereas those with hepatic illness do not.

A limited number of people using tirofiban have been documented to experience severe but reversible thrombocytopenia. It is thought that tirofiban binding causes a conformational shift in GpIIb-IIIa, and antibodies are produced against the recently exposed epitope[167], so inducing thrombocytopenia. Re-administration of tirofiban is not known to be safe, yet individuals who experienced thrombocytopenia following tirofiban exposure had elevated antibody titers[168].

EPTIFIBATIDE

Eptifibatide is a synthetic cyclic heptapeptide connected by a disulfide bond. It exhibits a strong selectivity for GpIIb-IIIa[169,170] and is designed after the KGD sequence present in the snake venom disintegrin (barbourin) isolated from Sistrurus miliarius barbouri. It is possible that the inhibitory effects of eptifibatide were overestimated due to the collection of blood samples into citrate, even though preliminary reports suggest that eptifibatide produces less prolongation of the bleeding time than other GpIIb-IIIa inhibitors at doses producing comparable inhibition of platelet aggregation[171].

Patients with renal impairment show extended suppression of platelet function after taking eptifibatide because the medication is excreted by the kidneys. It's unclear how much eptifibatide is appropriate for people with mild to severe renal insufficiency. The steady-state eptifibatide level is around 1,900 ng/mL at an infusion rate of 2 μg/kg per min, indicating that more than 50 eptifibatide molecules bind to each GpIIb-IIIa. As such, platelet transfusion might not counteract the medication's effects[172,173].

Efficacy and Current Scenario of GP IIb/IIIa Drugs

In patients with ACS without persistent ST-segment elevation, randomised trials have evaluated the safety and effectiveness of tirofiban, lamifiban (a nonpeptide GpIIb-IIIa blocker, whose development has been discontinued), eptifibatide, and abciximab in addition to conventional antithrombotic therapy[174,175,176]. According to these studies, the relative risk decrease in MI or mortality at 30 days ranged from 0% to 27%. The FDA has approved eptifibatide and tirofiban for the treatment of ACS, including patients who will be treated medically and those who are having PCI. Nevertheless, abciximab for 24 hours (0.25 mg/kg bolus followed by a 0.125 mg/kg per min infusion) or 48 hours was not helpful as first-line medical therapy in patients with ACS, according to the Global Utilization of Strategies to Open Occluded Arteries (GUSTO) IV-ACS study[175]. A 9 percent reduction in the probabilities of mortality or MI at 30 days is suggested by a meta-analysis of all significant randomised clinical studies with GpIIb-IIIa antagonists in patients with ACS who were not usually scheduled to receive early coronary revascularization[177].

Therefore, the benefit/risk profile of currently available GpIIb-IIIa antagonists is very problematic for patients with ACS who are not routinely planned for early revascularization. On the other hand, a good strategy for high-risk PCI patients to reduce the risk of thrombotic issues related to the procedure is to increase the dose of antiplatelet therapy and add an IV GpIIb-IIIa blocker.

Phase 2 studies using eptifibatide and abciximab in acute MI revealed that GpIIb-IIIa blockage may be beneficial when used in addition to thrombolysis. The GUSTO V trial evaluated 16,588 patients in the first six hours of developing ST-segment elevation MI to assess the safety and effectiveness of half-dose Reteplase and full-dose abciximab against standard-dose Reteplase. The two therapy groups' 30-day mortality rate was comparable as the major end point (5.6 percent vs 5.9 percent). Combination treatment consistently reduced the incidence of MI's subsequent consequences, such as reinfarction, albeit this was somewhat offset by a rise in extracranial haemorrhages. After a year, there was no mortality advantage from combination therapy; hence, combined therapy appeared to have little to no net benefit[178].

An updated meta-analysis[181] has critically re-evaluated the advantages and risks of this approach in light of the failure of several more recent randomised trials to show benefits of GpIIb-IIIa blockade among patients with ST-segment elevation MI treated with primary angioplasty and in patients treated with clopidogrel[179,180].

CONCLUSION

To summarise, platelets play an essential role in preserving the integrity of blood vessels and preventing the formation of blood clots. In order to put a stop to the bleeding, they clump together with the assistance of a number of different substances and receptors. In contrast, platelet hyperactivity may result in difficulties, particularly in cases of coronary artery disease. Antiplatelet drugs such as aspirin, clopidogrel, and GP IIb/IIIa inhibitors are able to reduce the likelihood of thrombosis by interfering with one or more phases of platelet release and aggregation. The utilisation of these, on the other hand, increases the likelihood of bleeding. Platelet activity may be measured in a variety of ways, including flow cytometry, platelet aggregometry, and global thrombosis tests, to name just 3 of the many possible methods.

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Review

A Review on Platelet Aggregation Inhibition Activity of Spices, Condiments and Nuts

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ABSTRACT

Platelet activation and aggregation play a crucial role in thrombosis and cardiovascular events. Anti-platelet agents are used to inhibit platelet activation and reduce the risk of these events. However, consumption of the synthetic anti-platelet agents has own adverse effects. Interestingly, spices and condiments along with nuts are rich sources of bioactive compounds that have potential anti-platelet effects. In view of this, an attempt was made to find out those spices and nuts which have shown platelet aggregation inhibition effects either in vitro or in vivo. Some of the spices having anti-platelet effect were turmeric, clove, onion, garlic, nutmeg, ginger, saffron, cumin, carom, coriander etc. Some of the nuts were chilgoza pine, walnut, baru almond, betel nut etc. These plants are rich in therapeutic compounds belonging to various secondary metabolites for example, phenolics, terpenoids, flavanoids, alkaloids, steroids etc. and may provide potential benefits for reducing platelet activation and the risk of cardiovascular events. However, further research is warranted to explore the efficacy, specific mechanisms and dosage of the compounds present in these spices and nuts through clinical studies to validate their anti-platelet activities.

KEYWORDS: *Curcuma longa,* Ginger, Chilgoza Pine, Cardamom, Walnut

INTRODUCTION

Platelets play a crucial role in haemostasis, the process of blood clot formation. However, excessive platelet activation and aggregation can lead to the formation of blood clots which could be harmful and increase the risk of cardiovascular events such as heart attack and stroke. Anti-platelet agents are used to inhibit platelet activation and aggregation, thereby reducing the risk of thrombosis[1-2]. Some of the common drugs used for this purpose are clopidogrel, aspirin, and ticlopidine which are associated with own side effects[3-4]. Therefore, need for natural anti-platelet agents seem appropriate. In this regard, plants are the best choice since time immemorial[5].

Spices, condiments and nuts have been a part of diet since ancient times. They are not only consumed for imparting flavour and aroma but also used for their nutritional as well as medicinal qualities[6]. Several nuts and spices have been shown to possess platelet aggregation inhibition activity as these are rich sources of bioactive compounds that include phenolic compounds, flavonoids, terpenes, and alkaloids. Various scientific studies have identified the mechanisms behind the inhibition of platelet activation and aggregation through spices and nuts. Some of the prominent mechanisms are inhibition of platelet adhesion and aggregation, suppression of thromboxane A2 (TXA2) production, inhibition of cyclooxygenase (COX) enzymes,

modulation of calcium signalling pathways, and interference with platelet signalling molecules etc.[7]. Besides, the protection provided through platelet aggregation inhibition, the bioactive molecules present in the spices and nuts also protect from various cardio-metabolic risk factors[8].

METHODOLOGY

In view of this, online literature search was conducted using the keywords for example, platelet aggregation, anti-platelet, anti-thrombotic, inhibition, activation, thrombosis, *in vitro, in vivo* combining with common as well as botanical names of various spices, condiments and nuts on the databases namely, Pubmed, Springer Link, Scopus, Science Direct, Google Scholar, and Research gate to find out the studies carried out on this aspect. Finally, the major findings of all the relevant papers selected for this review article is presented in following sections under the two separate headings; platelet aggregation inhibition activity of spices and condiments and nuts.

Platelet aggregation inhibition activity of spices and condiments

Wang *et al*.[9] reported that capsaicin; a bioactive compound of chilli peppers (*Capsicum frutescens;* Family - Solanaceae) effectively inhibits platelet aggregation induced by thrombin and collagen. However, marginal inhibition was observed on rat platelet aggregation induced by arachidonic acid (AA) and calcium ionophore A23187. An IC_{50} value of 85 µg/ml was found for collagen-induced platelet aggregation.

The anti-platelet activity of nutmeg (*Myristica fragrans;* Family - Myristicaceae) on rabbit platelets was investigated by Rasheed *et al*.[10]. Eugenol and isoeugenol were the two most active components found in nutmeg oil. Indomethacin was taken as a reference drug and platelet aggregation was induced by arachidonic acid. At the same concentration, indomethacin 1 µg/mL was substantially more effective than eugenol, whereas eugenol at a concentration of 1 µg/mL and 10 µg/mL showed a dose-response relationship ($P< 0.05$). Additionally, the pure oils demonstrated inhibitory efficacy against AA-induced aggregations, with an IC_{50} of around 10 µg/mL. Interestingly, recently the effects of *Myristica fragrans* ethanol extract (MF) on sepsis and sepsis-associated thrombocytopenia (SAT) have been studied by Jeong *et al*.[11] using flow cytometry, desialylation and activation of platelets treated with sialidase and adenosine diphosphate (a platelet agonist). Through the inhibition of bacterial sialidase activity in washed platelets, the extract inhibited platelet desialylation and activation. Furthermore, in a mouse model of sepsis generated by cecal ligation and puncture, MF decreased inflammation, enhanced survival, and decreased organ damage. By suppressing circulation sialidase activity, it also stopped platelet desialylation and activation while preserving platelet numbers. Hepatic JAK2/STAT3 phosphorylation and thrombopoietin mRNA expression are decreased when platelet desialylation is inhibited. This decreases hepatic Ashwell–Morell receptor–mediated platelet clearance. The study provides insight into sialidase-inhibition-based sepsis therapy approaches and paves the way for the creation of plant-derived medicines for SAT and sepsis.

According to Srivastava[12], ether extract of Carom seeds (*Trachyspermum ammi*; Family- Apiaceae) inhibited platelet aggregation in a concentration-dependent manner when induced with arachidonic acid (AA), epinephrine, and collagen. It worked best against aggregation induced by AA. It inhibited AA-induced aggregation in every blood sample at a concentration of > 60 µg/ml. At 100–200 µg/ml concentration, the second phase of epinephrine-induced aggregation was eliminated. However, relatively higher concentration (400 µg/ml) was required to prevent collagen-induced aggregation.

The anti-platelet property of Cumin (*Cuminum cyminum;* Family - Apiaceae) and Turmeric (*Curcuma longa;* Family - Zingiberaceae) were investigated by Srivastava[13]. Ether extracts of Turmeric and Cumin inhibited aggregation induced by arachidonic acid and 137 ± 37 µg/mL and 92 ± 73 µg/mL were the doses of platelet-rich plasma (PRP) that abolished the AA-induced aggregation by turmeric and cumin extracts, respectively. The two spice extracts were unable to inhibit collagen and calcium ionophore A23187 induced platelet aggregation and a significantly higher concentration was required for slight reduction in adenosine diphosphate (ADP)-induced aggregation. Interestingly, Ar-turmerone, an active constituent from the rhizome of *Curcuma longa* (Family- Zingiberaceae) has shown platelet aggregation inhibition property. It effectively inhibited platelet aggregation caused by arachidonic acid (IC_{50}, 43.6 µM) and collagen (IC_{50}, 14.4 µM) without any impact on the platelet aggregation caused by thrombin. Moreover, as compared to aspirin, Ar-turmerone was more potent platelet inhibitor against collagen-induced platelet aggregation[14].

Shah *et al*.[15] studied the platelet aggregation inhibitory effect of curcumin, extracted from turmeric. The platelet agonists arachidonic acid (AA; 0.75 mM), collagen (20 mg/mL), platelet-activating factor (PAF; 800 nM), epinephrine (200 µM), and ADP (4 µM) were used to induce platelet aggregation. The curcumin was found effective against PAF and AA with IC_{50} values of 25 and 30 mM, respectively, while higher concentration of curcumin was required to inhibit aggregation brought on by other platelet agonists. When curcumin was pre-treated with platelets, platelet aggregation caused by the calcium ionophore A23187 was inhibited (IC_{50}; 100 µM). However, aggregation caused by the protein kinase C (PKC) activator phorbol myrsitate acetate (1 µM) was not inhibited by curcumin up to a concentration of 250 µM. By utilising fura-2 acetoxymethyl ester, curcumin (100 µM) reduced the intracellular Ca^{2+} mobilisation produced by A23187. Moreover, curcumin also inhibited platelets from producing thromboxane A2 (IC_{50}; 70 µM).

Srivastava[16] demonstrated platelet aggregation inhibitory activity of two active compounds, eugenol and acetyl eugenol isolated from the oil of cloves (*Syzygium aromaticum;* Family - Myrtaceae). Eugenol and acetyl eugenol were shown to be more effective than aspirin in suppressing platelet aggregation caused by arachidonate, adrenaline, and collagen based on their

IC_{50} values. Eugenol inhibited platelet aggregation induced by AA, collagen and adrenaline with IC_{50} values of 0.8 ± 0.1 µM, 39 ± 11 µM, and 12 ± 3 µM, while acetyl eugenol inhibited aggregation of platelets with IC_{50} values of 2 ± 0.6 µM, 56 ± 27 µM and 11 ± 2 µM, respectively. The standard anti-platelet drug aspirin demonstrated the IC_{50} values as 28 ± 22 µM, 74 ± 28 µM and 50 ± 13µM against AA, collagen and adrenaline respectively.

Guh *et al*.[17] evaluated *in vitro* anti-platelet activity of gingerol, a bioactive component of *Zingiber officinale* (Family-Zingiberaceae) against different agonists inducing washed platelets from rabbit blood. Platelet aggregation produced by collagen and ADP was inhibited by gingerol at concentrations ranging from 0.5 to 20µM. Gingerol also decreased the production of prostaglandins D2 and TXB2 by Arachidonic acid at doses of 0.5–10µM. Interestingly, Ginger has also shown *in vivo* anti-platelet activity against ADP- and epinephrine-induced platelet aggregation after administration of its single dose (10 g) to CAD patients[18].

The *in vitro* platelet aggregation inhibitory action of aqueous extract of Cardamom (*Elettaria cardamomum*; Family - Zingiberaceae) on human platelets was investigated by Suneetha and Krishnakantha[19]. Epinephrine (2.5 mM), ADP (2.5 mM), calcium ionophore A 23187 (6 mM), collagen (10 mM), and ristocetin (1.25 mg/mL) were among the agonists used to stimulate human platelets. With epinephrine, ADP, calcium ionophore A 23187 and collagen, the IC_{50} values were 0.21, 0.49, 0.59 and 0.55 mg, respectively, and there was no platelet aggregation inhibition observed with ristocetin. At IC_{50}, the inhibitory effect was dose-dependent and time-dependent, with values varying from 0.14 to 0.70 mg.

Administration of 2.5 g Fenugreek powder (*Trigonella foenum-graecum*; Family - Fabaceae) twice daily to healthy individuals for 3 months did not affect platelet aggregation inhibition[18].

