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Effects of different in vitro anticoagulants on parameters of platelet function

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LIST OF ACRONYMS

ACS	acute coronary syndromes
ADP	adenosine diphosphate
CAD	coronary artery disease
DNA	deoxyribonucleic acid
GPCR	G-protein coupled receptor
LTA	light transmission aggregometry
MK	megakaryocyte
mRNA	messenger ribonucleic acid
MRR	mean relative radius
PA	platelet aggregation
PC	platelet count
PPP	platelet-poor plasma
PRI	platelet reactivity index
PRP	platelet-rich plasma
SPA	spontaneous platelet aggregation
VASP	vasodilator-stimulated phosphoprotein
vWF	von Willebrand factor
WB	whole blood

ABSTRACT

Light transmission aggregometry (LTA) is commonly applied in the diagnosis of platelet bleeding disorders and in the research setting to monitor platelet function in the evaluation and development of antiplatelet agents. The guidelines for the standardisation of LTA for the study of platelet aggregation (PA) published in 2013 by the SSC-ISTH were largely based on the consensus of experts, because methodological studies directly comparing different procedures were lacking.

We tested the cogency of some SSC-ISTH recommendations: 1) buffered citrate (109 or 129 mM) should be used as anticoagulant to help keep the pH stable; 2) after centrifugation, platelet-rich plasma (PRP) should sit at room temperature for 15 min before testing; 3) LTA studies should be completed within a maximum of 4h after blood sampling.

Blood from 10 healthy volunteers was collected into buffered and non-buffered citrate (109 and 129 mM). PA was measured by LTA in PRP stimulated by ADP (2 μM) immediately after preparation, 15 min thereafter (=1h after blood sampling), 2, 3 and 4h after blood sampling. Plasma pH and platelet count were measured at the same time points as LTA.

PA changed within the first 15 minutes after PRP preparation, then remained stable up to 2h after blood sampling, and decreased thereafter; 2) PA tended to be higher in 109 mM than in 129 mM citrate; 3) there was no difference in PA between buffered and non-buffered citrate; 4) pH in buffered samples was lower than in non-buffered samples, but increased in all samples to pH 8 at 4h; 5) platelet count in non-buffered citrate PRP was lower than in buffered citrate and decreased over time in all samples. The loss of platelets was inhibited

by addition of prostaglandin E1 (PG-E1, 2 μM) to whole blood upon collection.

We conclude that PRP should rest for 15 min before testing; buffering of citrate does not affect PA significantly but does affect platelet count: therefore, it is uncertain whether the use of buffered citrate is preferable over non-buffered citrate; LTA studies should be completed within 2h rather than 4h after blood sampling.

1. INTRODUCTION

1.1 PLATELET BIOLOGY

Platelets are small anucleate cells in mammals, primarily involved in haemostasis and coagulation. These functions include maintaining the integrity of vasculature, arresting bleeding and promoting wound healing. Additionally platelets have rudimentary roles in inflammation and defence; they are able to interact directly or via primary and secondary messenger molecules to communicate tissue damage or infection to other more specialised blood cells. These latter functions are thought to be remnants of the evolutionary past of platelets, as they diversified from more primitive yet multicompetent blood cell.[1]

Platelets are formed by cytoplasmic fragmentation of highly specialised polyploid progenitor cells, the megakaryocytes (MKs) that reside in the bone marrow.[2] MKs in turn originate from pluripotent stem cells and undergo multiple DNA replication steps without cell division (endomitosis) followed by formation of extensive internal demarcation membrane system (DMS), rapid cytoplasmic expansion, protein synthesis and reorganisation to form cytoplasmic protrusions called proplatelets that fragment to liberate platelets (Figure 1).[2] Newly formed platelets inherit most of their protein content from MKs, along with messenger ribonucleic acid (mRNA) and ribosomes that allow for protein synthesis in the absence of a nucleus. In addition platelets take up and store a vast array of proteins from plasma.

In humans platelets are released into the circulation at a rate of 100 billion per day and circulate for 7 - 10 days. Under equilibrium only a small portion of platelets is consumed in haemostatic processes, while the majority is cleared by the reticuloendothelial system in the liver and

spleen.[3] There is emerging evidence that an apoptotic program controls platelet survival and removal in vivo.[3]

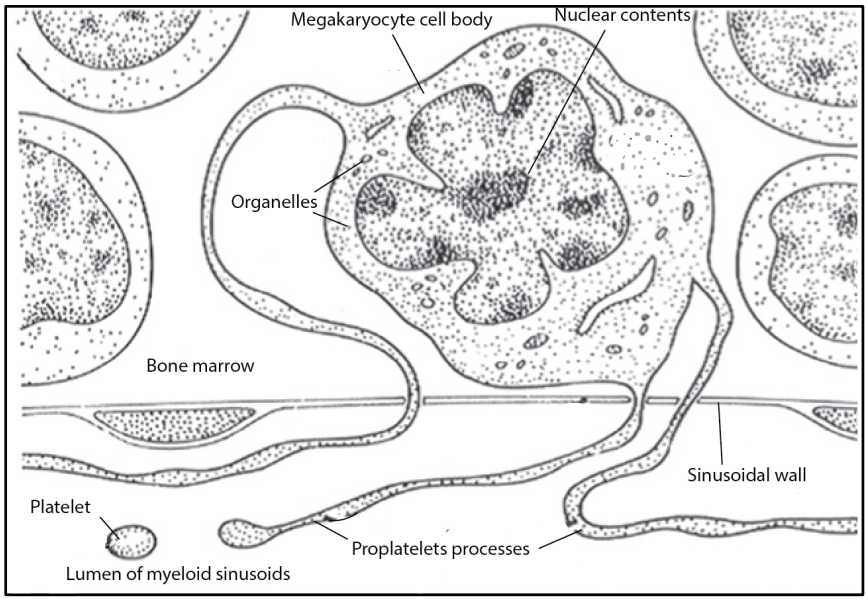


Figure 1. Schematic drawing of platelet formation: megakaryocytes residing within the bone marrow undergo endomitosis, grow an extensive demarcation membrane system and synthesizes vast amount of protein that is packaged into organelles and transferred to the proplatelet processes that extend via junctions in the endothelial lining of the sinusoidal wall into the sinusoidal lumen where platelets are released. Adapted from[4]

Platelets are the smallest of all circulating blood cells, with discoid shape measuring 2.0 - 5.0 µm in diameter and 0.5 µm in thickness (mean platelet volume of 6 - 10 femtolitres). Their shape and size causes platelets to be pushed towards the vessel wall under blood flow, where they can detect and respond to vascular injury. Platelets have highly specialised structure and physiology that supports their haemostatic functions.

1.1.1 Platelet structure

The platelet can be subdivided into 3 zones, known as the peripheral, sol-gel and organelle zones. The open canalicular system (OCS) connects extracellular space with the interior organelle zone via undulating channels. The OCS is thought to be involved in the release of granule contents during platelet activation and in the uptake of proteins from plasma into α-granules (e.g. fibrinogen) by circulating platelets.[5]

The peripheral zone includes the plasma membrane with its many small folds and openings of the surface-connected OCS, that provide the extra membrane needed during platelet shape change and spreading. Exterior to the plasma membrane is the glycocalyx, a dynamic coat of sensory molecule. It contains many glycoprotein receptors that mediate cell adhesion and cell signalling that activate platelets, promote aggregation and accelerate clot retraction at the site of injury. GPIb-IX-V and GPIIb-IIIa receptor complexes are the most abundant glycoprotein receptors in the glycocalyx (25,000 and 80,000 copies per platelet respectively) and are connected to the cytoskeleton. Structural filaments in the submembrane area supports platelet shape change and spreading, as well as the translocation of receptors.

The sol-gel zone refers to the gel matrix of fibrous components that support the platelet cytoskeleton within which organelles are suspended. Microtubules support the discoid shape of resting platelets, while shape change and internal contraction during platelet activation and degranulation are accompanied by polymerisation and reorganisation of fibres into microfilament structures.[6]

The organelle zone occupies the majority of the volume of the resting platelet and contains 3 major classes of secretory granules: α granules, dense (δ)-bodies and lysosomes. Other

organelles are a small number of mitochondria that support the metabolic needs of the platelet and glycosomes.

α-granules are the largest and most abundant organelles in the platelet, with 40 – 80 granules per cell. They contain over 200 different proteins that characterise the platelet secretome.[7] α-granules are important stores of adhesive proteins that promote cell-cell interactions, including fibrinogen, von Willebrand factor (vWF), thrombospondin, fibronection, plasminogen and P-selectin. Additionally α-granules contain proteins originating from parent MKs (synthesised or endocytosed) including epidermal growth factor, transforming growth factor-β, platelet factor 4 (PF4), proteins, factor V, IGF-binding protein-3, proteins endocytosed by MKs and platelets, such as albumin and immunoglobulins, and proteins endocytosed by circulating platelets.