Jessie and Krishnakantha[20] investigated the inhibitory effect of aqueous extract of saffron (*Crocus sativus;* Family - Iridaceae) on human platelets. Collagen (11 mg/mL), epinephrine (76 µM), ADP (61 µM), calcium ionophore A23187 (6 µM), and ristocetin (1.25 mg/mL) were used to stimulate human platelets in the presence and absence of saffron extract. The IC_{50} values for these agonists were 0.86, 0.35, 0.66, and 0.59 mg, respectively, and ristocetin showed no inhibition. Moreover, the inhibitory impact was time-dependent and dose-dependent, with values ranging from 0.16 to 0.80 mg.

Suneetha and Krishnakantha[21] examined the antiplatelet aggregation activity of aqueous extracts of curry leaf *Murraya koenigii* (Family- Rutaceae) and coriander leaf *Coriandrum sativum* (Family - Apiaceae) against human platelets using agonists such as adenosine diphosphate, epinephrine and collagen. After one minute of incubation, the IC_{50} values for platelet aggregation inhibition were found as 0.94 ± 0.049, 0.65 ± 0.042 and 0.58 ± 0.035 mg/mL for curry leaf and 0.55 ± 0.045, 0.66 ± 0.033 and 0.57 ± 0.031 mg/mL for coriander leaf with ADP, epinephrine, and collagen, respectively.

Park *et al*.[22] investigated the inhibitory effects of four acidamides, namely, were piperine, pipernonaline, piperoctadecalidine, and piperlongumine that were extracted from the fruits of *Piper longum* L. (Family - Piperaceae). Except the thrombin-induced aggregation, all four of the tested acidamides shown dose-dependent inhibitory effects on washed rabbit platelet aggregation induced by collagen, arachidonic acid, and platelet-activating factor (PAF). Piperlongumine demonstrated more potent inhibition of rabbit platelet aggregation produced by collagen, AA, and PAF compared to other acidamides. At the concentration of 300, 150, 30, and 10 mM, piperlongumine showed 100%, 100%, 49.8%, and 19.9% inhibitory effects on collagen induced platelet aggregation, respectively. Piperlongumine also exhibited 100%, 76.4%, and 12% inhibitory effects, respectively, at 300, 150, and 30 mM concentrations in an AA induced test as well as 100%, 100%, and 29.9% inhibition against PAF-induced platelet aggregation, respectively.

Piperine; an active molecule from *Piper nigrum* and *Piper longum* has also shown anti-platelet effects. Son *et al*.[23] have shown the mechanism behind anti-platelet action of piperine using Rabbit platelets and murine macrophage RAW264.7 cells. It was observed that piperine was able to significantly inhibit the liberation of AA liberation through diminishing $cPLA_2$ activity in collagen-stimulated platelets. Moreover, a significant inhibition of the activity of TXA_2 synthase, but not of COX-1, in platelets was also observed indicating that platelet aggregation was inhibited through attenuation of $cPLA_2$ and TXA_2 synthase activities by piperine, instead of through the inhibition of COX-1 activity. On the other hand, a significant suppression of lipopolysaccharide-induced generation of prostaglandin (PG) E_2 and PGD_2 in RAW264.7 cells was executed by piperin through inhibiting the activity of COX-2, without any effect on $cPLA_2$.

Jantan *et al*.[24] investigated platelet aggregation inhibitory activity of different phytoconstituents from Zingiberaceae family. Arachidonic acid, collagen and adenosine diphosphate were taken to induce platelet aggregation in human whole blood and aspirin was taken as a positive control. IC_{50} values of less than 84 µM were found for curcumin from *Curcuma aromatica*, cardamonin, pinocembrine, 5, 6-dehydrokawain from *Alpinia mutica*, and 3-deacetylcrotepoxide from *Kaempferia rotunda* when platelet aggregation was induced by AA. Curcumin was shown to be the most effective anti-platelet agent, with IC_{50} values of 37.5, 60.9, and 45.7 µM, against AA-, collagen-, and ADP-induced platelet aggregation, respectively.

In vitro human anti-platelet aggregation potential of active principles of various spices, such as,eugenol, capsaicin, piperine, quercetin, curcumin, cinnamaldehyde, and allyl sulphide, was demonstrated by Raghavendra and Naidu[25]. Various agonists were used *viz*., collagen (500mg/mL), ADP (50 µM), calcium ionophore A-23187 (20 µM) and arachidonic acid (1.0mM) to induce platelet aggregation. The most effective inhibitors of AA-induced platelet aggregation among the active principles examined were capsaicin and eugenol, with IC_{50} values of 14.6 µM and 0.5 µM, respectively and

eugenol was 29 times more effective than aspirin in inhibiting AA-induced human platelet aggregation.

Kim *et al.*[26] reported the anti-platelet activity of several phytocompounds isolated from *Cinnamomum cassia* (Family - Lauraceae) extract. The platelet aggregation induced by arachidonic acid was inhibited by coniferaldehyde, eugenol, cinnamic alcohol, amygdalactone, 2-methoxycinnamaldehyde, and 2-hydroxycinnamaldehyde with the values of 0.82, 3.8, 31.2, 5.16, 16.9 and 40.0 μM, respectively. Moreover, acetylsalicylic acid (reference drug) had an IC_{50} value of 60.3μM. Furthermore, epinephrine-induced platelet aggregation was inhibited with eugenol, cinnamic alcohol, amygdalactone, cinnamaldehyde, 2-methoxycinnamaldehyde, 2-hydroxycinnamaldehyde, and coniferaldehyde with IC_{50} values of 1.86, 37.7, 1.10, 25.0, 15.3, 16.8, and 0.57 μM, respectively as compared to acetylsalicylic acid (IC_{50} 50.0 μM). It was observed that the two most potent anti-platelet components of *C. cassia* were eugenol and coniferaldehyde.

Ro *et al*.[27] evaluated *in vitro* platelet aggregation inhibitory activity of onion (*Allium cepa*; Family - Amaryllidaceae) peel extract (OPE) on collagen-induced washed rat platelets. Quercetin, was found as an active component through HPLC analysis of OPE. The IC_{50} value for quercetin was found 65 μg/mL in collagen (5 μg/mL)-induced platelet aggregation.

González *et al*.[28] evaluated *in vivo* anti-platelet aggregation effect of aqueous extract of garlic (*Allium sativum*; Family - Amaryllidaceae) using various agonists such as collagen, arachidonic acid, adenosine diphosphate, and epinephrine. There were significant differences in platelet activation in response to every agonist ($P<0.05$). Arachidonic acid had the most promising anti-platelet effect, whereas a combination of collagen and arachidonic acid had the least.

Platelet aggregation inhibition activity of Nuts

Ghayur *et al*.[29] reported the anti-platelet activity of aqueous-methanol (70%) extract of betel nut (*Areca catechu;* Family - Arecaceae) in human platelet-rich plasma. Platelet aggregation was induced by different agonists such as adenosine diphosphate, arachidonic acid, epinephrine, platelet-activating factor (PAF) and Ca^{2+} ionophore. *A. catechu* crude extract (Ac.Cr) inhibited platelet aggregatory effect of various agonists in a dose-dependent fashion (0.07 to 2 mg/mL). Ac.Cr had shown the most significant inhibitory effect against Ca^{2+} ionophore and ADP-induced aggregation. The IC_{50} values were found 0.628, 1.590, 1.677, 1.902 and 0.987mg/mL for ADP, AA, epinephrine, PAF and Ca^{2+} ionophore, respectively. Moreover, acetylsalicylic acid, a reference agent revealed an IC_{50} value of 0.03mg/mL against AA-induced platelet aggregation.

Shanmuganayagam *et al*.[30] analysed *in vitro* anti-platelet activity of different phenolic fractions of seed and skin of grape (*Vitis vinifera;* Family - Vitaceae), unfractionated grape skin (GSK) and gallic acid. Dried fruits of *V. vinifera* are well known as Raisins and consumed especially in sweet dishes. Six GSK phenolic fractions were sequentially prepared with water (fraction 1), 50% water/ethanol (fraction 2), ethanol (fraction 3), 50% ethanol/methanol (fraction 4), methanol (fraction 5), and 80% aqueous acetone (fraction 6). Surprisingly, the collagen-induced platelet aggregation was enhanced by fractions 1, 2, and 3 by 3.8 ± 1.4% ($p< 0.05$), 7.2 ± 2.4% ($p< 0.05$), and 23.8 ± 4.2% ($p< 0.001$), respectively, whereas, platelet aggregation was considerably decreased by fractions 4, 5, and 6 by 48.4 ± 11.4% ($p< 0.005$), 89.6 ± 5.3% ($p< 0.001$), and 72.5 ± 8.3% ($p< 0.001$), respectively. Notably, fractions, 4-6 were enriched in polygalloyl polyflavan-3-ols. However, gallic acid showed no discernible impact, whereas unfractionated GSK exhibited slight decrease of platelet aggregation (12.0 ± 3.8%).

Park *et al*.[31] reported the anti-platelet activity of methanolic extract of *Pistacia chinensis* (Family - Anacardiaceae) bark in rat platelets induced by ADP under *in vitro* conditions. The plant extract inhibited the platelet aggregation in a concentration ranging from 2.5-20 μg/mL. Moreover, it also reduced the [Ca(2+)] i, ATP, and TXA2 release in ADP-activated platelets, but also enhanced cAMP production in resting platelets. This could be suggested that the other plant of the same family and genus, *Pistacia vera* may also possess anti-platelet activity and needs scientific investigations.

Meshkini and Tahmasbi[32] evaluated *in vitro* platelet anti-aggregation effects of hull extract of Walnuts (*Juglans regia*; Family - Juglandaceae). The wall hull extract inhibited thrombin-induced platelet aggregation by 50% at a concentration of 50 μg/mL and exhibited a dose-dependent action on platelet aggregation without causing any cytotoxic effects on platelets.

Rehman *et al*.[33] evaluated the impact of Chilgoza Pine (*Pinus gerardiana*; Family - Pinaceae) nut oil (PGNC) on *in vitro* platelet aggregation after activation induced by collagen and adrenaline. Platelet-rich plasma (PRP) was treated with 10μl PGNC, and the greatest impact was observed. When collagen was used to activate platelets, PGNC significantly ($p<0.001$) lengthened the time it takes for platelets to aggregate in a dose-dependent manner. On the other hand, when triggered by epinephrine, PGNC reduced the time for platelet aggregation in a concentration dependent manner significantly ($p<0.001$).

Baru (*Dipteryx alata;* Family - Fabaceae) is a nutritious nut from central-western area of the Brazilian Savanna; especially rich in tocopherols. The effect of Baru almond oil supplementation on vascular function, thrombus formation and platelet aggregation in the aorta arteries of Wistar rats was investigated by Silva-Luis *et al*.[34]. Both platelet aggregation and the generation of the superoxide anion radicals were significantly diminished ($p<0.05$) by Baru oil supplementation. Furthermore, vascular function of the aorta arteries was significantly ($p<0.05$) enhanced and antithrombotic effect was also observed with supplementation of Baru oil.

Interestingly, active constituents of some of the nuts have shown promising anti-platelet activity. For example, Morin hydrate; a flavonol which is an important constituent of Almonds (*Prunus dulcis*; Family – Rosaceae) has effectively

inhibited collagen (3 µg/ml) - and thrombin (0.05 U/ml)-induced human platelet aggregation in a dose-dependent manner. The IC_{50} value obtained against collagen-induced aggregation was 50 µM. The study further reported that the mechanism behind anti-platelet activity of morin hydrate is through down-regulation of TXA_2 production and integrin $\alpha_{IIb}\beta_3$ activation, as well as by upregulation of cAMP generation[35].

Despite this, there are some of the spices namely, asafoetida, star anise, cinnamon and nuts such as cashew nut, macadamia nut, hazel nut and apricot whose platelet aggregation inhibition activity could also be scientifically evaluated in order to know their health benefits in prevention of cardiovascular diseases.

CONCLUSION

Coronary heart disease and stroke are leading causes of mortality and morbidity in the human population. Platelet aggregation is one of the bases behind beginning of these athero-thrombotic events. Synthetic anti-platelet drugs are available but these are associated with adverse effects. Therefore, plant based anti-platelet drugs provides hope. Several of the nuts and spices possess significant anti-platelet activity. Moreover, these are enriched with therapeutic bioactive molecules which provide additional beneficial properties for example, antioxidant, anti-inflammatory, hypotensive, hypolipidemic, thrombolytic etc. Dietary consumption of these could be therefore, helpful in prevention of cardiovascular diseases. However, long term scientific studies are warranted to validate this.

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Review

Phosphodiesterase Inhibitors: A Review

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ABSTRACT

Phosphodiesterase Inhibitors are class of drugs, actively inhibiting the specific target Phosphodiesterase Enzymes (PDE).FDA approved to be used in myriad of disorders such as Chronic Obstructive Pulmonary Disease (COPD), Erectile Dysfunction (ED), Benign Prostatic Hyperplasia (BPH), Pulmonary Artery Hypertension (PAH), Bronchial Asthma (BA) etc. The primary mechanism of PDE Inhibitors is smooth muscle relaxation and bronchodilation by inhibiting Cyclic Guanosine Monophosphate (cGMP) and Cyclic Adenosine Monophosphate (cAMP) degradation by acting on Nitric Oxide (NO) led pathway. This role of PDE Inhibitors is beneficial in so many conditions, as cGMP and cAMP pathways are present in numerous areas. We present a review on current state uses of PDE Inhibitors and their potential future therapy of experimental uses having off label effects utilising the Nitric Oxide led pathways as the core basis.

KEYWORDS: Phosphodiesterase Enzyme (PDE), cAMP, cGMP, Nitric Oxide, Platelet aggregation, Sildenafil, Tadalafil.

INTRODUCTION

Henry Hyde Salter was a British physician who is considered to be the first to document Phosphodiesterase (PDE) inhibitor. In his book on asthma, he advised having a strong cup of coffee empty stomach in the morning to control asthmatic attack, which actually denoted the weak PDE inhibitory effect of caffeine[1].

It is understood that PDE Inhibitors are the drugs which acts by blocking the PDE Enzyme and its five subtypes. This in turn ceases the inactivation of the Cyclic Adenosine Monophosphate (cAMP) and Cyclic Guanosine Monophosphate (cGMP) which are the secondary messengers in the intracellular compartment.

HISTORY

The first documentation for the isolation of these enzymes are found in the year of 1970, which was done from rat brains[2]. Weiss and Hait first identified the pharmacological and therapeutic properties of PDE in 1977[3].

Beavo identified and classified the multiple subtypes of PDE and placed them under the PDE Super family umbrella. (Table – 1)[4]

Given the widespread tissue expression of PDE, a wide range of drugs are available targeting various conditions.

Table 1: Phosphodiesterase Inhibitors and Drug Examples

Group	Expressive Tissue	Inhibiting Drugs
PDE-1	Smooth muscles, lung, brain, heart	KS-505a, Vinpocetine
PDE-2	Platelets, Liver, Lung, Adrenal Gland	EHNA
PDE-3	Adipose tissue, Inflammatory precursors, Cardiac Cells	Milrinone, Cilostazol, Anagrelide
PDE-4	Sertoli cells, renal cells, nervous cells	Roflumilast, Cilomilast
PDE-5	Smooth muscle cells, vascular cells, platelets	Sildenafil, Zaprinast
PDE-6	Photoreceptor cells	Dipyridamole
PDE-7	Skeletal tissue, cardiac cells, pancreatic cells, T Immune cells (Lymphocytes)	IC242
PDE-8	Testicular cells, hepatic cells, ovarian cells	Zaprinast
PDE-9	Renal, hepatic, nervous, lung	BAY-73-6691
PDE-10	Testicular cells	-
PDE-11	Salivary glands, pituitary cells	-

INDIVIDUAL SUBTYPES AND MECHANISMS

The primary mechanism of the PDE is to isolate the phosphate molecule from the target ell and reduce the amount of cAMP / cGMP. Now when this enzyme is inhibited through selective blockade it stops from cAMP and cGMP to degrade further and in turn preserving their primary effect such as bronchodilation, vasodilation, smooth muscle relaxation etc.

PDE-3

PDE-3 inhibitors specifically target the cardiac cells, they increase the cAMP volume in the peripheral vasculature as well as myocardial cells and platelets, resulting in peripheral vasodilation, increased ionized calcium in myocardial cells and preventing platelet aggregation. These properties are effective in treatment of Peripheral Vascular Disease (Arterial) and heart failure. Milrinone, Cilastazole, Anagrelide are commonly employed[5].

PDE-4

PDE-4 has a degradative impact on the cAMP substrate. They are the most abundant of all the PDE subtypes. There are around 20 subtypes of PDE-4 Inhibitors. PDE-4 target hydrolyzation of cAMP in both nervous and immune cells[6]. PDE-4 inhibitors have multiple uses. Their cognition benefits are well known to improve long term memory[7] along with numerous other cognition benefits such as alertness[8]. They also prevent excitotoxic damage to neuronal cells[9]

There are distinct subvariants of PDE-4 inhibitors, differentiated on the basis of target action and mechanism.The prototype examples of PDE -4 Inhibitors:

- Cilomilast
- Crisaborole
- Ibudilast
- Roflumilast
- Rolipram

Apart from constitutional adverse effects such as vomiting, nausea, Roflumilast is notorious to cause urinary tract and upper respiratory tract disorders[10].

Table 2: Sub Variants of PDE 4

Sub Variants	Expression, Action
PDE4A, PDE4 D	Antidepressant
PDE4 -B	Antipsychotic
PDE4 -C	Expressed in peripheral circulation (Anti-Inflammatory), alcohol de-addiction
PDE4 -D	Area postrema – Potent emetic

PDE-5

The fifth subtype of PDE Inhibitors, PDE-5 is a cGMP related variant. It was first isolated from platelets in rat blood in the year of 1978, termed as cGMP-PDE[11]. The first PDE-5 Inhibitor to be given in humans was Zaprinast. It was given in patients suffering from exercise induced asthma, it was given for its bronchodilator effect[12]. PDE-5 Inhibitors were also considered as a promising agent in cardiovascular medicine due to their effects on vaso-relaxation and smooth muscle relaxation[13]. After being studied it's effect in rat blood, in 1992 Rajfer et al[14]. studied that similarly as in rat blood, the Nitric Oxide (NO) formation was stimulated by Electrical Field Stimulation (EFS) in human corpus cavernosum also, which in turn bringing relaxation. This effect was particularly diminished, or if not then it's reduced in impotent males. He further studied Zaprinast and demonstrated that it further stimulates the relaxation of corpus cavernosum induced by Nitric Oxide, giving us the most widely employed use of PDE-5 – Impotency[14].

PDE-5 Inhibitor agents such as Sildenafil Tadalafil and Vardenafil are used in the treatment of erectile dysfunction[15]. Due to their affinity for cGMP and effect on Nitric Oxide reducing pathway, PDE-5 inhibitors are also used in treating some variants of pulmonary hypertension and benign prostatic hyperplasia. In patients suffering from PAH, PDE-5 inhibitors have significant effect on mortality and also on substantial improvement in quality of life, more so in patients suffering from PAH due to right heart failure[16].

PDE-5 was initially discovered by a British physician named Henry Salter, who discovered the bronchodilator effects of caffeine when a bronchial asthma patient got relief from his symptoms after having a strong cup of coffee[17]. And for the beneficial effects of Angina, initial pre-clinical work was started by Pfizer. Current available marketable agents are Sildenafil, Tadalafil, Zaprinast, Vardenafil, Icaria etc[18].

PDE- 7, 9, 10

Quinazoline a type of PDE-7 inhibitor has been used for it's neuroprotective and anti-inflammatory effects[19].

The primary metabolite of caffeine – Paraxanthine, inhibits the PDE-9 receptor whose primary affinity is with cGMP. It is almost similar to PDE-5 in expression, especially in corpus cavernosum[20].

A common opioid alkaloid derivative – Papaverine is proven to inhibit the PDE-10 receptor. The PDE-10 is seen exclusively in the striatum, and they increase cAMP and cGMP following

inhibition by agents such as Papaverine. It was documented that PDE10-A inhibition increases the cAMP level, this effect correlates to the hypoactivity in brain. The determinant of this action is mainly related to the elevation in cAMP levels by the inhibitors and its capacity to do so[21].

improve attention, cognition, memory registration, inhibition, and processing information. The Long Term Potentiation (LTP) is a favourable property of these PDE Inhibitors. Hope lies on a potential isotope of PDE Inhibitor that helps in aged brain such as in Alzheimer which has acceptable rate of side effects[24].

Table 2: Summary of various PDE - Inhibitors

PDE Group	Disease Target
PDE – 2	Acute Respiratory Distress Syndrome, Sepsis[22]
PDE – 3	PVD, Heart failure etc.
PDE – 4	Depression, Alzheimer's disease, Memory Loss etc.
PDE – 5	Erectile Dysfunction, Pulmonary Hypertension, Premature Ejaculation, Renal Failure
PDE – 7	Anti – Inflammatory
PDE – 9	Psychostimulant
PDE – 10	Antipsychotic

NOVEL POTENTIAL USE

Neuroprotective Role

Numerous disquiets related to the central nervous system over the period of time end up tarnishing the structural format of the architecture. This leads to dysfunctional CNS and also a dysregulated and curtailed repair. To counteract the damage by nervous system disorders and trauma, the intracellular signals comprising mainly of cGMP and cAMP which regulates the inflammation, cell death (neuronal), immune response, neuroplasticity has to be altered. As we discussed earlier, PDEs have an inhibitory effect on these regulators.

In an event of neurotrauma or pathology, PDEs hydrolyse the cGMP and cAMP to 5'GMP and 5'AMP. This inhibits the ongoing process and promotes inflammation and destruction. Inhibiting this PDEs effect has a potential role in neuroprotective and repair process[23].

Out of the entire family, PDEs 2,4,5,9,10 has been proven to

Pleiotropism and Cardiovascular Medicine

The PDE-5 inhibitor Tadalafil, Sildenafil has been also proven to improve the left ventricular function when given with Doxorubicin (Anthracycline derivative – chemotherapeutic agent) and it also has an inhibitory effect in LV dysfunction and apoptosis caused by Doxorubicin. Inhibition has been led by the same cGMP and cAMP upregulation along with the reactive oxygen species (ROS) superoxide dismutase (SOD). This effect does not hamper the chemotherapeutic effect of the agent[25,26,27].

The ROS (Mitochondrial) has been reported to improve killing of neoplastic cells. Doxorubicin acts by increased production of ROS in cancer cells[26]. When combined with sildenafil, this production is further increased exponentially. This killings are independent of p53[28].

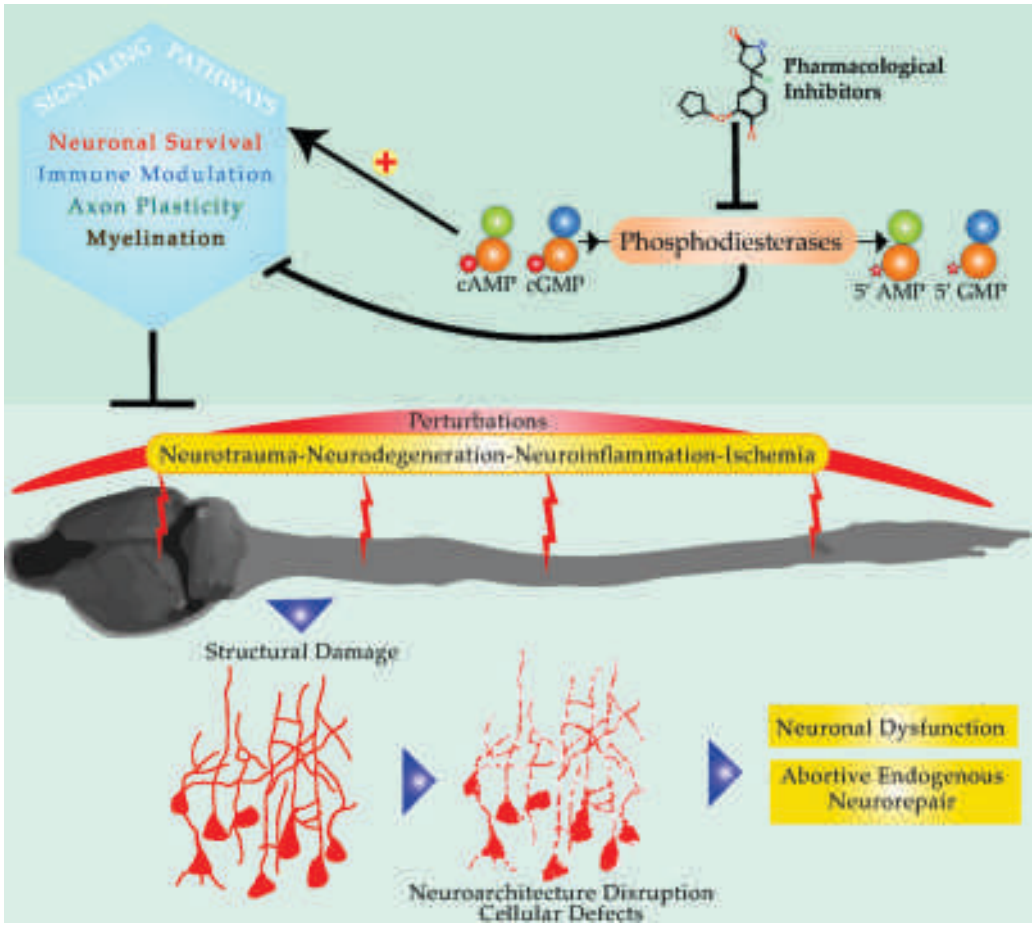

Figure 1: Role of PDEs in Neurodegeneration and therapeutic role of PDE Inhibitors [23]
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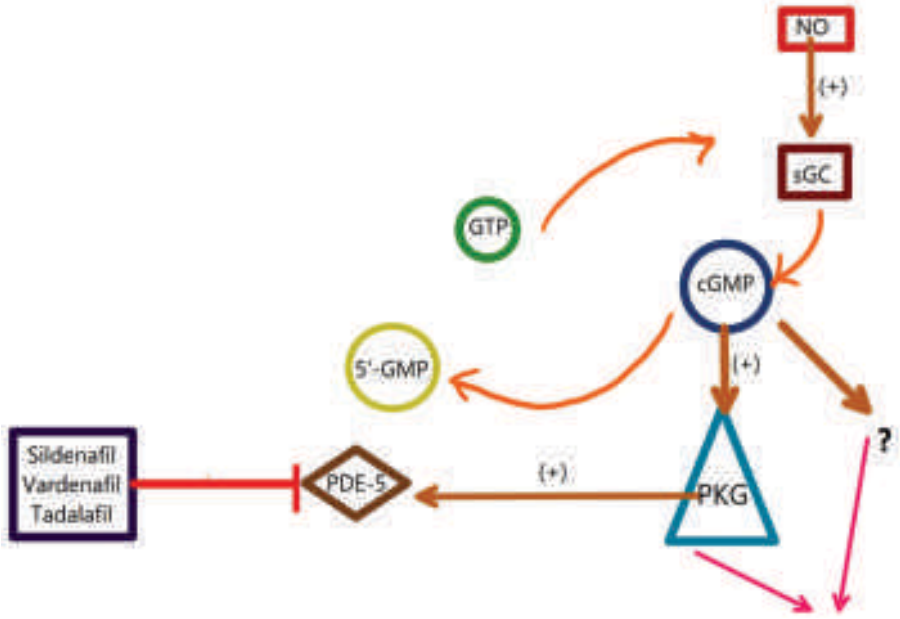

Figure 2: cGMP – PDE 5 Site of action Pathway
(Image redrawn from Gross G[29].)

The increase in cAMP could potentially affect cardiac function, vascularity of heart and the tonicity. They have been proven useful also in stroke (cerebral and cardiac), peripheral vascular disorders and severe heart failure[30]. Pentoxifylline was the first PDE inhibitor to be approved in treatment of limb claudication. However, studies were inconclusive for the same[31,32] The most recent addition in the similar use-case is Cilastazole. The therapeutic benefits are much better compared to Pentoxifylline[33]. It is reported that Cilastazole reduces plasma triglyceride (TGL) levels, and upsurges High Density Lipoproteins (HDL)[34]. Cilastazole has also been shown to reduce the occurrence of coronary restenosis post Percutaneous Transluminal Coronary Angioplasty (PTCA)[35]. Current state of PDE Inhibitors in cardiovascular disease is limited to two approved agents. Pentoxifylline and Cilastazole for intermittent claudication in oral form and Milrinone for acute congestive heart failure intravenously[36].

shown to reduce the serum Lactate Dehydrogenase (LDH) levels with substantial increase lymphocyte count[39]. Milrinone has also shown to improve cardiac function and beneficial in immune dysregulation in septic conditions when given with Esmolol[40]. Reduction in pulmonary arterial pressure and mean arterial pressure is seen in early stages of ARDS induced by severe COVID-19 with Sildenafil[41].

The world entered in a stage of Pandemic because of the havoc created by the deadly SARS CoV -2. The viral pneumonia caused by SARS CoV -2 virus led to severe immune response and dysregulated cytokine recruitment leading to severe ARDS and residual deadly fibrosis. The effect on NO led cGMP cAMP axis of PDE-5 is being studied as a phase three trial given the fact that PDE5 primarily has pulmonary expression. They reduce the cytokine recruitment and alveolar necrosis[42].