Platelet δ-granules are smaller and less numerous; they are rich in the adenine nucleotides adenosine diphosphate (ADP) and adenosine triphosphate (ATP), serotonin, pyrophosphate, calcium and magnesium.[5] Upon release these prothrombotic and procoagulant molecules take part in clot formation.[5]

Lysosomes are relatively few in platelets (0 – 3 organelles per cell) and contain over 13 different acid hydrolases along with other digestive enzymes; lysosomes are only released upon very strong stimulation.

1.1.2 Haemostatic function of platelets

Under steady state platelet function is under tight negative control by 3 endothelium-mediated mechanisms. The arachidonic acid-prostacyclin ($PG\text{-}I_2$) pathway activates adenylyl cyclase and

raises cyclic adenosine monophosphate (cAMP) levels, which is the strongest known inhibitor of platelet function. The L-arginine-nitric oxide pathway inhibits phosphodiesterases that break down cAMP, while the endothelial ecto-adenosine diphosphatase (ecto-ADPase) degradation of adenine nucleotides ADP and ATP removes these mild agonists from circulation.[8] Together these mechanisms keep platelets in check to prevent unwarranted activation and thrombosis.

Damage to the vessel exposes the adhesive subendothelial matrix proteins collagen and von Willebrand factor (vWF). Under high shear of arterial circulation the GPIb component of the GPIb-IX-V complex binds vWF. The ability of the engaged glycoprotein complex to translocate though the glycocalyx softens and prolongs the initial contact between the platelet and the vessel wall. Cytoskeletal changes initiated by GPIb-IX binding to vWF occur as the platelet begins to spread; its 2 collagen receptors GPVI and integrin $\alpha 2\beta 1$ engage with subendothelial collagen fibres, reinforcing the initial platelet tethering. Platelet shape change involves extension of pseudopods and platelet spreading to cover the injured site. It is accompanied by internal contraction and centralisation of granules whose contents are secreted via the OCS. Secreted granular contents including thrombin and ADP, and freshly synthesised thromboxane A_2 (TxA_2) act in autocrine and paracrine manner to reinforce platelet activation via G-protein coupled receptors (GPCRs) and recruit further platelets from the circulation. This ultimately leads to activation of GPIIb-IIIa, whose active form binds fibrinogen and fibronectin exposed at site of injury, and allows for cross-linking of platelets by fibrinogen or vWF. Integrin and contact signalling further stabilise the haemostatic plug.

Additionally activated platelets support leukocyte migration to injured tissue and release messengers that stimulate the immune response, wound healing and growth of new vasculature.

The key signalling events involved in platelet activation are summarised in Figure 2.

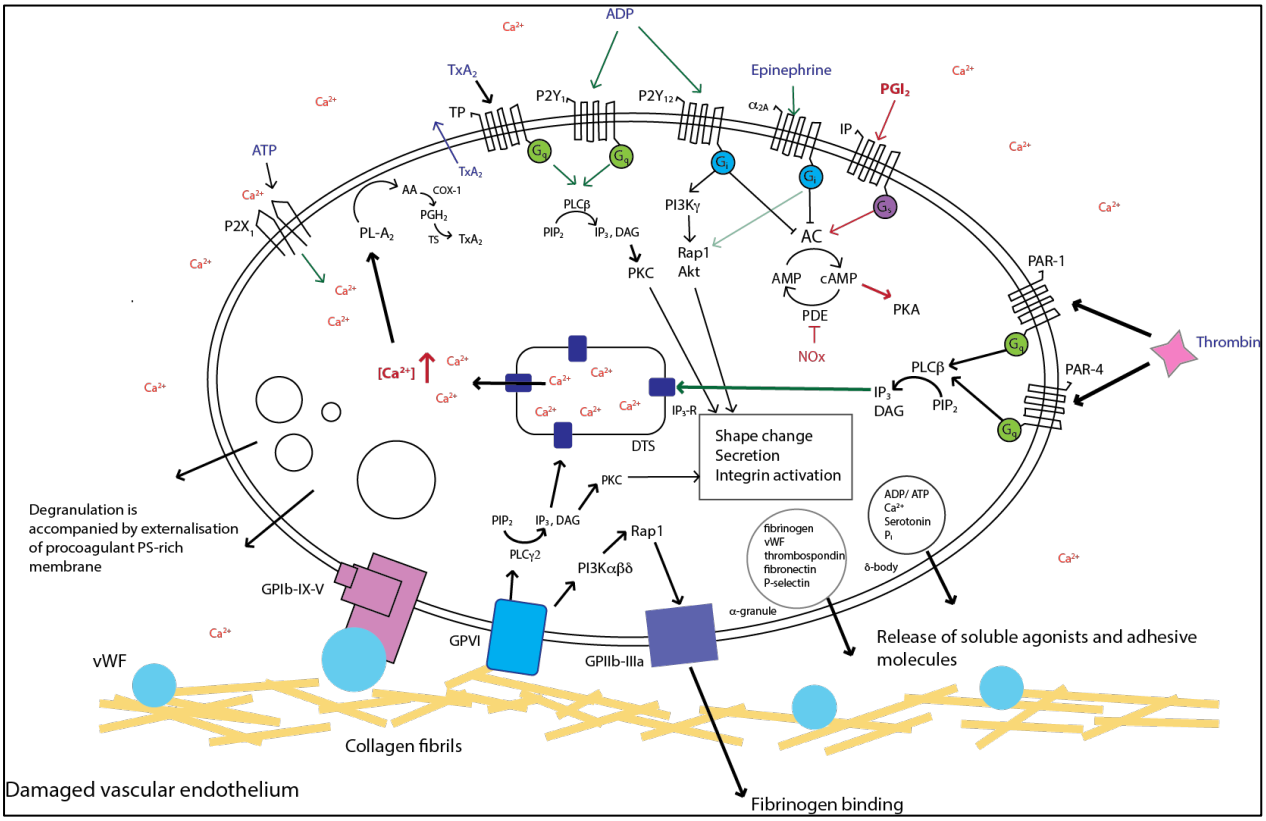


Figure 2. Key signalling events of platelet activation at site of vascular injury: Platelets adhere to the damaged vessel wall by binding vWF via GPIb-IX-V and collagen via GPVI. Soluble agonists released from injury site, including thrombin and ADP, and freshly synthesised TxA_2 stimulate platelets in autocrine and paracrine manner via G-protein coupled receptors (GPCRs) on the platelet surface.

Thrombin is the strongest of these agonists and stimulates platelets via PAR-1 and PAR-4 receptors which are G_q or G_i coupled. Strong coupling to G_q activates phospholipase C (PLC), which hydrolyses membrane phosphatidylinositol-4,5-biphosphate (PIP_2) to give inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds its receptor (IP_3-R) on the surface of the dense tubular system (DTS), releasing internal calcium stores and raising cytosolic calcium concentration from 0.1 to 1 μM, leading to phospholipase A_2 (PLA_2) activation, which liberates arachidonic acid (AA) from the phospholipid membrane. AA is converted to prostaglandin-H_2 in a 2-step reaction by cyclooxygenase-1 (COX-1); thromboxane synthase (TS) converts prostaglandin-H_2 to thromboxane A_2 (TxA_2). TxA_2 diffuses out of the platelet and promotes platelet activation.

Thrombin signalling via G_i-coupled receptors inhibits adenylyl cyclase (AC) and reduces cAMP, which potentiates platelet activation.

Although only a weak agonist, ADP is an important mediator of platelet activation; platelets express 2 GPCRs for ADP, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is G_q-coupled and leads to mobilisation of internal calcium stores via activation of PLC β-isoform, which results in platelet shape change and TxA_2 synthesis. $P2Y_{12}$ is G_i-coupled and inhibits AC. It also activates PI3K and Akt, which lead to integrin activation, and PLC generating IP_3 and DAG that stimulate internal calcium efflux and PKC that promotes secretion. Co-activation of both platelet P2Y receptors is necessary for normal platelet function in response to ADP.[9] On its own ADP is able to elicit platelet shape change and reversible (transient) aggregation, however, secretion of α– and δ–granule contents and release of TxA_2 stimulate positive feedback pathways that can lead to a stable aggregation response.

The platelet $P2X_1$ receptor is an ATP-gated cation channel that regulates permeability of the platelet membrane to monovalent and divalent cations and effectively raises intracellular calcium levels during platelet activation.

Procoagulant role of platelets

Platelets are intimately involved in the coagulation system. During platelet activation the release reaction leads to externalisation of the negatively charged phospholipid phosphatidylserine (PS), typically expressed only at the cytoplasmic surface of the plasma membrane.[10] PS provides a catalytic surface for the assembly of tenase and prothrombinase complexes, responsible for formation of factor Xa and thrombin, respectively.[3,11] PS externalisation is enhanced during strong activation by thrombin or collagen, and is dependent on high levels of intracellular calcium (intracellular flux from internal stores, and influx from outside cell).

Tissue factor (TF) is a membrane protein expressed by fibroblasts and pericytes that is exposed to blood on vessel damage and binds serine protease factor (F) VIIa in circulation to form the

extrinsic Xase complex, which in turn activates FIX and FX. Once activated the serine protease FXa associates with FVa expressed by activated platelets in a calcium dependent manner on the procoagulant surface of the platelet, to form the prothrombinase complex that catalyses the conversion of prothrombin to thrombin. Thrombin is a strong platelet agonist that activates further platelets and activates additional FV and FVIII. FVIIIa associates with FIXa in calcium dependent manner to form the intrinsic Xase complex, which activates FX at a much higher rate than the extrinsic Xase (TF-bound FVIIa). The procoagulant role of platelets is illustrated in Figure 3.