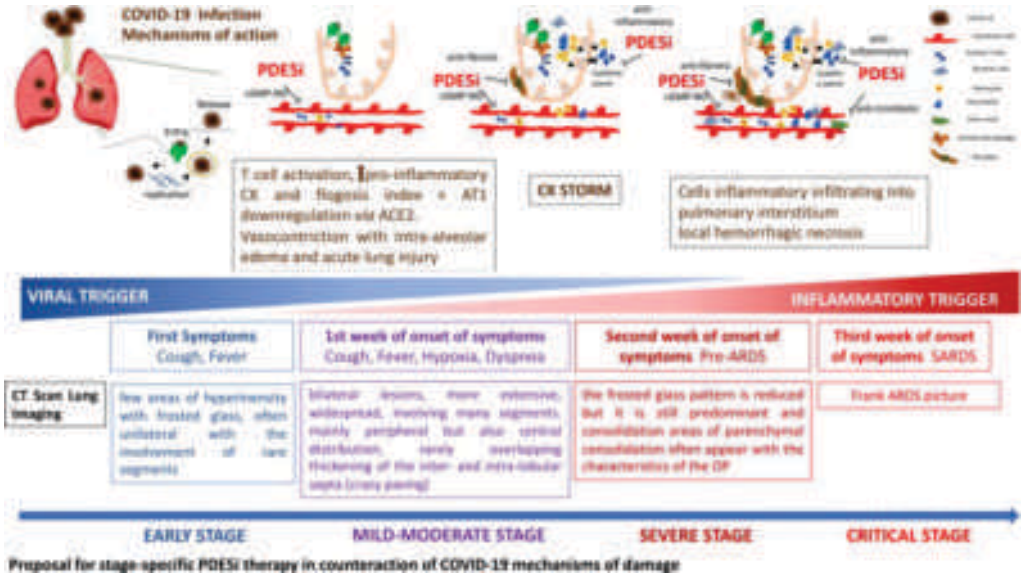

Figure 3: A proposal for PDE Inhibitor in COVID -19
(Image re-used under public health emergency permission. Image by Isadora AM *et al*[42])

Acute Lung Injury and COVID -19

The story of PDE Inhibitor started with beneficial effect of caffeine in bronchial asthma patients due to its bronchodilator properties[1]. But in Acute Lung Injury especially such as ARDS, pneumonia or acute exacerbations of COPD, the pathology is mainly due to dysregulated immune response and cytokine recruitment. In a study done by Salari et al. it was reported that Aminophylline significantly reduced Epidermal Growth Factor Levels (EGF) when introduced in mechanically ventilated patients with PEEP. Both in conjunction reduced the overall Acute Physiology and Chronic Health Evaluation (APACHE) Pi score[37]. Experimental evidence shows that Pentoxifylline improves the oxygen transport and hemodynamic stability in critically ill patients suffering from septic shock[38]. Pentoxifylline was also

Anti-Platelet Therapy

Platelet aggregation is a vital step in the coagulation cascade. The pivotal messengers cAMP and cGMP do exert a robust inhibition on the essential platelet function. The basic principle behind the potential role of PDE Inhibitors in platelet aggregation inhibition is increasing the platelet activating nucleotide which in turn interferes with the cytoskeletal fibrinogen activation, recruitment of pro-inflammatory mediators and degranulation. This affect can be achieved by activating the quintessential cAMP and cGMP pathways[47].

Caffeine was administered as a 250mg oral dose thrice a day for a week in healthy volunteers, they all showed reduced platelet aggregation, upregulation of platelet adenosine receptors and increased cAMP[48]. This characteristic of caffeine as a platelet

inhibitor was first seen in 1967[49]. Only PDE2, PDE3 and PDE5 have been seen being secreted from platelets[50].

- **PDE-2**

PDE-2 acts by hindering the thrombin led platelet aggregation due to nitroprusside[51].

Currently numerous thienylacyl hydrazone derivatives are studied as a PDE-2 representative for their platelet aggregating characteristic[52].

- **PDE-3**

PDE3A is the primary subtype of PDE3 exerted by platelets.[53] Anagrelidehas been found to be causing thrombocytopaenia in humans[54] even though it is a platelet aggregator inhibitor[55]. Another agent Cilastazole also hinders platelet aggregation. It more commonly acts on platelet aggregation led by ADP, collagen fibres and arachidonic acid[56,57]. An enhanced antiplatelet effect was seen when Cilastazole was given in combination with aspirin plusclopidogrelin primary PTCA patients of Myocardial Infarction[58,59]. Along with this Cilostazol has been used in preventing stent restenosis also[60].

Cilastazole has been widely lauded as an excellent agent to prevent stroke and myocardial infarction. Numerous studies including Cilastazole Stroke Prevention Study (CSPS 1&2) shows that Cilastazole reduces the stroke risk significantly along with that it is proven better than Aspirin prophylaxis alone[61,62].

After being discovered to inhibit platelet aggregation in rabbits,[63] all eyes turned to Dipyridamole as another potential antithrombotic element of PDE3 + PDE5 inhibitor especially in stent restenosis[64]. Dipyridamole enhances the Nitric Oxide inhibition on rabbit as well as human platelets[65]. However, there is scarce clinical literature to support the evidence that Dipyridamole can be used as a singular anti platelet agent. But combination use has been promising. The two large scale ESPS2 & ESRIT trial concluded that when used with low-dose of aspirin in cerebrovascular stroke patients, the outcome is more favourable than aspirin alone[66,67]. The American College of Chest Physicians (ACCP) took charge over these studied and in 2008 started recommending dual Anti-platelet therapy with Dipyridamole in stroke or ischaemic event patients[68]. Not just with aspirin, dipyridamole has been proven to be more efficacious in thrombus prevention in patients with artificial heart valves with warfarin prophylaxis[69].

- **PDE -5**

A very potent PDE-5 inhibitor Sildenafil is widely used in treatment of Erectile Dysfunction. But along with this, there is promising role of Sildenafil as anti platelet agent. Very few clinical studies have been employed for the use-case. In a clinical study of healthy volunteers, sildenafil hindered the collagen led platelet aggregation (in doses of 100mg), with enhanced effect if given in conjunction with nitrates[70].

In a study of 30 healthy male volunteers Sildenafil was administered in doses of 50 and 100mg in two randomised groups to see platelet aggregation. It was seen that Sildenafil did in fact improve platelet aggregation ex-vivo, more so on 100mg dose. However ADP led platelet aggregation remained unaffected[71].

This led to research gap in PDE-5 Inhibitor's effect on ADP Induced platelet aggregation. Following up on that, In another study done on 30 male healthy individuals, different PDE-5 agent Tadalafil was administered in a single dose of 10/20 mg to observe its effect on platelet aggregation. The study observed that Tadalafil is actually an effective platelet aggregation inhibitor when induced by ADP and Collagen. The 20mg dose proved to be more effective.

CONCLUSION

PDE Inhibitors are diverse class of drugs having significant therapeutic benefit in numerous diseases. Because of its affect on cAMP and cGMP pathways, they do have a promising role in so many new areas, which are still yet unknown. Be it from using it in ED, Acute Lung Injury or Platelet aggregation, the future looks promising for PDE Inhibitors and appropriate clinical backing is required to prove the theoretical claims.

CONFLICTS OF INTEREST: None

FINANCIAL SUPPORT: None

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Review

A Scopic Review on Reactive Thrombocytosis

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ABSTRACT

Thrombocytosis, also called thrombocythemia, is generally defined as platelet count greater than a defined upper limit of normal. The most common cut off for normal is <450,000/µl. Elevated platelet counts are often an incidental or unexpected finding on a complete blood count conducted to evaluate an unrelated condition. The causes of thrombocytosis are separated into two categories: autonomous (primary) thrombocytosis and reactive (secondary) thrombocytosis. Autonomous thrombocytosis occurs as a result of myeloproliferative disorders, myelodysplastic disorders, or rarely as a result of a hereditary condition. Reactive thrombocytosis is most often a normal physiologic response to coexistent chronic inflammatory conditions. Distinction between these two categories is important since autonomous thrombocytosis is associated with a significantly increased risk for thrombotic or hemorrhagic complications whereas reactive thrombocytosis is not. The most common reason for an elevated platelet count is reactive thrombocytosis. The present review will discuss about the association of reactive thrombocytosis with different clinical conditions and the possible underlying mechanism.

KEYWORDS: Thrombocythemia, Autonomous thrombocytosis, Platelet granules

INTRODUCTION

Historical Aspect - Discovery of Platelet

Brewer traced the history of the discovery of the platelet[1]. Although red blood cells had been known since van Leeuwenhoek (1632–1723), it was the German anatomist Max Schultze (1825–1874) who first offered a description of the platelet in his newly-founded journal Archiv für mikroscopische Anatomie[2]. Max Schultze describes "spherules" to be much smaller than red blood cells that are occasionally clumped and may participate in collections of fibrous material. He recommends further study of the findings.

Giulio Bizzozero (1846–1901), building on Schultze's findings, used "living circulation" to study blood cells of amphibians microscopically in vivo. He is especially noted for discovering that platelets clump at the site of blood vessel injury, a process that precedes the formation of a blood clot. This observation confirmed the role of platelets in coagulation[3].

PLATELET[4]

Platelets have been described as the smallest cell fragment in the human body[5] (Jurk & Kichrel, 2005). The normal platelets are small, disc-shaped cells without a nucleus, normally measuring 1 to 2 µm in diameter and 0.5 to 1.0 µm in thickness with a volume of about 6µl. Platelets are derived from the cytoplasm of megakaryocyte, primarily located in the bone marrow. Normally, a platelet is released to the blood stream and circulates for about 10 days before its removal, largely by the spleen. Platelets circulate freely without adhesion to the vessel wall or aggregation with other platelets. If stimulated, platelets become spherical, extend pseudo pods, and adhere to vessel walls and to each other. It participates with the blood vessel, coagulation factors, and other platelets in the initiation of haemostasis.

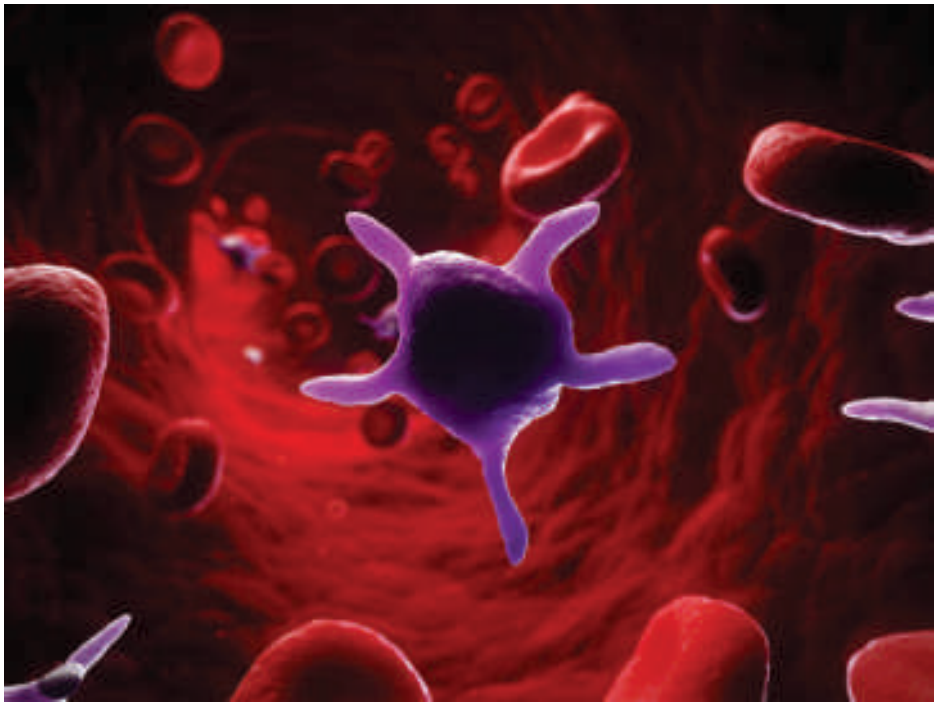

Figure 1: Platelet

Platelet Production and Release[4]

The megakaryocyte, parent cell of the platelet, is derived from pluripotential cells in the bone marrow. Individual megakaryocyte have been estimated to produce as many as 1000 platelets per cell, and apparently very efficient system facilitated by the absence of nuclei in platelets. IL-6 and IL-11 are thought to increase platelet production by megakaryocyte. There are two possible mechanisms whereby platelet achieves the transition from being stationary constituents of megakaryocyte cytoplasm in the bone marrow to circulation cells in the bloodstream. One theory is that megakaryocyte themselves are released from the bone marrow and are carried to the pulmonary capillaries, where they fragment into individual platelet. Another is that the bone marrow endothelium has special properties that encourage formation of pseudopods extending from mature megakaryocyte to bone marrow sinuses and thereby directly release platelets into the blood.

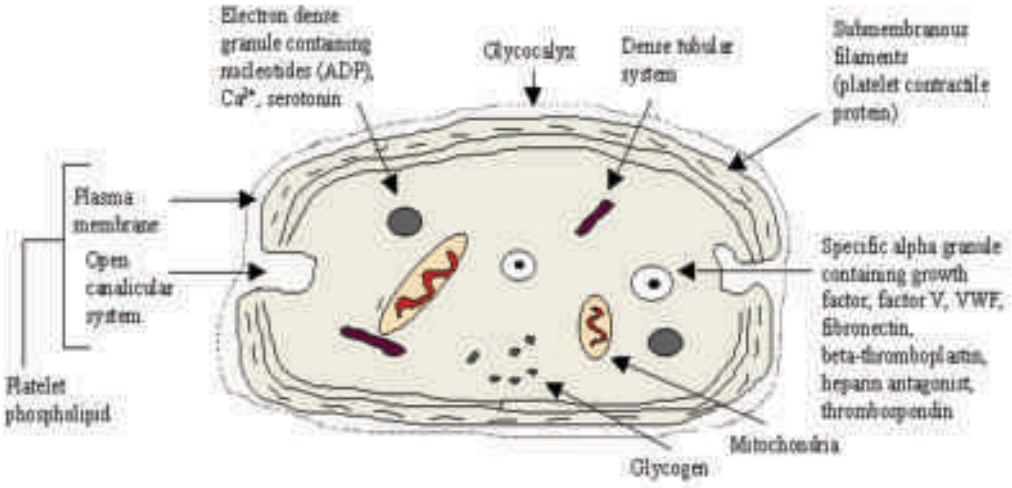

Figure 2: Ultra Structure of Platelet

Platelet Structure[4]

Platelets are composed of three principal components: membrane structures, microtubules, and granules. Platelet membrane, overlying glycocalyx, and submembrane structures mediate responses to platelet stimulation and express specific antigenic characteristics. The surface glycoprotein's variously serve as receptors, facilitate platelet adhesion, contraction, and determine expression of specific platelet antigens. Platelet canalicular system is created by numerous invaginations of the platelet surface and, interspersed among these structures; a set of narrower channels termed the dense tubular system. The canalicular system provides a direct connection between the interior and the surface of the platelet, providing entrance of plasma ingredients into the platelet as well as exit of its own ingredients in connection with the release reaction. The dense tubular system, on the other hand, is entirely enclosed and is the major site for storage of Ca^{2+} and the location of cyclooxygenase, the critical enzyme for conversion of membrane-derived arachidonic acid to unstable endoperoxide precursors of prostaglandins and thromboxanes. The major inner structures of the platelet are the cytoskeleton, the microtubules, and a system of contractile proteins. The cytoskeleton provides a framework to anchor the platelet membrane and allow signal transduction to take place. Furthermore, it is a framework against which the contractile proteins of the platelet can operate to initiate shape change and protrusion of pseudopodia at the onset of spreading. Actin, actin-binding protein, talin, vinculin, stectrin, a-actinin, and several membrane glycoproteins make up the cytoskeleton. Actin-binding protein binds both actin and GPIb-IX. In resting platelet this maintains the discoid shape of the platelet. With activation and calcium influx, calpain is activated, severing the link of actin-binding protein to GPIb-IX. The microtubules are arranged in the form of an inner ring beneath the surface of the platelet and are distinct from the canalicular and dense tubular systems of the membrane zone. The microtubules provide structural support of the platelet, maintain its discoid shape in the resting state, and influence the character of its contractile functions. Contractile proteins largely consist of myosin and submembrane actin filaments that are anchored to the surface of the platelet by the Tran membrane glycoprotein a-actinin. On stimulation of the platelet, the cytoplasmic concentration of Ca^{2+} rises and calmodulin is activated and combines with myosin light-chain kinase; this enzyme phosphorylates myosin, leading to the combination of myosin with actin to form contractile act myosin, which mediates the initial changes in shape of the platelet and ultimately, retraction of the formed clot.

The three types of storage granules dominate the central cytoplasm are the dense granules, alpha granules and lysosomal granules.

In general, platelet aggregation is associated with release but at least in vitro certain "strong" agonists, i.e., collagen and thrombin, can trigger release without aggregation. P selectin or GMP140 is a component of the a-granule membrane. Release involves the granules nearest the platelet surface being transported to the platelet membrane and fusing with it so that a

Content of Platelet Granules[6]

Dense Granules	Alpha Granules	Lysosomal Granules
Adenosine triphosphate	PF-4	Galactosidase
Adenosine diphosphate	Beta-thromboglobulin	Fucosidase
Glucuronidase	Fibrinogen	Hexaminidase
Calcium	Factor V	Thrombospondin
Serotonin	Fibronectin	Cathepsin
Pyrophosphate	Plasma inhibitors	
P-selectin(CD-62)	P-selectin(CD-62)	
Transforming growth factor beta-1	Platelet derived growth factor inhibitor(PDGF)	
Catecholamines	Alpha-2 macroglobulin	
Nor-adrenaline/adrenaline		
Guanosoine-5diphosphate		
GDP/guanosine-5(GTP)		
Triphosphate		

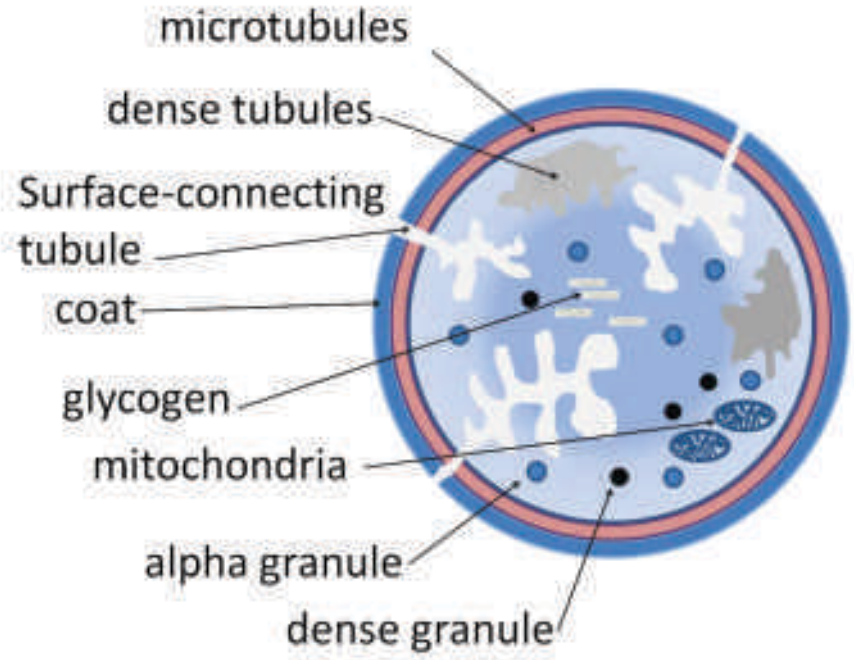

Figure 3: Platelet Structure

small portion of the post release external platelet membrane is made up of the inner membrane of theca-granule, including GMP140.

Dense bodies are granules characterized by high electron density and are fewer in number than a-granules. These structures serve as a depot for non-metabolic substances that are extrinsic to the platelet and may be picked up or released as indicated. On their release, these substances are particularly critical to platelet aggregation.

Lysosomal granules are also present in platelets, perhaps representing the original role of the platelet as a white blood cell. These granules contain at least seven acid hydrolyses. These enzymes may contribute to the intracellular effects of phagocytosis of may create an uncertain amount of damage extracellularly at the site of platelet release.

The contents and functions of the non-granular organelles of the platelet may be summarized as follows: mitochondria contain enzymes for oxidative metabolism and thereby provide a major source of energy through the generation of ATP, and peroxisomes contain catalase, which protects the platelet from oxidative damage in connection with periodically intense metabolic activity. Platelets also contain occasional ribosomal particles and small amounts of RNA.

On films made from blood anticoagulated with the strong calcium chelating agent ethylenediamine tetra acetic acid (EDTA) and stained with Wright's stain, platelet appears as small bluish-gray, oval-to-round with several purple-red granules. When anticoagulated blood is used to prepare blood films, platelet undergo variable activation and spreading and thus platelet aggregate are commonly seen: platelet from specimens may demonstrate three to four very long and thin processes extending out from the body of the platelet (filopodia).

Normal Range, Life Span and Physiological Variation of Platelet Count

The platelet count in the peripheral blood is maintained at a fairly constant level, which is in the range 150,000 to 450,000/cmm of blood in normal subjects. A somewhat lower range is seen in the newborn, normal adult level being achieved by about 3 months. Considerable fluctuation may occur during the course of menstrual cycle, lowest level being found at or just prior to menstruation. Heavy exercise and adrenergic stimulation tends to increase the platelet count transiently, possibly by mobilization of the splenic pool. There are some racial differences in platelet count for e.g. Mediterranean races' platelet count is as low as 80,000/ cmm of blood are sometimes found in normal individuals.

However in such cases the mean platelet volume is increased so that over all platelet mass is unaltered. Apart from this so called "Mediterranean Macrothrombocytopenia"[13] which is not clinically significant. There may be sex difference: thus in women, the platelet count have been reported to be about 2% higher than man. There is no evidence that oral contraceptives affect the platelet count. There are some ethnic differences and in healthy west Indians and Africans platelet counts may on average be 10-20% lower than those of Europeans living in the same environment. Strenuous exercise causes a 30-40% increase in platelet count. The normal life span of platelet ranges between 8 to 14 days[7].

Platelet Morphology and Number in Peripheral Blood Smear

Platelet appears in normal stained blood as small blue or colourless bodies with red or purple granules. Normal platelet average about 1-3 micrometer in diameter but show wide variation in shape, from round to elongated, cigar shaped forms. A rough estimate of the platelet count can be made by observation of the stained blood film. If the platelet count is normal, approximately 8 to15 platelet (individual or in small clumps) should be visible in each oil immersion field. There should be one platelet for every 10-30 erythrocytes[8].

The occurrence of giant platelets or platelet masses may indicate a myeloproliferative disorder, or absence of the spleen or improper collection of blood sample. Estimation of platelet concentration is best determined from EDTA anticoagulated blood, where the platelets generally do not aggregate[8].

Giant platelets and abnormal platelet granulation are characteristic features of idiopathic myelofibrosis[9].

Platelet morphology show large, pale-staining, hypo-granular platelet in essential thrombocythemia in peripheral smear. Characteristic morphological platelet features are seen in two platelet inherited disorders associated with bleeding. The Bernard- Soulier syndrome in which there are giant platelet with defective ristocetin response and Gray Platelet syndrome in which platelet lacks granules and have ghost like appearance on blood stained film[10]. In about 1% of patient EDTA anticoagulated blood causes platelet clumping and thus resulting in pseudo-thrombocytopenia.

Role of Platelet in Infection and Inflammation

Blood platelets are presented as active players in antimicrobial host defence and induction of inflammation and tissue repair in addition to their participation in hemostasis. Megakaryopoesis is inhibited after the acute infection with viruses or bacteria. In addition chronic inflammation is often associated with reactive thrombocytosis. Platelets can bind and internalize pathogens and release microbicidal proteins that kill certain bacteria and fungi. By making cell-cell contacts with leukocytes and endothelial cells, platelets assist white blood cells in rolling, arrest and transmigration. On stimulation by bacteria or thrombin, platelets release the content of their alpha granules, which include an arsenal of bioactive peptides, such as growth factors for endothelial cells, smooth muscle cells and fibroblast. This integral to innate immunity, the tiny little platelets may become bombshells when irritated by pathogens[11].

Thrombocytosis

Thrombocytosis is the presence of an abnormally high number of platelets in the circulating blood. It may result from various physiological stimuli and pathological processes[12].

Classification

A) Primary Thrombocytosis (Essential thrombocytosis) - This is due to a failure to regulate the production of platelets (autonomous production) and is a feature of a number of myeloproliferative disorders. About a third of patients are asymptomatic at the time of diagnosis[13].

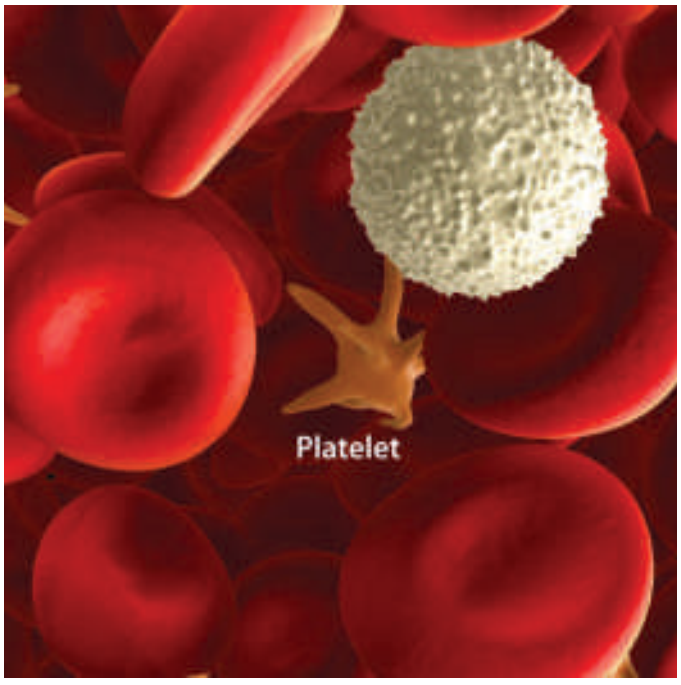

Figure 4(a): Low Platelet Count

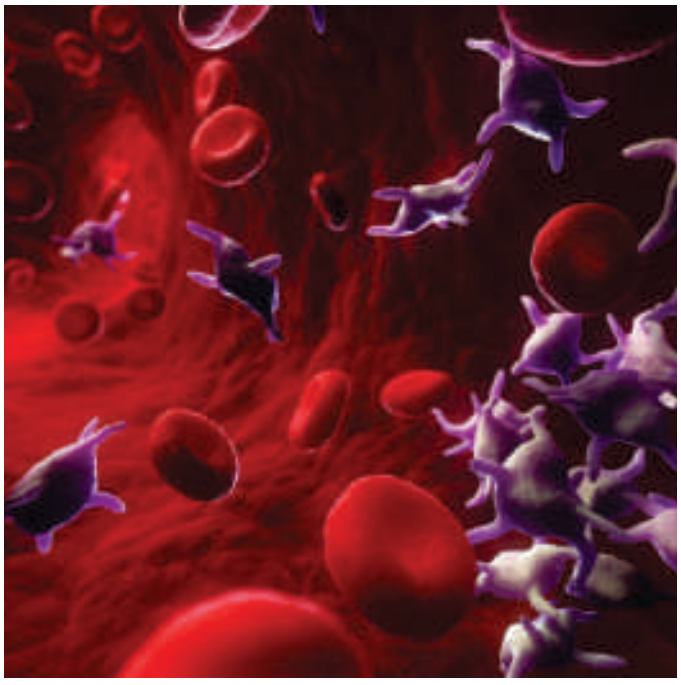

Figure 4(b): High Platelet Count

B) Secondary Thrombocytosis (Reactive thrombocytosis) - This can be secondary to a number of conditions. It is an exaggerated physiologic response to a primary problem, such as an infection. The trigger factor (e.g. infection) results in the release of cytokines which mediate an increase in platelet production. It is often a transient phenomenon which disappears when the underlying cause is resolved[14].

C) Non-specific Thrombocytosis - A recent "expert panel" has recommended that a platelet count of 400-450,000 needs no further evaluation.[15] Any platelet count > 450,000 does need evaluation. If there is no evidence of a "reactive" thrombocytosis, then Janus Kinase 2 mutations (JAK-2) testing should be done. A bone marrow biopsy should also be done, which would include testing for the Ph+ chromosome. Commonly, if these tests are negative, the individual platelet count is between 450,000/µl and 600,000/µl, and no evidence of reactive process then the individual is labeled "non-specific thrombocytosis."

Causes of Thrombocytosis[12]

I. Physiological: Exercise, Parturition, and Epinephrine

II. Pathological:

A) Primary Thrombocytosis

1) Myeloproliferative disorders.
   - Polycythemia vera
   - Chronic myeloid leukemia (CML)
   - Chronic idiopathic myelofibrosis
   - Essential thrombocytosis

2) Myelodysplastic disorders

3) Hereditary thrombocytosis

B) Secondary Thrombocytosis

1) Infection
   - Meningitis,
   - Infections of the upper and lower respiratory tract,
   - Urinary tract infections,
   - Gastroenteritis,
   - Septic arthritis,
   - Osteomyelitis and Generalised sepsis.

2) Chronic inflammations and vasculitis
   - Rheumatoid arthritis,
   - Kawasaki syndrome,
   - Henoch-Schonlein purpura,
   - Inflammatory bowel disease.

3) Tissue damage
   - Postsurgical,
   - Burns,
   - Trauma,
   - Fracture.

4) Rebound thrombocytosis
   - Iron deficiency anemia,
   - Bleeding,
   - Cancer chemotherapy,
   - Recovery phase of idiopathic thrombocytopenic purpura (ITP)

5) Postsplenectomy

6) Haemolytic anemia

7) Renal disorders (for example nephrotic syndrome, nephritis)

8) Malignancy (especially soft tissue sarcoma, osteosarcoma)

9) Low birth weight/ preterm infants.

Pathophysiology of Reactive Thrombocytosis[12]

Reactive thrombocytosis is usually mediated by increased release of numerous cytokines in response to infections, inflammation, vasculitis, tissue trauma, and other factors. Thrombopoietin (TPO), the primary cytokine for platelet production and maturation, and interleukin (IL)-6 levels are usually initially elevated in response to the primary events mentioned earlier; they stimulate an increase in platelet production.

It may be due to the overproduction of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-11, that occurs in chronic inflammatory, infective, and malignant states. The presence of elevated IL-1, IL-6, C-reactive protein (CRP), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) in individuals with these conditions suggests that these cytokines may be involved in secondary thrombocytosis (reactive thrombocytosis).

However, serum or plasma levels of these cytokines do not seem to be correlated with degree of thrombocytosis. Other cytokines may participate in the stimulation of platelet production. They include IL-3, IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin. These cytokines are directly or indirectly released during the primary events. When the original stimulation stops, the platelet count then returns to the reference range.

In severe infections, such as bacterial meningitis, one of the causes may be a rebound phenomenon after initial thrombocytopenia due to rapid consumption of platelets. This most commonly occurs in neonates and infants, indicating the labile nature of platelet count control in these subjects.

The most common infection associated with thrombocytosis is

pneumonia. In some instances, such as chronic haemolytic anemia, the stimulus (hypoxia) to produce cytokines persists, causing long-term elevation of platelet counts. Although thrombocytosis in association with iron-deficiency anemia is well documented, the mechanism remains unclear. Although elevated erythropoietin levels are observed in patients with thrombocytosis who have iron-deficiency anemia, a recent study showed that these elevated levels had no correlation with platelet count. Levels of other cytokines potentially responsible for thrombocytosis, such as IL-6 and TPO, were not elevated.