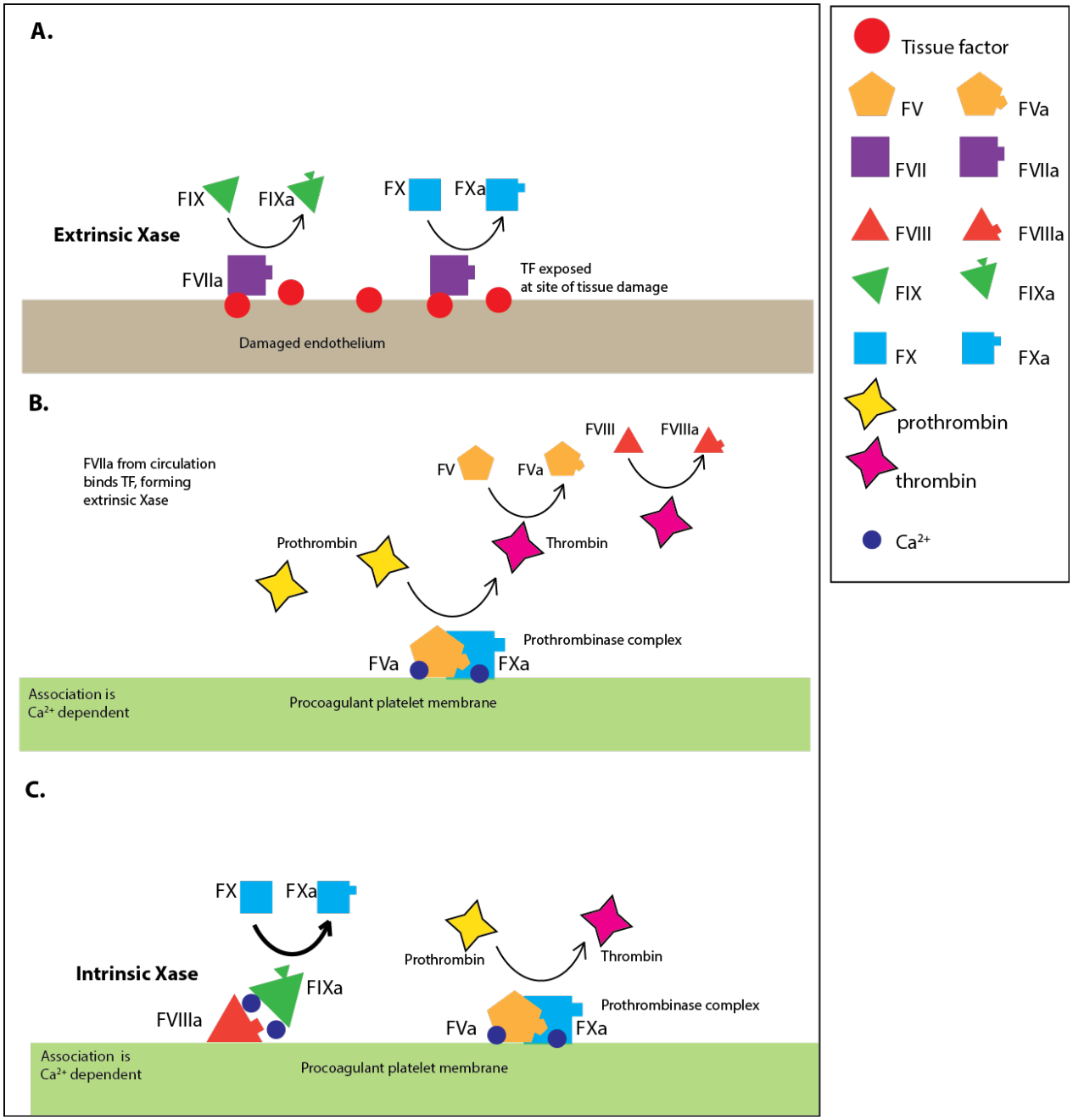


Figure 3. Schematic representation of the procoagulant role of platelets. (A) Tissue factor (TF) expressed at site of damaged vascular endothelium binds serine protease factor VIIa (FVIIa) in circulation to form the extrinsic Xase complex, which activates FIX and FX. (B) Once activated the serine protease FXa associates with FVa expressed by activated platelets in a calcium dependent manner on the procoagulant surface of the platelet, to form the prothrombinase complex that catalyses the conversion of prothrombin to thrombin. Thrombin activates additional FV and FVIII. (C) FVIIIa associates with FIXa in calcium dependent manner to form the intrinsic Xase complex, which activates FX at a much higher rate than the extrinsic Xase (TF-bound FVIIa).

1.2 PATHOPHYSIOLOGY OF PLATELETS

1.2.1 Platelet bleeding disorders

Bleeding diathesis associated with impaired platelet function can be due to congenital or acquired platelet defects. The severity of symptoms associated with this heterogeneous group of bleeding disorders varies greatly and depends on the affected mechanisms of platelet function.[12,13] Following the initial steps of collecting medical history, peripheral blood smear and complete cell count are performed, followed by laboratory evaluation of platelet function, which typically involves the use of light transmission aggregometry to assess platelet responses to a range of agonists.[14-16]

1.2.2 Atherothrombosis

Unlike the relatively rare platelet bleeding disorders, atherothrombosis constitutes the leading cause of mortality in the developed world. It involves the disruption of atherosclerotic plaque coupled with thrombus formation. Atherothrombosis can lead to acute coronary syndromes, which comprise a range of myocardial ischaemic events.[17] Platelets are intimately involved in both atherosclerotic plaque formation and thrombosis and are therefore key targets of therapy used in prevention of ischaemic events.[18,19] Characterisation of platelet function is essential for the development and evaluation of new and improved antiplatelet agents.[20]

1.3 PLATELET FUNCTION TESTING

Platelet aggregation is probably the most widely studied functional response of platelets. It is of clinical significance because the in vivo processes of haemostatic plug formation and arterial thrombosis both involve platelet clumping. By measuring platelet aggregation *ex vivo* one can glean information about real (patho-)physiological functions of platelets.[21]

The development and fine-tuning of turbidimetric or light transmission aggregometry (LTA) by O'Brien and Born enabled the laboratory study of platelet aggregation.[22,23] Detailed characterisation of platelet activation mechanisms has been achieved in the 50 years that followed in large part via the application of LTA. The only significant improvement to the technique involved integration of the luminescence assay of ATP and ADP which allows simultaneous measurement of dense granule secretion.[24] Whole blood aggregometry methods have been developed including impedance aggregometry[25] and platelet counting[26], however LTA remains the gold standard reference and most popular method of platelet aggregation testing worldwide due to its robustness and reproducibility. [21,27,28] The main use of LTA is in the assessment of bleeding disorders[27]; although it is also used in research for the evaluation of new and existing antiplatelet agents.[20]

Light transmission aggregometry (LTA)

To perform LTA anticoagulated whole blood is centrifuged to remove the heavy opaque red (and white) blood cells, which yields relatively translucent plasma rich in platelets (PRP). PRP can be used directly in aggregation experiments or processed further to obtain gel-filtered or washed platelets suspended in a carefully controlled medium.[29] In LTA a narrow light beam is

shone though a sample of PRP or platelet suspension, which is continuously stirred at 37^{oC}, and a detector measures the light that passes. Single platelets scatter photons in all directions so the light arriving at the detector is initially low. After a platelet agonist is added and as platelets aggregate, less light is scattered and more reaches the detector. The increase in light transmission through the sample is indicative of the extent of platelet aggregation, and is usually expressed as a percentage of the total range of light transmission of the sample, which is defined by the minimum (initial transmission of PRP or platelet suspension before stimulation) and the maximum (transmission of platelet poor plasma (PPP) or suspending buffer).[21]

A panel of 7 platelet agonists is commonly used to study platelet function and includes ADP, epinephrine, collagen, a thromboxane mimic, a thrombin receptor agonist, arachidonic acid and ristocetin.[15] Aggregation curves can yield information about shape change and the kinetics of primary and secondary aggregation waves; lumiagrregometry allows to monitor the release reaction and identify storage and release defects.[15] It is important to consider the mechanisms responsible for platelet aggregation.

The ultimate step responsible for platelet aggregation is the activation of GPIIb/IIIa integrin complex, which binds bipolar fibrinogen molecules that cross link platelets and lead to aggregate formation. Another mechanism of platelet association is ristocetin-mediated activation of vWF that, once activated, can bind GPIb platelet receptor and cross-link platelets. During the initial phase of this process, known as agglutination, platelets are metabolically passive; once associations form platelet activation and GPIIb/IIIa mediated aggregation follows.[21,27] Additionally thrombin-induced conversion of fibrinogen to fibrin can lead to entrapment of platelets in a fibrin mesh without an active role from platelets. All these platelet

associations lead to a change in light transmission of samples, but the underlying mechanisms are different.[21]

The relationship between light transmission of PRP and the amount of aggregation is also not straightforward. Small changes in light transmission can be caused by shape change or release of platelet granules, as well as platelet aggregation; conversely LTA can be insensitive to small changes in aggregation.[21] Microaggregate formation of 2 – 8 platelets can go undetected by LTA; changes in light transmission are detectable when small aggregates associate to form larger ones.[30] Single platelet counting to measure loss of platelets as a result of platelet aggregation can be more suited to the detection of micro-aggregate formation.