The spleen is the major organ for the destruction of platelets; therefore, after splenectomy, a sharp rise in the platelet count is routinely observed, although the count subsequently slowly decreases to the reference range. Similarly, functional asplenia that may occur after splenic artery embolisation results in thrombocytosis.

Laboratory Findings in Reactive Thrombocytosis

There was no specific laboratory finding in patients with reactive thrombocytosis, and the diagnosis ultimately depends on diagnosing the underlying problem. Serum IL-6 concentrations measured by activity assay of ELISA are increased in majority of patients believed to have reactive thrombocytosis and in of the patient with clonal megakaryopoesis, but these tests are not clinically available. Hollen found increased serum concentrations in 80% to 100% of his patients with malignancy, inflammation or recent surgery. Only 50% of the anaemic patients had increased concentration of IL-6 and only one of five patients were in this group[12].

Elevated fibrinogen levels are found in patients of reactive thrombocytosis, presumably as a part of acute-phase reaction, and may be helpful in differentiating primary from secondary thrombocytosis[16].

Serum obtained from patient with thrombocytosis may contain elevated concentration of acid phosphates or potassium. In blood samples containing increased number of platelets, the PaO_2 may be significantly reduced due to consumption of oxygen by the platelet, particularly if the blood sample is stored at the room temperature[16].

In one study done by Perez Encinas et al[36] in 1995, they found the most potent stimulator for the hepatic synthesis of C-reactive protein is interleukin-6. Also, interlukin-6 is endowed with thrombopoietic activity and its level increases in most of the reactive thrombocytosis where as they remain normal in primary thrombocytosis. They had concluded that Quantitation of C-reactive protein could thus prove useful in differentiating between primary and reactive thrombocytosis[17].

Differential Diagnosis of Thrombocytosis

Clinical and laboratory features that distinguish between primary (ET) and secondary thrombocytosis (RT)[12].

Features	ET	RT
1. Thrombosis and haemorrhage	+	-
2. Splenomegaly	+	-
3. Increased acute-phase reactants (IL-6, CRP and plasma fibrinogen)	+	-
4. Bone marrow reticulin fibrosis	+	-
5. Bone marrow megakaryopoesis clusters	+	-
6. Clonal haematopoiesis	+	-
7. Spontaneous colony formation	+	-
8. Abnormal cytogenetic	+	-

Prognosis

Most patients with reactive thrombocytosis do not have significant problems caused by thrombocytosis, and the prognosis of the basic disease is not usually significantly affected.

Thrombocytosis in Childhood[18]

Thrombocytosis is a frequent finding in hospitalized and ambulatory children due to the widespread use of automated blood cell counters. Reactive thrombocytosis is very common and is due to a variety of conditions

Causes of Secondary or Reactive Thrombocytosis in Children[18]

1. Infections (e.g., of the respiratory tract, gastrointestinal tract, urinary tract infections central nervous system, skeleton and others)
2. Iron deficiency anemia, haemolytic anemia
3. Bleeding
4. Connective tissue diseases (juvenile rheumatoid arthritis, small and large vessel vasculitis including Wegener's granulomatosis, polyarteritis nodosa and others)
5. Kawasaki's disease
6. Inflammatory bowel diseases
7. Langerhan's cell histiocytosis
8. Malignancies (mostly solid tumours, such as hepatoblastoma, hepatocellular carcinoma, neuroblastoma, and rarely acute lymphoblastic leukaemia)
9. Drugs (adrenaline, corticosteroids, vinca alkaloids, iron, miconazole, antibiotics, haloperidol, narcotics, and non-narcotic psycho pharmaceutical agents)
10. Trauma, burns, tissue injury
11. Intense exercise
12. Splenectomy (surgical or functional e.g., sickle cell anemia)

It seems to affect up to 15% of hospitalized children[19-25]. It is more common in neonates, particularly premature ones, and infants up to 2 years of age and less common in older children. In most children with reactive thrombocytosis, platelet counts

are moderately elevated up to 700,000/µl. Moderate thrombocytosis (platelets between 700,000 and 899,000/ µl) occur in 6-8% of children with reactive thrombocytosis, while platelets >1,000,000/µl occur in less than 2-3% of children with reactive thrombocytosis[22].

Presently, **infections** of the respiratory tract account for 60-80% cases of secondary thrombocytosis in children[20,22-27], followed by infections of the urinary[28] and gastrointestinal tracts, and of the bones[22-24,27,29].

From the **non-infectious** causes of secondary thrombocytosis, iron deficiency anemia is a common one, since it is the single most common nutritional deficiency worldwide[30,31]. The fact that thrombocytosis is more frequent in children up to 2 years of age is partly due to the higher incidence of iron deficiency in this age group.

In patients with **systemic-onset JRA,** serum IL-6 levels correlate with platelet counts and with the extent and severity of joint involvement[32]. Regarding **Kawasaki's disease (KD),** thrombocytosis typically occurs in the second week of the illness, and it is therefore not helpful in making a timely diagnosis. Moreover, the absence of thrombocytosis during convalescence does not exclude the disease. TPO in conjunction with IL-6 contributes to the thrombocytosis of patients with KD. TPO serum levels are also increased in patients with inflammatory bowel diseases, irrespective of disease activity, platelet counts and clinical characteristics of the patients[33].

The association between **liver tumours** and thrombocytosis is likely due to the increased production of hepatic TPO in these patients. Reactive thrombocytosis has also been described in children with other small, blue round cell tumours of childhood, such as **neuroblastoma**[34].

Reactive thrombocytosis can also be related to treatment with several drugs. **Adrenaline and corticosteroids** are known to cause transient thrombocytosis, as a result of release of stored platelets from the spleen into the blood circulation[35]. Various antibiotics such as **carbapenems and cephalosporins** are also claimed to cause thrombocytosis in children[36-43]. In the first week, when platelet counts are normal, circulating TPO concentrations rise and then gradually decrease. When platelet counts peak during convalescence, TPO concentrations are back to normal. Hence, the development of thrombocytosis during the recovery phase after appropriate antibiotic therapy for an infection is consistent with the bone marrow response to TPO and not the result of the antibiotic[43,44].

Miconazole, ciprofloxacin and tazobactam/ piperacillin caused thrombocytosis in a single patient18 since the platelet count started to increase immediately after initiation and dropped immediately after discontinuation of the drug[39].

Neonatal reactive thrombocytosis has been described from **maternal narcotic drug abuse,** but may also occur in infants born to mothers treated during pregnancy with non-narcotic psycho pharmaceutical agents[45,46]. Finally, reactive thrombocytosis may be due to multiple, simultaneous, causative factors. In one paediatric series, 9% of cases of secondary thrombocytosis were multi-factorial[47].

Reasons for reactive thrombocytosis in different clinical conditions:

1) Inflammations and Infections:

Thrombopoietin (TPO), the primary cytokine for platelet production and maturation, and interleukin (IL)-6 levels usually stimulate an increase in platelet production. Thus, it may be due to the overproduction of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-11, that occurs in chronic inflammatory, infective, and malignant states.

2) Iron Deficiency Anemia:

Reactive thrombocytosis is usually of mild to moderate degree. Extreme thrombocytosis is not so common but in some patients it can occur. Thrombopoietic growth factors including interlukin-6(IL-6), tumour necrosis factor-alpha, and thrombopoietin have been implicated as the cause of reactive thrombocytosis. Several clinical and the laboratory observation support the possible pathogenic role of elevated IL-6 in reactive thrombocytosis. An alteration of the bone marrow megakaryocyte count in iron deficiency anemia is not mentioned except for the two reports. In these reports authors mentioned that the bone marrow megakaryocyte count was increased and the plausible explanation for the thrombocytosis must be increased production of the platelet. The mechanism causing reactive thrombocytosis in iron deficiency anemia is unknown.

3) Tissue damage from trauma or surgery (postoperative):

The platelet count increases when a relatively large amount of body tissue is damaged either intentionally following surgery or after an accident. This is because of body natural defence mechanism to ensure adequate clot formation and prevent fatal bleeding.

4) Blood loss:

In event of an injury, the response of the bone marrow to blood loss is to produce more red blood cells and more platelets.

5) Post-splenectomy:

The splenectomised patients are expected to have high postoperative platelet counts because of reduced platelet storage in the spleen. The increase may remain for a long time, but usually it settles back into the normal range.

6) Haemolytic anemia:

Haemolytic anemia is another frequent cause of thrombocytosis. Sickle cell anemia is a congenital haemolytic anemia associated with thrombocytosis due to increased bone marrow platelet production, but also due to functional asplenia from the repetitive splenic autoinfarcts. Patients with sickle cell anemia and thrombocytosis are at

increased risk for vaso-occlusive complications, such as brain infarcts, painful crises, while they have highly impaired full scale IQ[48,49].

7) Malignancy:

Malignancy causes high platelet count either by causing damage to tissues, causing blood loss (for example from the bowel) or by erroneously producing a response from the immune system that stimulates the bone marrow to produce platelets.

8) Tuberculosis:

The pathogenesis of reactive thrombocytosis in tuberculosis is not clear. It has variously been attributed to increase thrombopoietin[50] or production of platelets in pulmonary vasculature by fragmenting proplatelets[51].

CONFLICT OF INTEREST: None

FINANCIAL SUPPORT: None

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Review

Role of Platelets in Connecting Depression with Cardiovascular Diseases – A Brief Review of Current Literature

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ABSTRACT

Cardiovascular diseases (CVD) are known to be the most common cause of death worldwide and have long been associated with various psychosocial factors. Depression on the other hand is conceptualised as a systemic illness with neurobiological mechanisms overlapping with various medical disorders including cardiovascular and related diseases. The comorbidity between depression and coronary heart disease is a recognised risk factor for cardiac events including mortality. The relationship between the two however, is multifaceted and seen to be bidirectional as per existing research. Although the exact underlying mechanisms remain poorly understood, several diverse processes such as neuroendocrine dysregulation, inflammation, and autonomic system and platelet activity alterations have been proposed to play a role in complicating the prognosis of comorbid depression and CVD. This article focuses on reviewing current literature to study recent evidences and explore the dynamics between these two disorders through the mediation of platelets.

KEYWORDS: Platelets, Depression, Serotonin, Cardiovascular

INTRODUCTION

As per World Health Organisation, cardiovascular diseases (CVD) remain the most common cause of death globally and an important target for health reforms. Amongst psychosocial factors in CVD, the role of specifically depression is multifaceted and found to be bidirectional[1]. Studies have not clearly deciphered if this association could be causative or only temporally related. Behavioural factors along with activation of the hypothalamo-pituitary and adrenal (HPA) axis, dysregulation of autonomic, serotonin and neurotrophic pathways, oxidative stress, and platelet activation with endothelial dysfunction are the various proposed underlying mechanisms[2-7]. Depression, now conceptualized as a systemic illness with varied neurobiological mechanisms explaining its influence on other medical illnesses, increases the risk of and accelerates the progression, and gives poorer treatment response for numerous medical disorders, including cardiovascular[8].

This article intends to summarise the various factors involved in the intricate dynamics of CVD and depression and review the recent literature findings outlining the specific role of platelets in the same. The relationship between the medical and psychiatric aspects of this disorder is discussed under domains of overlapping endocrine,

behavioural ang genetic components as well as the many proposed neurochemical mechanisms. The role of platelets via serotonin pathways is specifically mentioned along with potential biomarkers indicating further research directions.

FINDINGS

Relationship between CVD and Depression

Many investigators studying potential biological and behavioural explanations for increased association between CVD and Depression mention several plausible potential mechanisms. These include behaviours like medication noncompliance, cigarette smoking, and physical inactivity, apart from biological factors[9,10]. The coexistence of these two conditions is more lethal than either diagnosis alone[11,12].

HPA axis in CVD and Depression

Chronic or severe stress activates HPA axis through CRF (corticotropin releasing factor) induced glucocorticoid activation and thus causes hyperglycaemia. This results in insulin resistance, leading to diabetes mellitus with potential cardiovascular events[13]. Elevated CRF stimulates the production of IL-1, IL-6, and TNF-alpha (pro-inflammatory cytokines) by immune cells in bloodstream, which cross blood-brain barrier, causing neuroinflammation, and producing depression-like symptoms[14].

Lifestyle in CVD and Depression

Patients with depression commonly exhibit a lifestyle which involves use of alcohol, cigarette smoking, junk food, poor compliance to medication, and reduced physical activity. This results in significant weight gain, and poor resultant health outcomes. These patients are less likely to change their lifestyle towards adoption of healthy behaviours[15]. Literature shows the mportance of higher calorie foods containing saturated fat, energy drinks and high glycemic foods to cause a rise in peripheral inflammatory markers. On the other hand, a diet of fruits, vegetables, and high fibre reduces inflammation, free radical production and the levels of pro-inflammatory cytokines[16].

Genetics in CVD and Depression

The genetic association between these two ailments is mediated through the interaction of brain derived neurotrophic factor (BDNF) and steroid hormones (stress-induced glucocorticoid elevation). Recent literature states that glucocorticoids reduce the BDNF-dependent upregulation of excitatory glutamate receptors by suppressing microRNA-132 expression. This is thought to play a role in the pathophysiology of various neurological diseases including psychiatric disorders, Alzheimer's disease, and Parkinson's disease[17].

Platelets

Platelets are known to be an essential component of our haemostatic process with additional physiological and immunomodulatory roles[18]. Biochemical agonist binding (collagen, thrombin, arachidonic acid, epinephrine) or disruption of blood vessels mechanically causes the initiation of haemostatic responses in local vascular endothelium. This causes the start of thrombus formation where platelets adhere to the damaged endothelium, become activated, and aggregate together, followed by serotonin granule secretion into extracellular space. This process is mediated through intracellular chemical signals (P selectin and glycoprotein IIb/IIIa)[19].

Serotonin

Evidence supports that serotonin (5-HT) plays a key role in the psychopathology of depression. The cerebrospinal fluid concentration of 5-hydroxyindoleacetic acid (5-HIAA), a major metabolite of 5-HT is found to be low in suicidal patients[20]. A whopping 99% of the body's serotonin is stored in the dense granules of platelets[21]. Release of serotonin from these granules at the damaged endothelial sites plays a major role in promoting aggregation of platelets during thrombus formation. This gives a logical mechanism of how platelets link depression with CVD[22].

Platelets in Depression

Depressed patients have been shown to exhibit increased reactivity, and increased P selectin and activated glycoprotein IIb/IIIa expression in platelets[23]. Thus, increased susceptibility for platelet activation looks to be a possible mechanism behind depression posing as a significant risk factor for CVD. The popular SADHART trial examined the effects of sertraline (SSRI-selective serotonin reuptake inhibitor) treatment for 24 weeks in patients with acute coronary syndrome and depression. It found that SSRIs supress platelet activation (based on diminished release of P-selectin and β thromboglobulin from platelets)[24].

Other platelet pathways seen to exhibit significant changes in depressed patients are reduction in platelet adenosine response, increase in platelet thrombin response, increase in expression of glycoprotein Ib, and decrease in BDNF levels in platelets[25]. Several other molecular mechanisms interlinked with the physiological functioning of platelets and potentially contributing to increased aggregability are discussed below.