LTA has some disadvantages in that it is time consuming, requires technical training and is highly dependent on pre-analytical and analytical variables.

1.4 PRE-ANALYTICAL VARIABLES

The results of LTA depend on many pre-analytical and analytical factors including food, alcohol, cigarette or drug consumption, the level of exercise or stress of the subjects prior testing, method of blood collection, the anticoagulant used, sample handling and PRP preparation, as well as the aggregometer and materials used. Additionally temperature and time after blood collection have a strong effect of results of platelet aggregation and must be carefully controlled. Finally, agonist concentrations and interpretation of aggregation curves, including the choice of parameters to report can affect results and conclusions.[21,31,32]

1.5 IN VITRO ANTICOAGULANTS

Addition of an anticoagulant to blood upon blood collection is essential for nearly all subsequent platelet function tests. It prevents blood from clotting, but inevitably alters blood composition and influences cell and protein function, potentially generating in vitro artefacts. Sodium citrate is the most commonly used anticoagulant for platelet function studies. It works by lowering plasma levels of ionic calcium, a vital cofactor to a large number of enzymes that control key reactions of the coagulation cascade; its removal irreversibly prevents blood clotting within collection tube.

Alternative anticoagulants include the thrombin inhibitors hirudin (protein synthesised in the salivary gland of leeches) or its synthetic mimetic polypeptide PPACK, as well as the naturally occurring biomolecule heparin. These maintain physiological levels of calcium, but perturb cell function in other ways.

Sodium citrate

Citrate addition reduces the level of ionised calcium in plasma from 1.2 mM to about 0.1 mM; it also reduces the level of magnesium ions.[33] This low calcium ion concentration prevents blood or derivative PRP from clotting, but is sufficient to support platelet aggregation and release reaction in response to platelet agonists.[34]

It is known that citrate enhances platelet responses to ADP relative to hirudin and heparin anticoagulants. Typically samples PRP samples anticoagulated with hirudin or heparin give reversible aggregation to ADP, whereas 2 waves of aggregation are observed with citrate; the second wave is mediated by TxA_2 production induced by ADP, and associated release of granules.[33] Citrate potentiation of TxA_2 production is due reduced plasma calcium levels. [35,36]

The release reaction during 2nd wave of aggregation induced by ADP in PRP was not inhibited by in vitro addition of thrombin inhibitors heparin[37] or hirudin;[37,38] on the other hand addition of sodium citrate to heparin[34,38] or hirudin[37,39,40] PRP potentiated the release reaction as measured by serotonin (δ-granule) release assay. [37,38] Maximum release occurred when citrate was added to heparin PRP at a concentration of 10 – 15 mM, or when 0.1 or 0.5 mM calcium was added to PRP depleted of ionic calcium or magnesium.[40] 2nd wave and release were blocked by aspirin, inhibitor of COX-1-mediated TxA_2 synthesis.[38]

Citrate also enhanced the degree of inhibition of platelet aggregation by GPIIb/IIa antagonist integrilin. This was explained by increased affinity of the drug for the receptor under conditions of lower plasma calcium. External ionic calcium was proposed to have 3 roles in platelet aggregation: maintenance of GPIIb/IIIa complex, the conversion of this complex to active form capable of binding fibrinogen and in fibrinogen binding; magnesium able to substitute calcium only in fibrinogen binding.[41]

As in EDTA, platelets in blood samples anticoagulated with sodium citrate have been found to adopt a spherical shape and swell over 1-2 hours, increasing in volume by 3 – 10 % depending on anticoagulant concentration. Platelet loss and spontaneous aggregation in vitro have also been reported with sodium citrate anticoagulant.[42,43]

1.6 STANDARDISATION OF LTA

Given the sensitivity of LTA results to pre-analytical and analytical factors, the variability in the methodology of platelet aggregation testing between laboratories has been a cause for concern, as it impedes interpretation and comparison of reported findings.[32,44] The platelet physiology subcommittee of the Scientific and Standardization Committee (SSC) of the International Society

for Thrombosis and Haemostasis (ISTH) conducted a worldwide survey of LTA practices for platelet function testing, which confirmed very high variability in practice and evidenced the need for standardisation.[28] This effort was followed by the elaboration of consensus guidelines for the standardised LTA procedure.[31] The guidelines were compiled based on expert opinion where studies comparing different procedures were absent from the literature. The guidelines included recommendations that blood be collected "into a buffered anticoagulant to help keep the pH stable during processing and testing" and that sodium citrate anticoagulant with a citrate concentration of 109 or 129 mM should be used. Although 5 experts expressed preference for one of two suggested concentrations, the final recommendation is that either concentration of sodium citrate is acceptable.[31] A further recommendation was that LTA studies should be completed within a maximum of 4 hours after blood collection.

3. PART I:

THE EFFECTS OF CONCENTRATION AND PH OF SODIUM CITRATE ANTICOAGULANT ON PLATELET AGGREGATION MEASURED BY LIGHT TRANSMISSION AGGREGOMETRY

3.1 AIM:

To test the cogency of some of the ISTH guidelines for LTA practice, in particular:

- To test whether buffering the anticoagulant is necessary, efficient at keeping the pH of PRP samples stable, and affects platelet aggregation measured by LTA

- To test whether the recommended concentrations of sodium citrate anticoagulant (109 and 129 mM) are equivalent in terms of platelet aggregation response measured by LTA

- To evaluate the time dependence of platelet aggregation induced by ADP in buffered and non-buffered sodium citrate anticoagulant at the recommended concentrations

3.2 MATERIALS AND METHODS I:

Population

A group of 16 healthy volunteers (7 women, median age of 24 and range [21, 65] years) free of any medication known to affect platelet function for 10 days prior to blood collection were recruited from the staff and students of the hospital. All subjects provided informed consent in accordance with the Declaration of Helsinki.

Blood collection

Blood was collected in the morning from an antecubital vein using a 21-gauge butterfly needle and a tourniquet, which was released soon after needle insertion. The first 3 ml of blood were collected into K_3EDTA (Sarstedt, Verona, Italy) for blood cell count by Coulter Haematology Analyser (Beckman Coulter, Milano, Italy) and subsequent blood was drawn with a syringe and transferred immediately into four polypropylene (PP) tubes containing different citrate anticoagulant solutions (1:9 v/v, final volume 10 ml). Sodium citrate anticoagulant solutions were prepared in-house molecular biology grade sodium citrate tribasic dihydrate and citric acid (Sigma, Milan, Italy), as follows:

C3.2 - non-buffered sodium citrate 3.2 % (109 mM Na_3Citrate, pH 8.45),

BC3.2 - buffered sodium citrate 3.2 % (89 mM Na_3Citrate, 20 mM citric acid, pH 5.6),

C3.8 - non-buffered sodium citrate 3.8 % (129 mM Na_3Citrate, pH = 8.45),

BC3.8 - buffered sodium citrate 3.8 % (109 mM Na_3Citrate, 20 mM citric acid, pH = 5.6).

Upon collection blood samples were gently mixed and allowed to rest for 15 minutes at RT. WB samples were assayed for platelet count (Coulter Haematology Analyser, Beckman Coulter, Milano, Italy) and pH (pH meter Basic 20 with microelectrode, Crison Strumenti, Modena, Italy) before centrifuging to obtain PRP.

PRP preparation

Blood samples were centrifuged (Eppendorf R7105, Milano, Italy) for 10 minutes at 200 x g and RT, as this PRP preparation protocol strikes a good balance between favourable removal of contaminating red blood cells and undesired removal of platelets.[45] In addition this relatively low centrifugation speed helps to maintain mean platelet volume nearer native values.[46] The supernatant PRP was collected and carefully transferred to PP tubes using plastic pipette, and kept capped at RT until analysis.

Platelet count of PRP samples was not adjusted to a pre-determined value using autologous PPP, as this can inhibit PA and introduce artefacts,[47] and PA measured by LTA was shown to be independent of platelet count in the range of 150 - 500 platelets nl^{-1} (and only slightly reduced for more extreme values of platelet count of 75 and 750 nl^{-1}).[48]

Platelet aggregation (PA)

PA was measured by LTA using the two-channel ALAT-2 aggregometer (Biola Ltd., Moscow, Russia). Measurements were made at the following times after blood collection: 45 minutes (immediately on PRP preparation), 60 minutes (after the suggested 15 minute resting time of PRP following its preparation), 120, 180 and 240 minutes.

PRP was gently mixed by inversion of the capped tubes 3 times. A sample of PRP (294 μL) was pipetted into a siliconised cuvette (Biodata, Horsham, PA, USA) and pre-incubated for 3

minutes at 37°C. A magnet (Biodata, Horsham, PA, USA) was gently introduced into the cuvette and stirring of the sample was initiated (800 RPM). After 1 minute, PRP was stimulated by addition of ADP (6 μL, final concentration 2 μM, Sigma Aldrich, Milano, Italy). Changes in light transmission were recorded for 5 minutes. PA responses were reported as maximum aggregation in 5 minutes (LTA max) and final aggregation (LTA 5 min).

Platelet count and pH measurement

In parallel to PA measurements, aliquots of PRP (300 μL) were assayed for platelet count using the Coulter Haematology Analyser (Beckman Coulter, Milano, Italy) and pH (pH meter Basic 20 with microelectrode, Crison Strumenti, Modena, Italy).

Statistical analysis

Data was analysed using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, California, USA). Distribution of data was evaluated by D'Agostino & Pearson normality test with a significance level, $\alpha = 0.05$, descriptive statistics and distribution plotting. For data satisfying conditions for parametric tests, 1-way ANOVA for repeated measures and Sidak's post-test were used to compare groups; alternatively, Wilcoxon's signed rank test or Friedman and Dunn's post-test were applied.

3.3 RESULTS I:

pH of WB and PRP

pH measurements were normally distributed for each anticoagulant at every time-point assayed. The pH in WB or PRP samples was not different between 3.2 and 3.8 % sodium citrate anticoagulant at any time, however the pH of WB and PRP was significantly lower in buffered compared to non-buffered anticoagulant ($p < 0.0001$), irrespective of citrate concentration. In all anticoagulants pH of PRP increased significantly over 3 hours (1-way ANOVA, $p < 0.0001$), to reach a similar final value (Figure 1). Table 1 shows pH values in WB 20 minutes after blood collection and in PRP 60 and 240 minutes after blood collection.

pH	**Non-buffered citrate**		**Buffered citrate**	
	3.2 %	**3.8 %**	**3.2 %**	**3.8 %**
WB 20 mins	**7.65 ± 0.09**	**7.64 ± 0.09**	**7.41 ± 0.10**	**7.40 ± 0.09**
PRP 60 mins	7.81 ± 0.09	7.79 ± 0.06	7.63 ± 0.09	7.60 ± 0.07
PRP 240 mins	8.13 ± 0.09	8.08 ± 0.10	8.07 ± 0.08	8.00 ± 0.11

Table 1. pH in WB 20 minutes after blood collection and in PRP 60 and 240 minutes after blood collection

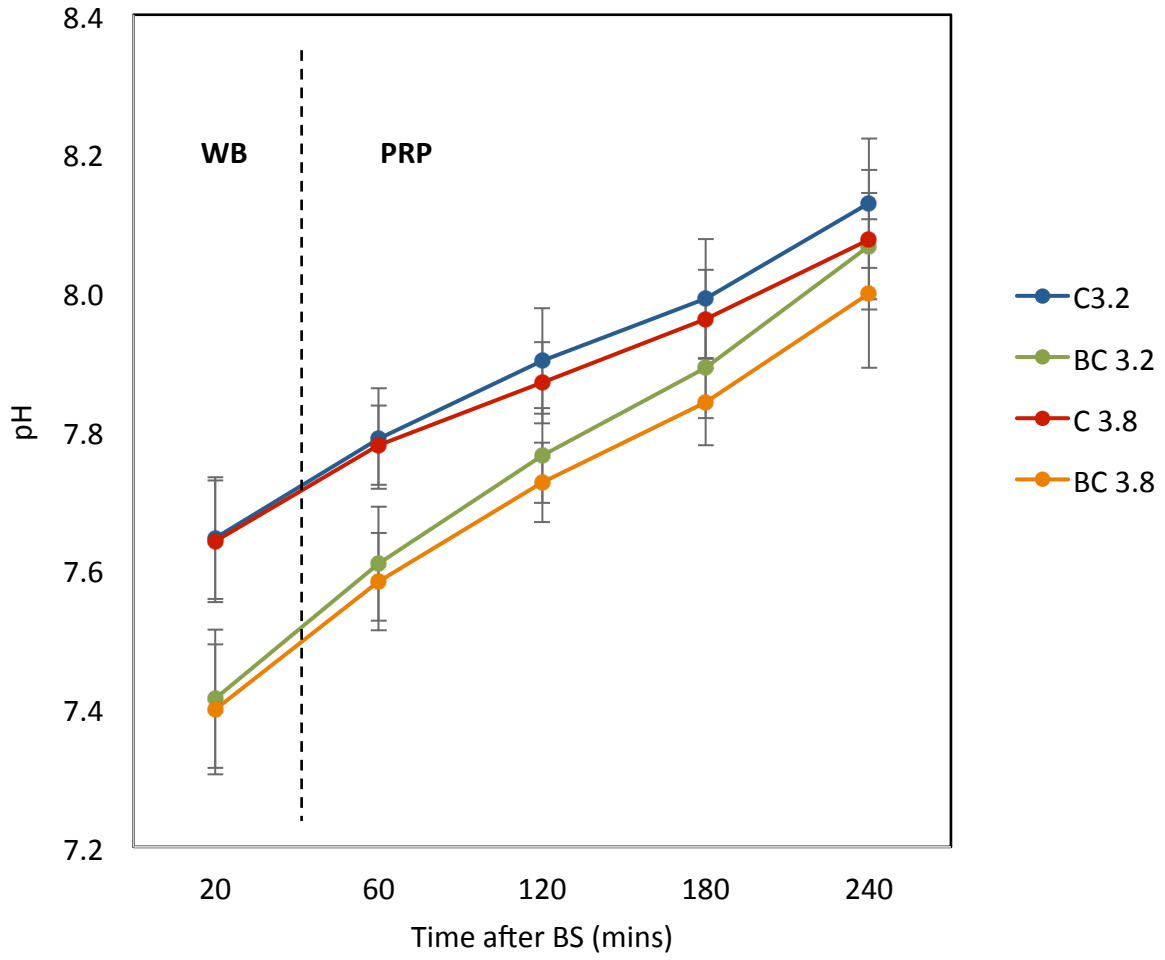


Figure 1. The pH in WB anticoagulated with sodium citrate 3.2 and 3.8 % (C3.2 and C3.8) and its buffered equivalents (BC3.2 and BC3.8) 20 minutes after blood collection and in derivative PRP 1, 2, 3 and 4 hours after blood collection. Mean and S.D. values are plotted and 1-way ANOVA and Sidak's post-test were used to compare groups. There was no significant difference in pH of WB or PRP anticoagulated with 3.2 and 3.8 % citrate at any time. Buffered citrate anticoagulant resulted in lower, more physiological, pH of WB and PRP, despite the use of buffer pH in PRP increased with time for all anticoagulants.

Platelet aggregation induced by ADP

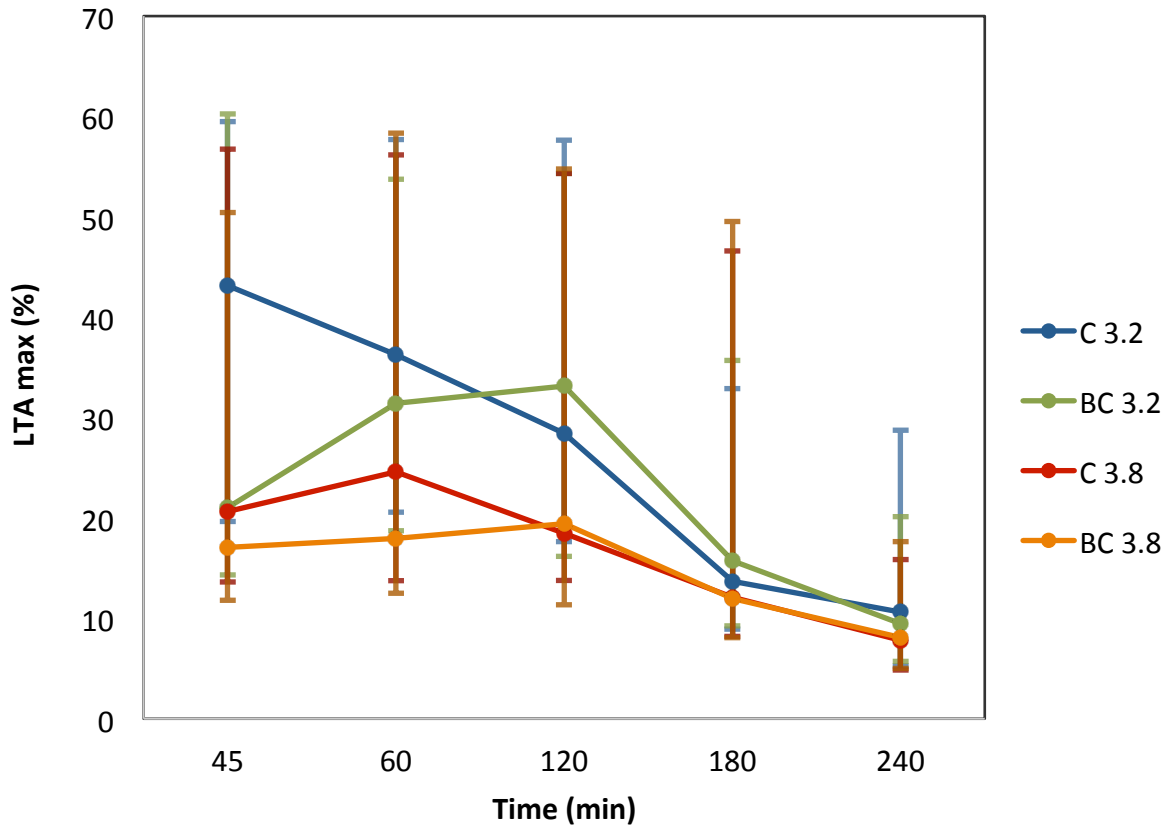


Figure 2. Graph shows the platelet aggregation response to ADP measured by LTA in PRP anticoagulated with non-buffered sodium citrate 3.2 or 3.8 % (C3.2 and C3.8) or their buffered equivalents (BC3.2 and BC3.8); mean and I.Q.R. values are plotted. Immediately upon PRP preparation response is variable; for all anticoagulants stable maximum response is observed 1 and 2 hours after blood collection, with decreased response at 3 and 4 hours.