Platelets and Catecholamines

Platelets express dopaminergic as well as adrenergic receptors, modulating thrombopoiesis through them. Low level of serum catecholamines potentiates the effects of thrombin and collagen (coagulation agonists). High serum catecholamines can independently induce platelet aggregation, and secretion of granules along with release of platelet markers (beta-thromboglobulin (BTG) and Platelet Factor 4 (PF4))[26].

Platelets and Leptin

Peptide hormones receptors (long form of leptin receptor (LEPRL)) on platelet surface underlies the link between altered leptin levels in depression and possibly altered platelet response contributing to cardiovascular complications. Leptin is known to increase thrombotic processes by potentiating platelet aggregation, and increasing platelets adhesion[27].

Platelets and Adiponectin

Adiponectin receptors (AdipoR1 and AdipoR2) on platelet surface has also been found in mice, where deletion of adiponectin increased thrombus formation in arterial injury induced photochemically. Anti-thrombotic effect of adiponectin seems to be related to leukocytes (reduced titre of aggregates of polymorphonuclear leukocytes/ or monocytes with platelets), and the inhibition of macrophage-related Tissue Factor (TF), which impairs coagulation cascade[28,29].

Platelets and Neurotrophins

Platelets contain both Nerve Growth Factor (NGF) and BDNF. While NGF can induce aggregation by binding to platelet surface, binding of BDNF can cause internalization of specific receptor sites[30].

Platelets and Low-Density Lipoprotein

Lipid alterations contribute to acute thrombotic events through platelet activated thrombosis. Low Density Lipoproteins are seen to increase platelet sensitivity to stimulation from agonists which makes them to respond faster and more extensively[31].

Platelets and Reactive Oxygen Species (ROS)

Extracellular ROS is known to promote thrombotic events. Activated platelets generate ROS which in turn activate more platelets. Intracellular ROS in platelets promotes secretion of dense exocytosis and increases platelet receptor sensitivity[32].

Serotonin in Haemostasis and Thrombosis

The role of platelets in haemostasis and thrombus formation is undisputed. Serotonin influences platelets in multiple pathways, including their binding at von Willebrand-Factor (vWF) on damaged endothelial sites in the vasculature. Platelets adhere on vWF via their surface glycoprotein (GP Iβα). This allows collagen to interact with GP IIb/IIIa further allowing intracellular signalling and dense granule exocytosis[33].

SSRIs have been shown to play a role venous thrombotic phenomenon. Depressed patients have reported higher incidence of venous thromboembolism (VTE). Also, the clinical use of tricyclic antidepressants other antidepressants is independently seen to be associated with increased risk of VTE[34,35].

BDNF

Evidence suggests BDNF may be useful as a biomarker in depression for the purpose of diagnostic clarity and prognostic implications[36]. Platelets – a major source (99%) of peripheral BDNF, are important for mediating the survival and activation of endothelial cells and playing a role in angiogenesis[37,38]. Low levels of BDNF are seen in patients with major depressive disorder and these levels have been shown to increase with long term antidepressant treatment. Increasing evidence has reiterated the presence of prothrombotic endophenotype in patients suffering from depression. Some authors have proposed the occurrence of epi-phenomenon (upregulation of BDNF receptors) resulting in altered BDNF metabolism and/or variation in peripheral BDNF titres during treatment with antidepressant medications in patients of depression[39,40].

CONCLUSION

Recent literature findings reiterate the fact that platelet dysfunction is a major connecting link between depression and cardiovascular pathology, contributing to increase in morbidity and mortality. Despite substantial evidence, the complex molecular mechanisms and mediators involved in this process need to be disentangled in order to formulate clear and robust associations with each other. There is an interesting role of neurotropic factors in possible epigenetic modulation affecting gene expression and subsequent presentation of depression in medical illnesses.

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New Drug Approvals

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
1.	Ulcerative Colitis	Velsipity (etrasimod) Tablets	Pfizer Inc.	Velsipity (etrasimod) is a sphingosine-1-phosphate (S1P) receptor modulator indicated for the treatment of moderately-to-severely active ulcerative colitis (UC) in adults.	October 12, 2023
2.	Generalized Myasthenia Gravis	Zilbrysq (zilucoplan) Injection	UCB, Inc.	Zilbrysq (zilucoplan) is a peptide inhibitor of complement component 5 (C5 inhibitor) for the treatment of generalized myasthenia gravis in adult patients who are anti-acetylcholine receptor (AChR) antibody positive.	October 17, 2023
3.	Hyperphos-phatemia of Renal Failure	Xphozah (tenapanor) Tablets	Ardelyx, Inc.	Xphozah (tenapanor) is a sodium hydrogen exchanger 3 (NHE3) inhibitor used to treat hyperphosphatemia in adults with chronic kidney disease.	October 17, 2023
4.	Presbyopia	Qlosi (pilocarpine hydrochloride) Ophthalmic Solution - formerly CSF-1	Orasis Pharma-ceuticals	Qlosi (pilocarpine hydrochloride ophthalmic solution) 0.4%, is a low dose formulation of the approved cholinergic agonist pilocarpine indicated for the treatment of presbyopia in adults.	October 17, 2023

Continued ...

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
5.	Postoperative Pain	Combogesic IV (acetaminophen and ibuprofen) Injection	AFT Pharma-ceuticals Ltd.	Combogesic IV (acetaminophen and ibuprofen) is an analgesic and nonsteroidal anti-inflammatory drug (NSAID) combination for the management of postoperative pain.	October 17, 2023
6.	Plaque Psoriasis	Bimzelx (bimekizumab-bkzx) Injection	UCB, Inc.	Bimzelx (bimekizumab-bkzx) is a humanized interleukin-17A and interleukin-17F antagonist for the treatment of moderate to severe plaque psoriasis in adults who are candidates for systemic therapy or phototherapy.	October 17, 2023
7.	Diabetes, Type 2	Liqrev (sildenafil citrate) Oral Suspension	Zydus Pharma-ceuticals USA Inc.	Zituvio (sitagliptin) is a dipeptidyl peptidase-4 (DPP-4) inhibitor indicated as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus.	October 18, 2023
8.	Meningococcal Disease Prophylaxis	Penbraya (meningococcal groups A, B, C, W, and Y vaccine) Injection	Pfizer, Inc.	Penbraya (meningococcal groups A, B, C, W, and Y vaccine) is indicated for active immunization to prevent invasive disease caused by Neisseria meningitidisserogroups A, B, C, W, and Y in individuals 10 through 25 years of age.	October 20, 2023

Continued ...

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
9.	Acne	Cabtreo (adapalene, benzoyl peroxide and clindamycin phosphate) Topical Gel	Bausch Health Companies Inc.	Cabtreo (adapalene, benzoyl peroxide and clindamycin phosphate) is a triple-combination for the topical treatment of acne vulgaris in adult and pediatric patients 12 years of age and older.	October 20, 2023
10.	Crohn's Disease, Ulcerative Colitis	Zymfentra (infliximab-dyyb) Subcutaneous Injection	Celltrion USA	Zymfentra (infliximab-dyyb) is a tumor necrosis factor (TNF) blocker used for the maintenance treatment of ulcerative colitis and Crohn's disease in adults.	October 20, 2023
11.	Duchenne Muscular Dystrophy	Agamree (vamorolone) Oral Suspension	Catalyst Pharma-ceuticals, Inc.	Agamree (vamorolone) is a corticosteroid indicated for the treatment of Duchenne muscular dystrophy in patients 2 years of age and older.	October 26, 2023
12.	Ulcerative Colitis	Omvoh (mirikizumab -mrkz) Injection	Eli Lilly and Company	Omvoh (mirikizumab-mrkz) is an interleukin-23 antagonist indicated for the treatment of moderately to severely active ulcerative colitis in adults.	October 26, 2023

Continued ...

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
13.	Nasopharyngeal Carcinoma	Loqtorzi (toripalimab-tpzi) Injection	Coherus Biosciences, Inc.	Loqtorzi (toripalimab-tpzi) is a programmed death receptor-1 (PD-1)- blocking antibody used for the treatment of nasopharyngeal carcinoma.	October 27, 2023
14.	Plaque Psoriasis, Psoriatic Arthritis, Crohn's Disease, Ulcerative Colitis	Wezlana (ustekinumab-auub) Injection	Amgen, Inc.	Wezlana (ustekinumab-auub) is a human interleukin-12 and -23 antagonist interchangeable biosimilar to Stelara used for the treatment of plaque psoriasis, psoriatic arthritis, Crohn's disease, and ulcerative colitis.	October 31, 2023
15.	Erosive Esophagitis	Voquezna (vonoprazan) Tablets	Phathom Pharma-ceuticals, Inc.	Voquezna (vonoprazan) is a potassium-competitive acid blocker (P-CAB) for the treatment of erosive esophagitis.	November 1, 2023
16.	Diabetes, Type 2	Zituvimet (metformin hydrochloride and sitagliptin) Tablets	Zydus Pharma-ceuticals USA Inc.	Zituvimet (metformin hydrochloride and sitagliptin) is a biguanide and dipeptidyl peptidase-4 (DPP-4) inhibitor combination indicated as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus.	November 3, 2023

Continued ...

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
17.	Weight Loss (Obesity/ Overweight)	Zepbound (tirzepatide) Injection	Eli Lilly and Company	Zepbound (tirzepatide) is a glucose-dependent insulinotropic polypeptide (GIP) receptor and glucagon-like peptide-1 (GLP-1) receptor agonist used for chronic weight management in adults.	November 8, 2023
18.	Colorectal Cancer	Fruzaqla (fruquintinib) Capsules	Takeda	Fruzaqla (fruquintinib) is a highly selective and potent oral inhibitor of VEGFR-1, -2 and -3 for the treatment of previously treated metastatic colorectal cancer.	November 8, 2023
19.	Thrombotic Thrombocytopenic Purpura	Adzynma (ADAMTS13, recombinant-krhn) Lyophilized Powder for Injection	Takeda	Adzynma (ADAMTS13, recombinant-krhn) is a human recombinant "A disintegrin and metalloproteinase with thrombospondin motifs 13" (rADAMTS13) indicated for prophylactic or on demand enzyme replacement therapy (ERT) in adult and pediatric patients with congenital thrombotic thrombocytopenic purpura.	November 9, 2023
20.	Chikungunya Disease Prevention	Ixchiq (chikungunya vaccine, live) Injection - formerly VLA1553	Valneva SE	Ixchiq (chikungunya vaccine, live) is a live-attenuated, single dose vaccine indicated for the prevention of disease caused by chikungunya virus (CHIKV) in individuals 18 years of age and older who are at increased risk of exposure to CHIKV.	November 9, 2023

Continued ...

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
21.	Prevention of Catheter-Related Bloodstream Infections	DefenCath (taurolidine and heparin) Catheter Lock Solution	CorMedix Inc.	DefenCath (taurolidine and heparin) catheter lock solution is a thiadiazinane antimicrobial and anticoagulant combination indicated to reduce the incidence of catheter-related bloodstream infections in adult patients with kidney failure receiving chronic hemodialysis (HD) through a central venous catheter (CVC).	November 15, 2023
22.	Non-Small Cell Lung Cancer	Augtyro (repotrectinib) Capsules	Bristol Myers Squibb	Augtyro (repotrectinib) is a tyrosine kinase inhibitor (TKI) for the treatment of patients with ROS1-positive locally advanced or metastatic non-small cell lung cancer (NSCLC).	November 15, 2023
23.	Breast Cancer	Truqap (capivasertib) Tablets	AstraZeneca	Truqap (capivasertib) is an AKT inhibitor used in combination with fulvestrant for the treatment of advanced hormone receptor-positive breast cancer.	November 16, 2023
24.	Neutropenia Associated with Chemotherapy	Ryzneuta (efbemalenograstimalfa-vuxw) Injection	Evive Biotech	Ryzneuta (efbemalenograstimalfa-vuxw) is a leukocyte growth factor to used to reduce the duration of febrile neutropenia in patients treated with chemotherapy for certain types of cancer.	November 16, 2023

Continued ...

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
25.	Desmoid Tumors	Ogsiveo (nirogacestat) Tablets	Spring Works Therapeutics, Inc.	Ogsiveo (nirogacestat) is a gamma secretase inhibitor indicated for adult patients with progressing desmoid tumors who require systemic treatment.	November 27, 2023
26.	Paroxysmal Nocturnal Hemoglobinuria	Fabhalta (iptacopan) Capsules	Novartis	Fabhalta (iptacopan) is a first-in-class, complement factor B inhibitor indicated for the treatment of adults with paroxysmal nocturnal hemoglobinuria.	December 5, 2023
27.	Colorectal Cancer, Non-Small Cell Lung Cancer, GlioblastomaMultiforme, Renal Cell Carcinoma, Cervical Cancer, Ovarian Cancer, Fallopian Tube Cancer, Peritoneal Cancer	Avzivi (bevacizumab-tnjn) Injection	Bio-Thera Solutions, Ltd.	Avzivi is a vascular endothelial growth factor inhibitor biosimilar to Avastin used for the treatment of colorectal cancer, non-small cell lung cancer, glioblastoma, renal cell carcinoma, cervical cancer, and epithelial ovarian, fallopian tube, or primary peritoneal cancer.	December 6, 2023
28.	Sickle Cell Disease, Beta Thalassemia	Casgevy (exagamglogeneautotemcel) Suspension for Intravenous Infusion	Vertex Pharmaceuticals Incorporated and CRISPR Therapeutics	Casgevy (exagamglogeneautotemcel) is a CRISPR/Cas9 genome-edited cell therapy for the treatment of sickle cell disease and transfusion-dependent beta-thalassemia.	December 8, 2023

Continued ...

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
29.	Sickle Cell Disease	Lyfgenia (lovotibeglogen eautotemcel) Suspension for Intravenous Infusion	Bluebird Bio, Inc.	Lyfgenia (lovotibeglogeneautotemcel) is an autologous hematopoietic stem cell-based gene therapy indicated for the treatment of patients 12 years of age or older with sickle cell disease and a history of vaso-occlusive events.	December 8, 2023
30.	Glaucoma, Open Angle, Glaucoma/ Intraocular Hypertension	iDose TR (travoprost) Intracameral Implant	Glaukos Corporation	iDose TR (travoprostintracameral implant) is a long-duration prostaglandin analog indicated for the reduction of intraocular pressure (IOP) in patients with open-angle glaucoma (OAG) or ocular hypertension (OHT).	December 13, 2023
31.	Neuroblastoma	Iwilfin (eflornithine) Tablets	US World Meds, LLC	Iwilfin (eflornithine) is an ornithine decarboxylase inhibitor used to reduce the risk of relapse in adult and pediatric patients with high-risk neuroblastoma (HRNB).	December 13, 2023
32.	Primary Immunodeficiency Syndrome	Alyglo (immune globulin intravenous, human-stwk) Liquid for Intravenous Injection	GC Biopharma USA, Inc.	Alyglo (immune globulin intravenous, human-stwk) is a 10% immune globulin liquid for intravenous injection indicated for the treatment of primary humoral immunodeficiency (PI) in adults.	December 15, 2023

Continued ...