Platelet aggregation response to ADP did not satisfy normality (LTA response to low concentration does not seem to come from a Gaussian distribution, rather a bimodal one, with some high responders and some low responders). Friedman's and Dunn's multiple comparison tests were used to compare responses recorded for each anticoagulant.

Effect of buffer on platelet aggregation

When data from 3.2 and 3.8 % citrate was pooled together, no difference in LTA response to ADP between buffered and non-buffered anticoagulant was observed at any time-point tested, except at 45 minutes (immediately after PRP preparation, Wilcoxon $p < 0.001$), when platelet aggregation in non-buffered anticoagulant was higher than in buffered anticoagulant (Figure 3).

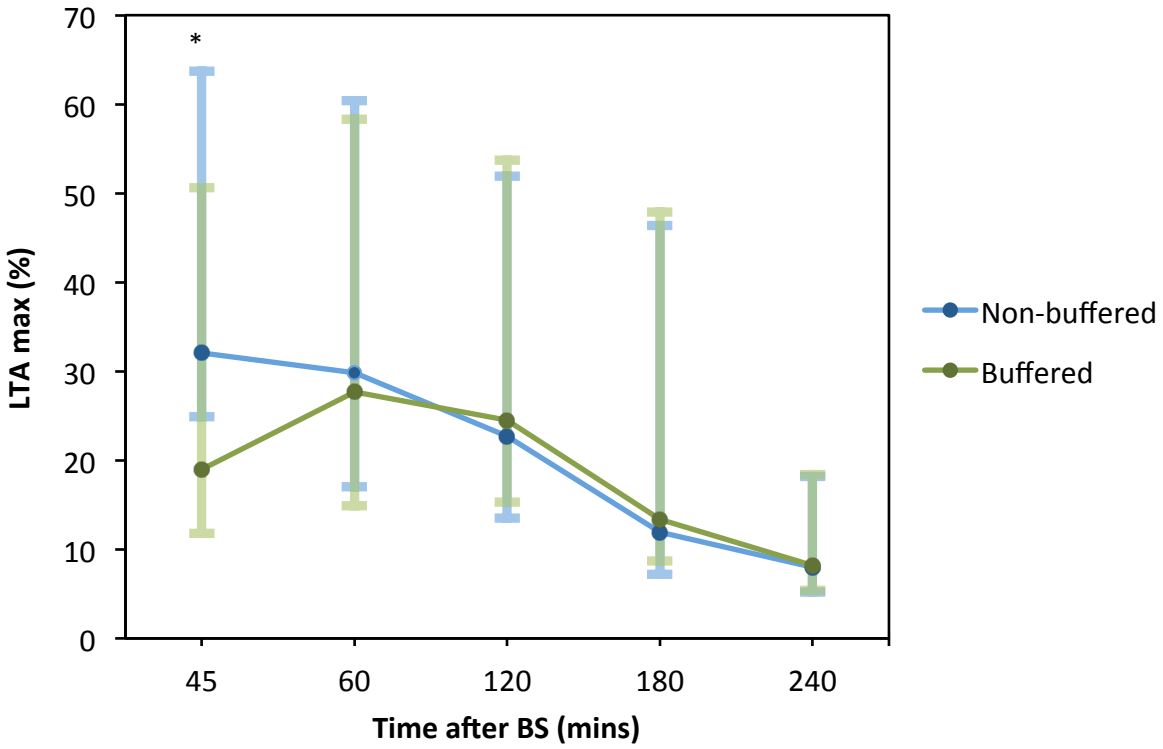


Figure 3. Platelet aggregation response to ADP (median and IQR) of 16 subjects, with pooled 3.2 and 3.8 % sodium citrate data. PA response was lower in buffered citrate immediately after PRP preparation (45 minutes after blood collection, Wilcoxon, $p < 0.001$), at all other time assayed response was equivalent between buffered and non-buffered anticoagulant.

Effect of citrate concentration on platelet aggregation:

When data for buffered and non-buffered sodium citrate was pooled together for each citrate concentration, LTA response to ADP was higher in 3.2 % relative 3.8 % citrate at all times assayed (Figure 4). Median platelet aggregation response was almost double in magnitude in 3.2 % relative to 3.8 % citrate 1 and 2 hours after blood collection, and the difference was statistically significant at all times except 3 hours (Wilcoxon $p < 0.001$ at 45, 60 and 120 minutes, $p = 0.136$ at 180 minutes, and $p < 0.01$ at 240 minutes) (Figure 4).

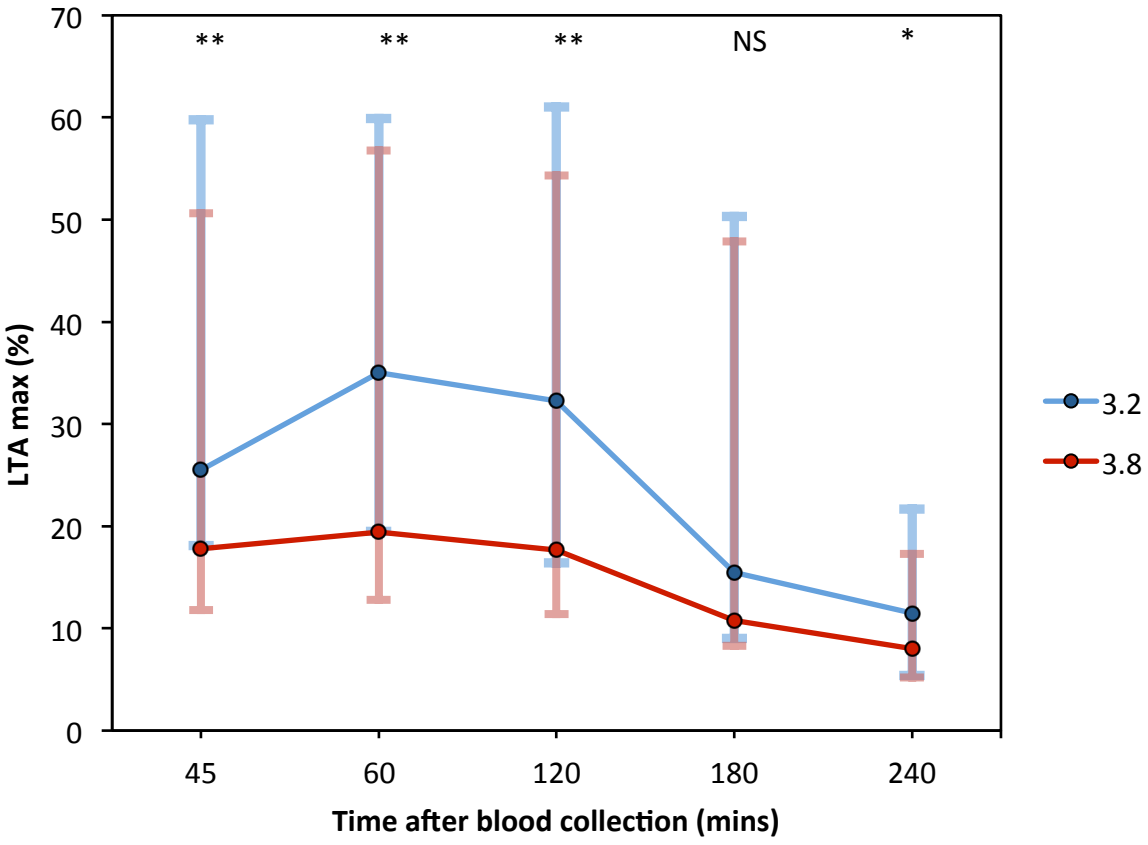


Figure 4. Platelet aggregation (PA) response to ADP (median and IQR) of 16 subjects, with non-buffered and buffered anticoagulants pooled for each citrate concentration. PA was higher in 3.2 % relative to 3.8 % citrate at all times (Wilcoxon, $p < 0.001$ at 45, 60 and 120 minutes, $p = 0.136$ at 180 minutes, $p < 0.01$ at 240 minutes).