S. No.	Treatment Indication	Drug's Name	Company	Description	Date of FDA Approval
33.	Epidermolysis Bullosa	Filsuvez (birch triterpenes) Topical Gel - formerly Oleogel-S10	Chiesi Global Rare Diseases	Filsuvez (birch triterpenes) is a topical birch bark extract indicated for the treatment of wounds associated with dystrophic epidermolysisbullosa and junctionalepidermolysisbullosa in adult and pediatric patients 6 months of age and older.	December 19, 2023
34.	Hereditary Transthyretin-Mediated Amyloid Polyneuropathy (ATTRv-PN)	Wainua (eplontersen) Injection	Ionis Pharma-ceuticals and AstraZeneca	Wainua (eplontersen) is a transthyretin-directed antisense oligonucleotide indicated for the treatment of the polyneuropathy of hereditary transthyretin-mediated amyloidosis in adults.	December 21, 2023

(Ravindra Bangar)
Editor

Call for Papers

Pacific Journal of Medical and Health Sciences (ISSN: 2456-7450) is a quarterly journal of the Pacific Group of Institutions in the Medical and Health Sciences. The subject areas for publication include, but are not limited to, the following fields: Anatomy, Anesthesia, Biochemistry, Biomedical Sciences, Physiology, Pharmacology, Cancer, Cardiology, Community Medicine, Dermatology and Venereal Diseases, Diabetes, Endocrinology, Epidemiology and Public Health, Forensic Science, Gastroenterology, Geriatric Medicine, Hematology, Immunology, Infectious Diseases, Internal Medicine, Microbiology, Nephrology, Neurology, Neurosurgery, Obstetrics and Gynecology, Ophthalmology, Orthopedics, Otorhinolaryngology, Pediatrics, Pathology, Psychiatry, Pulmonary Medicine, Radiology, Toxicology, Dentistry, Nursing, Health Informatics, Occupation Safety and Health. Its key aims are to provide interpretations of growing points in medical knowledge by trusted experts in the field, and to assist practitioners in incorporating not just evidence but new conceptual ways of thinking into their practice.

Invitation for Manuscripts

The *Pacific Journal of Medical and Health Sciences* invites original research based papers, medical case studies and paper reviews. The manuscripts received are sent to referees and are accepted on their recommendation only.

Guidelines for Authors

The *Pacific Journal of Medical and Health Sciences* is keen to promote high quality original research based papers, medical case studies and paper reviews based on sound evidence. Sufficient information should be given in the paper for it to be capable of reproduction by other authors and added to as more data become available.

Your paper should be approximately 8-15 pages in length, including abstract, all figures and tables and references.

Preparation of Manuscript

Please remember that your article should be an original piece of work in its own right and be written without the extensive reuse of previously published material. All source material should be fully acknowledged and referenced.

As part of the Cross-check initiative to detect and prevent plagiarism, the *Pacific Journal of Medical and Health Sciences* screens all accepted manuscripts. Plagiarism, including duplicate publication of the author's own work, in whole, or in part without proper citation is not accepted by the journal.

References

Number references consecutively in the order in which they are first mentioned. Identify references in text, tables, and captions by Arabic numerals superscripted above the line.

Abbreviations and Units

Only use standard abbreviations. SI units should always be used.

Trade Units

These should be marked with ® and proprietary drug names should be capitalised e.g. Cifran.

Manuscript Order

- TITLE page
    - Full title of the article
    - Initials (or first name) and surname of each author as they should appear in the chapter (Degrees and appointments will NOT be included)
    - Department and institution to which the work should be attributed
    - Name, full postal address, telephone and fax numbers and email address of author responsible for correspondence

- STRUCTURED ABSTRACT of no more than 150 words. The abstract headings should include:
    - Introduction or background
    - Sources of data
    - Areas of agreement
    - Areas of controversy
    - Growing points
- KEY WORDS: a minimum of 3 key words which reflect the content of the review
- SHORT TITLE
- TEXT to follow a similar general format to the abstract. Authors should ensure that technical language used is understandable to a scientific but general readership. A glossary may be a useful addendum where appropriate.
- DISCUSSION OR CONCLUSIONS, which gives more detail of areas of agreement, controversy, growing points and areas timely for developing research.
- ACKNOWLEDGMENTS
- REFERENCES listed in numerical sequence according to their order of appearance in the text. Avoid using abstracts as references.

Journals

If there are more than 6 authors of a paper, abbreviate to the first 3 names and then add 'et al'. Use abbreviated journal title as given in Index Medicus.

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- Pail KN, Smith ADF, Manners M et al. Coagulation mechanisms. J Cell Biol 1993;430: 200-30

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Authors and title of chapter are followed by the editor(s) of the book, title of book, main town of publisher, publisher's name (omit 'Press', '& Sons', 'Inc' etc), year and page range.

Electronic Source (Website/Web Page/Online Journal Article)

The publication is listed first followed by the article title, web address, publication date, and the date last accessed.

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- Acorn AD, Management of rheumatoid arthritis. In: Brwon CC, Davies GH. (eds) Inflammatory diseases. 3rd edn. London: Apple, 1992;203-30
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Public Health Laboratory Service. Antimicrobial Resistance in 2000: England and Wales. http://www.hpa.org.uk./infections/topics_az/antimicrobial_resistance/amr.pdf (7 January 2004, date last accessed).

Figures

The use of figures is strongly encouraged where they can assist the reader in the understanding of the article and replace lengthy passages of text. Number figures consecutively and, where figures are related, number them 1(a), 1(b), 1(c) etc.

Photographs

These should be of sufficiently high quality with respect to detail, contrast and fineness of grain.

Tables

Number tables consecutively and place a descriptive heading above each table. Give each column a short heading. Explain in footnotes all non-standard abbreviations used in a table.

Figure Captions

Captions should be brief descriptions of each figure or illustration (e.g. Fig. 1 The diagram shows…). Where relevant, captions should also include definitions for all symbols used.

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Format of Manuscript

Submission of manuscript must have a cover letter showing the full name of author(s) along with correspondence address including e-mail and contact numbers. The title should appear on the first page of the manuscript, as we use peer-review process, so that we can remove the identity of the author(s) before sending it to referees.

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Guidelines for Formatting the Paper

Paper be typed	MS. Word
Font	Times New Roman
Font Size	16pt. and Bold for Title of the Paper, 14pt and Bold for heading in the paper, 12pt. for text.
Line Spacing	1.5
Margin	1 inch on all sides.
Layout	Use a single column layout with both left and right margins justified.
Language	English and Hindi
The title page	It should contain title of the paper, followed by name(s) of author(s), Designation, affiliation, e-mail, phone, fax with STD code and Postal Address, Authors should not write their name and affiliations anywhere else in the paper.
Tables, Graphs and charts etc.	In the text, the references for table should be mentioned as Table-1 and so on, not as above table. Same should be followed in case of graphs and charts. Each table, Graph and chart should have its own heading and source.
Abstract	500 Words
Full length paper	5000 Words
References	APA with hanging format.

(Editorial Team)

Peer-Review Policy
Double-blind Peer Review Process

INTERNATIONAL PEER REVIEWED JOURNAL

Peer-review is the system used to assess the quality of a manuscript before it is published. Independent researchers in the relevant research area assess submitted manuscripts for originality, validity and significance to help editors determine whether the manuscript should be published in their journal.

In cases where the journal is unable to find sufficient peer reviewers, the Editorial Board may identify suitable reviewers and provide reports to avoid further delays for authors. Manuscripts submitted to Pacific Journal of Medical and Health Sciences are first assessed by our editors.

The aim and objective of the Pacific Journal of Medical and Health Sciences is to ensure the high standards of the original and scientific research papers and articles. With our Journal, a double-blind peer review system is in operation.

In the case of proposed publications, our editorial board will judge and evaluate the proposed manuscript on certain parameters like relevance of the submitted work with the aims and scope of the journal, scientific quality the work and contribution of the work in respective branch of knowledge. If, the proposed work found suitable in quick review by the editorial board than editor will forward copies of an author's work to two experts ("referees" or "reviewers") in the respective field by e-mail or through a web-based manuscript processing system.

These referees or reviewers will return an evaluation of the proposed work to the editor in prescribed format along with weaknesses, problems, and suggestions for improvement. Further, this evaluation will be forwarded by editor after reviewing the comments of referees in context with the scope of the journal to the author for consideration and improvement of the proposed work.

Referees' evaluations usually include an explicit recommendation of what to do with the manuscript or proposed work as per the options available in the prescribed format.

During this peer review process, the role of the referees is advisory, and the editor is typically under no formal obligation to accept the opinions of the referees. Moreover, in the process of scientific publication, the referees do not communicate with each other, do not act as a group, and are not aware of each other's identities or comments.

In particular situations, where the referees disagree considerably about the quality of a manuscript, there are a number of strategies for reaching a decision. When the editor receives positive and negative reviews for the same manuscript by two different reviewers, the editor will ask for one or more additional reviews or on the basis of comments of one reviewer, the edit may take his/her decision about the respective manuscript.

Reviewers' Guidelines

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We are committed to upholding the integrity of the work we publish. Pacific Journal of Medical and Health Sciences takes issues of copyright infringement, plagiarism or other breaches of best practice in publication very seriously. We seek to protect the rights of our authors and we always investigate claims of plagiarism or misuse of published articles. Equally, we seek to protect the reputation of our journal against malpractice. Submitted articles may be checked with duplication-checking software. Where an article is found to have plagiarized other work or included third-party copyright material without permission or with insufficient acknowledgement, or where the authorship of the article is contested, we reserve the right to take action including, but not limited to: publishing an erratum or corrigendum (correction); retracting the article (removing it from the journal); taking up the matter with the Head of Department or Dean of the author's institution and/or relevant academic bodies or societies; banning the author from publication in the journal in question or appropriate legal action.

We recommend that if reviewers suspect any of the following problems with any article that they are reviewing that they contact the journal editor to discuss the situation without delay. Reviewers should keep all information about such matters confidential and not discuss them with colleagues other than the journal editor.

1. If you suspect that the paper has been either published or submitted to another journal.
2. If you suspect that the paper is duplicating the work of others.
3. If you suspect that there might be problems with the ethics of the research conducted.
4. If you suspect that there might be an undeclared conflict of interest attached to the paper (Editors might have more information about this than you do so it is best to check).

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PURPOSE OF PEER REVIEW

It is widely accepted that Peer Review is the most valid form of research evaluation and it is a cornerstone in the process of bringing academic research to publication in the following ways:

Evaluation - Peer review is an effective form of research evaluation to help select the highest quality articles for publication.

Integrity - Peer review ensures the integrity of the publishing process and the scholarly record. Reviewers are independent of journal publications and the research being conducted.

Quality - The filtering process and revision advice improve the quality of the final research article as well as offering the author new insights into their research methods and the results that they have compiled. Peer review gives authors access to the opinions of experts in the field who can provide support and insight.

TYPE OF PEER REVIEW OF JOURNAL

Double blind peer review - names are hidden from both reviewers and the authors.

HOW TO REVIEW ARTICLES

Referees are sent invitations to review papers by journal editors. These requests are made via email. If you are asked to provide a review, in order to avoid delays, we would be grateful if you could let us know as soon as possible if you are unable to complete it at the time or if a problem arises after the invitation has been accepted. Suggestions for alternative reviewers are always gratefully received!

Below we present some advice and guidance about how to conduct a review and put together a reviewer report that will be effective and beneficial to authors:

ETIQUETTE

Timeliness - We understand that our reviewers are busy so it won't always be possible for invitations to be accepted. Please let us know as soon as possible if they need to refuse a review or if a problem arises after the invitation has been accepted. Most journal editors are grateful to receive suggestions about someone else that might be suitable to do the review if you have to decline the invitation.

Conflict of Interest - it is important to highlight to the journal editor any conflict of interest that you feel might occur if you review the paper. Please do so as discretely and as quickly as possible.

Discussion -- it is important to discuss with the journal editor any concerns that you have about the paper or their specific requirements for review if you are being invited to review for the first time. Editors are usually open to discussing their expectations and journal requirements with reviewers.

Ethics -Refer ethics and responsibility related to peer review.

INDIVIDUAL JOURNAL REVIEWER GUIDELINES

These guidelines include a list of questions and will usually offer the reviewer the chance to make general comments

- Read the paper very carefully.
- Relevance to the publication (most editors will reject at submission those articles that do not match the aims and scope of the journal, but it is worth considering this as you read the paper).
- Significance of the research within the field.
- Originality of the work conducted. It is also important to consider whether the author has ever published a substantially similar paper elsewhere (if you suspect the work may not be original, please view our ethics page for information about how to deal with a variety of situations).
- The methodology employed during the research.
- Technical accuracy.

STRUCTURE AND COMMUNICATION

- Accuracy of references
- Overall Structure of the paper, communication of main points and flow of argument
- Quality of written language and structure of the article
- Effectiveness of the article abstract and introduction (some journals will request
- Whether the argument is clear and logical and the conclusions presented are supported by the results or evidence presented
- Whether the title of the article is suitable or effective
- Whether the abstract is a good summary of the article
- Whether the work meets with the article types accepted by the journal

The accessibility of the paper to a broad readership

Whether the paper is internally consistent

FEEDBACK IN YOUR REVIEWER REPORT - GIVING ADVICE TO AUTHORS AND SUGGESTING REVISIONS

- Be as objective as possible in your comments and criticisms and avoid making negative comments about work referenced in the article

- Be specific and as constructive as possible in your criticism. Be clear about what needs to be added or revised.
- If relevant, make suggestions about additional literature that the author might read to enrich or improve their arguments
- You should ensure that you are clear which of your comments you are happy for the author to see and which are meant specifically for the journal editor in order to avoid confusion or bad feeling
- While peer reviewers should feel free to make general comments on written quality and make suggestions about how articles might be improved by broadening reading of other literature, it is not the job of the peer reviewer to rewrite articles or suggest detailed changes to wording

MAKING A DECISION

> Recommend whether a paper should be accepted, rejected or revised (major or minor revisions)

> Most importantly, keep all activity, content and comments relating to the paper confidential

Most important - keep all activity, content and comments relating to the paper confidential.

Publication Ethics and Publication Malpractice Statement

Our publication ethics and publication malpractice statement is mainly based on the Code of Conduct and Best-Practice Guidelines for Journal Editors (Committee on Publication Ethics, 2011).

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Publication Decisions

The editor is responsible for deciding which of the papers submitted to the journal will be published. The editor will evaluate manuscripts without regard to the authors' race, gender, sexual orientation, religious belief, ethnic origin, citizenship, or political philosophy. The decision will be based on the paper's importance, originality and clarity, and the study's validity and its relevance to the journal's scope. Current legal requirements regarding libel, copyright infringement, and plagiarism should also beconsidered.

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The peer-reviewing process assists the editor and the editorial board in making editorial decisions and may also serve the author in improving the paper.

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Any selected referee who feels unqualified to review the research reported in manuscript or knows that its prompt review will be impossible should notify the editor and withdraw from the review process.

Confidentiality

Any manuscripts received for review must be treated as confidential documents. They must not be disclosed to or discussed with others except as authorized by the editor.

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Reviews should be conducted objectively. Personal criticism of the author is inappropriate. Referees should express their views clearly with supporting arguments.

Disclosure and Conflict of Interest

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Authors will submit only entirely original works, and will appropriately cite or quote the work and/or words of others. Publications that have been influential in determining the nature of the reported work should also be cited.

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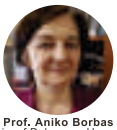
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