Effect of time on platelet aggregation

Platelet aggregation response to ADP was highly time-dependent for all citrate anticoagulants tested, with significantly reduced response 3 and 4 hours after blood collection ($p < 0.0001$) (Figure 5). When data from all citrate anticoagulants were pooled together, as for individual anticoagulants, maximum response was observed 1 and 2 hours after blood collection and decreased significantly (more than halved) at 3 and 4 hours (Friedman $p < 0.0001$) (Figure 5). Immediately after PRP preparation PA response was slightly reduced (Dunn's $p = 0.03$), although this is not representative of unbuffered citrate 3.2 %, which gave enhanced response immediately after PRP preparation, as shown in Figure 2.

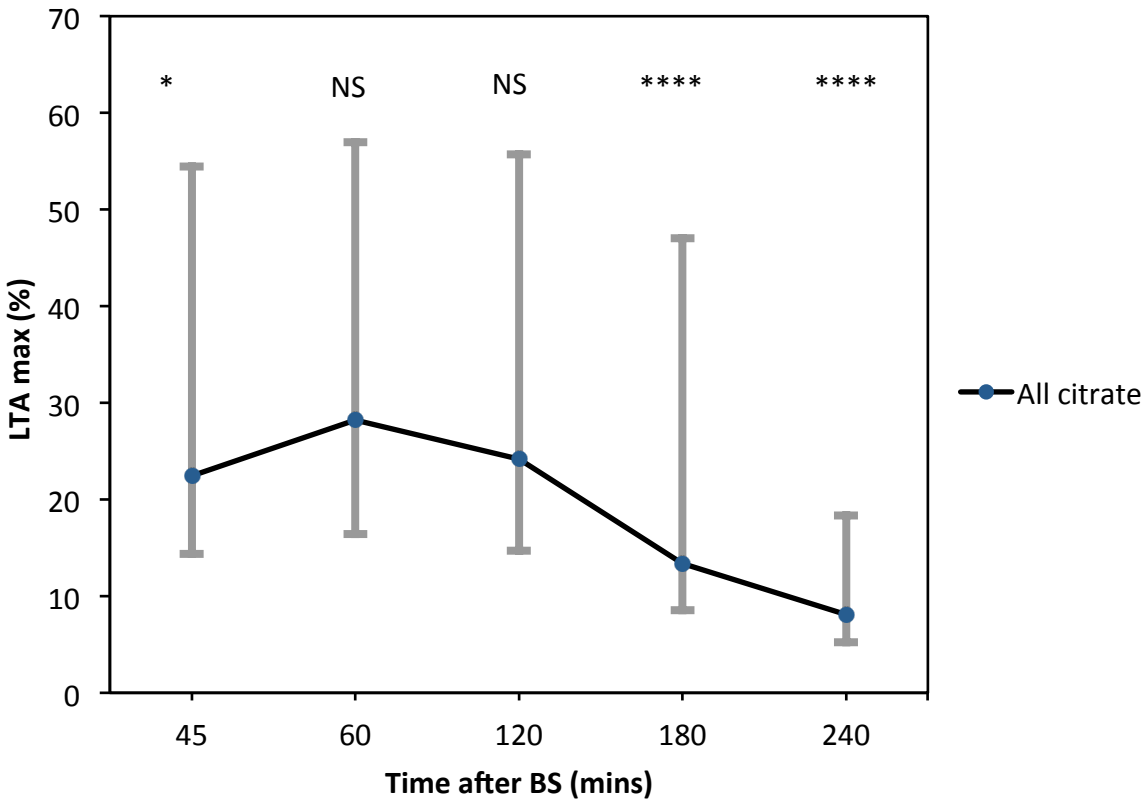


Figure 5. Effect of time on platelet aggregation for all citrate anticoagulants: platelet aggregation induced by ADP (median and IQR) was highly time-dependent for each citrate (N

= 16) and all citrates pooled together (N = 64) (Friedman, p<0.0001). At 45 min response is reduced (Dunn, p < 0.05), reaches a stable maximum 1 and 2 hours after blood sampling, and decreases at 180 and 240 min (Dunn, p < 0.0001).

Platelet count

Platelet count was normally distributed with a mean value of 209 ± 57 10 nL^{-1} in EDTA WB 20 minutes after blood collection. Table 2 shows platelet count in PRP on PRP preparation and 3 hours later. For each anticoagulant platelet count in PRP decreased significantly (by 10 – 15 %) with time after blood collection, dropping by about 40 – 60 nL^{-1} within 3 hours (Figure 6).

Platelet count (nL^{-1})	**Non-buffered citrate**		**Buffered citrate**	
	3.2 %	**3.8 %**	**3.2 %**	**3.8 %**
PRP 60 mins	371 ± 97	367 ± 98	394 ± 100	394 ± 99
PRP 240 mins	324 ± 90	331 ± 87	333 ±85	348 ±83

Table 2. Platelet count in PRP 60 and 240 minutes after blood collection

When buffered and non-buffered citrate data was pooled, concentration had no significant effect on platelet count in PRP at any of the four time-points assayed (Wilcoxon p > 0.05), although loss of platelets is somewhat lower in 3.8 % citrate than in 3.2 % citrate. When 3.2 % and 3.8 % data was pooled, buffered anticoagulant had significantly higher platelet count at all times (Wilcoxon p < 0.05).

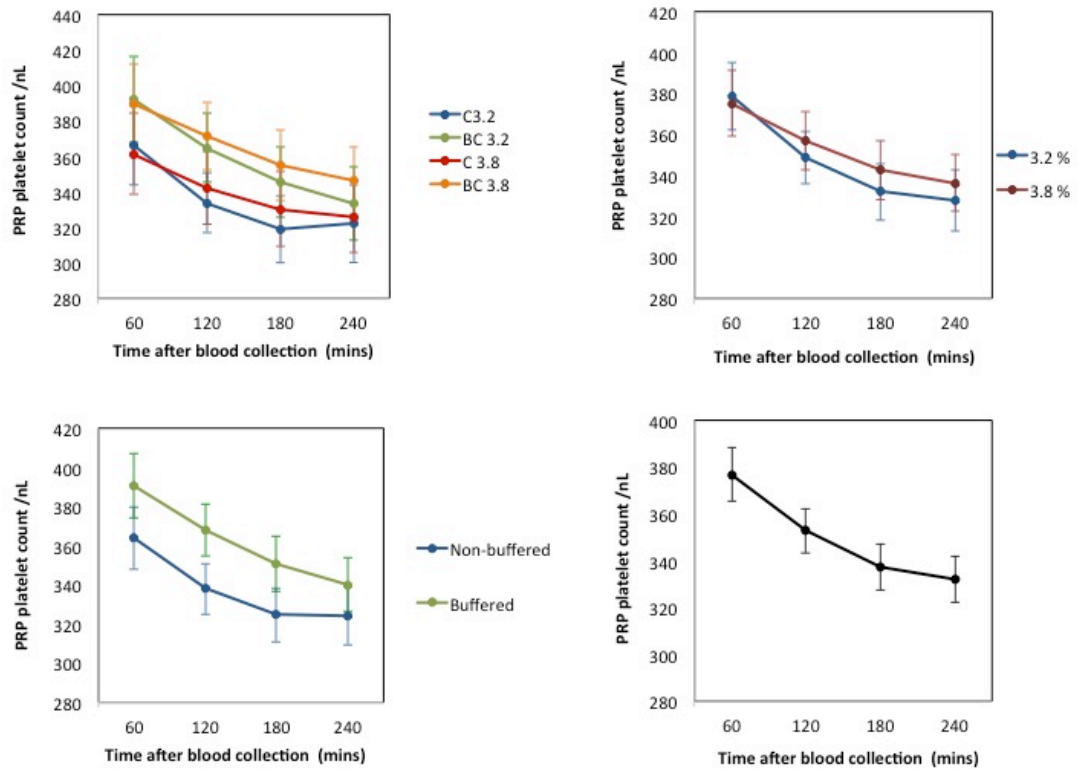


Figure 6. Platelet count in PRP (mean and SEM, N=16) was higher by 20 – 30 platelets **nL^{-1}** in buffered relative to non-buffered citrate; in all anticoagulants PRP platelet count decreased significantly over time (top left). Citrate concentration had no significant effect on platelet count (top right), whereas the use of buffer maintained platelet count at consistently higher than the non-buffered anticoagulant (bottom left). Pooling data from all four anticoagulants, the time dependent loss of platelets can bee seen (bottom right).

3.4 DISCUSSION I:

Use of buffered compared to non-buffered sodium citrate anticoagulant maintained the pH of samples in whole blood and PRP at a more physiological value. Buffering the anticoagulant did not prevent the rise of pH over time in PRP, as pH rose to a value of 8 within 4 hours of blood collection with all anticoagulants tested.

The anticoagulant is buffered with citric acid to lower anticoagulant pH so that the pH of collected blood is near physiological value. Sodium citrate/ citric acid mixture has a buffering range of 3.0 – 6.2, during blood collection 9 parts of blood with a pH of 7.35 – 7.45 are added to 1 part anticoagulant. The main component of the system is WB so the resultant pH of the mixture is that of WB.

The use of buffered anticoagulant has no effect on LTA response to ADP at any time after blood sampling, except immediately upon PRP preparation (before allowing samples to rest for 15 min, as recommended by guidelines), when the response is enhanced in non-buffered citrate and reduced in the buffered equivalent. The initially lower platelet aggregation in buffered citrate is consistent with the fact that platelets are inhibited at lower pH. The higher platelet aggregation in non-buffered citrate immediately upon PRP preparation may be due to activation and loss of platelets during the centrifugation and PRP handling.

Sodium citrate 3.2 % (109 mM) allows significantly higher platelet aggregation response to low concentration ADP than sodium citrate 3.8 % (129 mM). This is likely due to stronger chelation of calcium ions, which support platelet aggregation, by 3.8 % sodium citrate.

Platelet aggregation induced by ADP is highly time-dependent in all citrate anticoagulants tested, and diminishes significantly 3 hours after blood sampling. Hence, studies with ADP should be completed within 2 hours of blood collection.

Platelet count in PRP decreased over time in all anticoagulants, although the net loss of platelets was relatively small, about 10 – 15 % over 3 hours. The fact that non-buffered sodium citrate samples had consistently lower platelet counts and that samples in 3.8% citrate tended to have higher platelet counts suggest that the decrease in platelet count is attributable to in vitro platelet activation, and formation of platelet aggregates. In order top test this hypothesis, we performed the experiments in Part II.

4. PART II:

DOES THE IN VITRO INHIBITION OF PLATELET FUNCTION WITH PGE-1 REDUCE PLATELET LOSS AND SPONTANEOUS PLATELET AGGREGATION IN WHOLE BLOOD AND PRP SAMPLES ANTICOAGULATED WITH SODIUM CITRATE DURING LTA STUDIES?

4.1 AIM II:

To test whether the loss of platelets observed in PRP samples anticoagulated with sodium citrate during LTA studies is reduced by addition of PGE-1, and hence is due to platelet activation and micro-aggregate formation.

4.2 MATERIALS AND METHODS II:

7 healthy subjects (6 women) with median age 32 years and range [27, 55] who had not taken any non-steroidal anti-inflammatory drugs for 10 days prior to testing were enrolled.

Blood was collected with venepuncture as before, with the first 3 ml collected into KEDTA for cell count and the following blood drawn with plastic syringes and transferred to four tubes containing sodium citrate anticoagulant 3.2 % non-buffered or buffered solution, prepared as above. PGE-1 (final concentration of 2 μM) or saline was added to WB immediately upon blood collection.

Following the procedure previously described to prepare PRP, platelet aggregation studies were performed as before, but with addition of saline instead of ADP. Spontaneous platelet aggregation (SPA) was monitored by LTA and mean relative radius (MRR) for five minutes 45, 60, 120, 180, and 240 minutes after blood collection.

Additionally platelet count and pH were measured in WB and in PRP at each time point.

All subjects provided informed consent in accordance with the Declaration of Helsinki.

Data was analysed in GraphPad Prism, mean and S.D. values are reported unless otherwise stated. Student t-test or 1-way ANOVA were used to compare groups.

4.3 RESULTS II:

Platelet count in WB and PRP

We measured platelet count and pH in WB and PRP from 7 healthy subjects, as well as spontaneous platelet aggregation (SPA) in PRP by LTA and MRR. The mean platelet count in WB anticoagulated with K-EDTA was 246 ± 89 nl^{-1} and consistent with those in citrate WB, if 9:1 dilution on citrate addition is considered. Platelet counts in WB and PRP are shown in Table …. Addition of PGE-1 had no effect on initial platelet count in citrate WB.

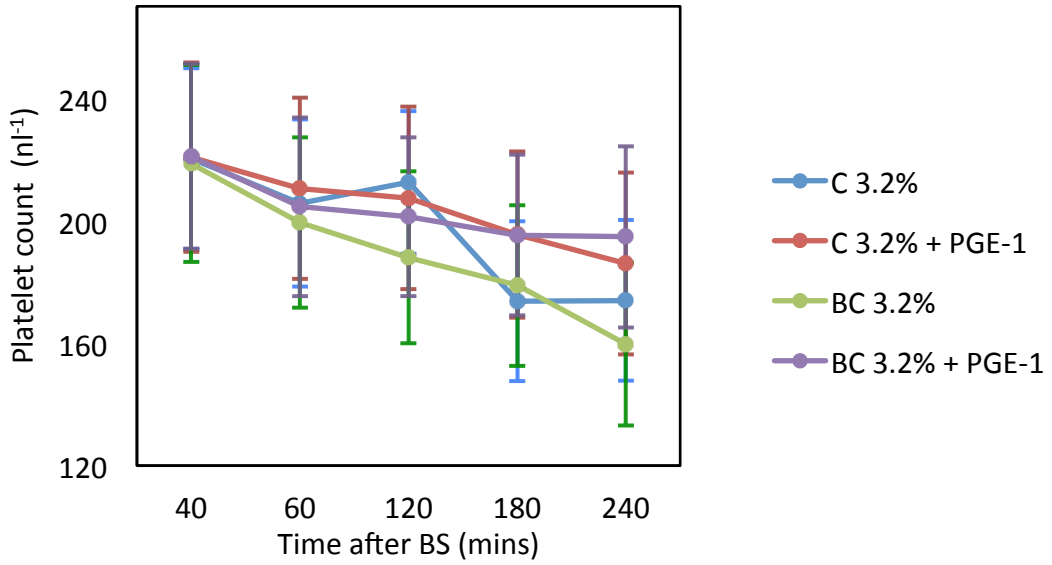
Over 4 hours following blood collection mean platelet count decreased significantly, 21 and 27 % for non-buffered and buffered sodium citrate WB respectively. In presence of PGE-1, loss of platelets was partially inhibited.

Platelet count (nL^{-1})	**Sodium citrate 3.2 %**		**Sodium citrate 3.2 % + PGE_1 2 (μM)**	
	Non-buffered	**Buffered**	**Non-buffered**	**Buffered**
WB 40 mins	220 ± 78	219 ± 85	221 ± 82	221 ± 80
WB 240 mins	174 ± 70	160 ± 70	186 ± 79	195 ± 78
PRP 60 mins	391 ± 125	408 ± 130	401 ± 115	415 ± 125
PRP 240 mins	359 ± 123	381 ± 112	407 +/- 124	422 ± 129

Table 2. Platelet count in WB and PRP at sample preparation and at the end of LTA studies.

In presence of PGE-1 initial platelet count in PRP was slightly higher and increased slightly over 4 hours, indicating a small degree of disaggregation. Figure 7 shows platelet count in WB and PRP over the time tested.

A.



B.

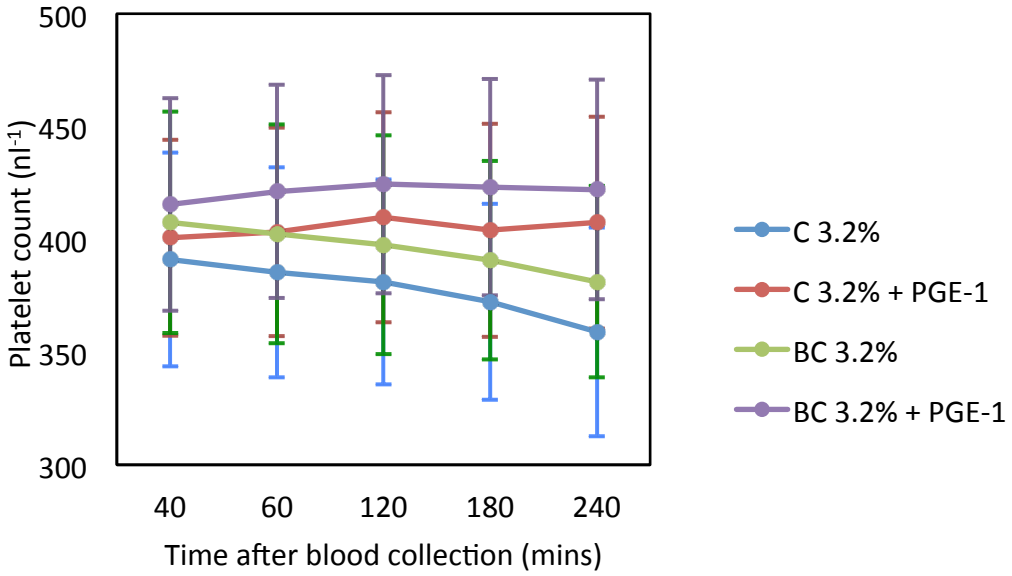


Figure 7. Platelet count in (A) WB and (B) PRP in non-buffered sodium citrate (C3.2) or the buffered equivalent (BC3.2) in presence and absence of PGE-1, mean and S.E.M. are shown.

Spontaneous platelet aggregation measured by mean relative radius and LTA

Varying degree of spontaneous platelet aggregation was observed in non-buffered and buffered sodium citrate anticoagulants. Initial SPA measured by LTA was 1.6 ± 1.3 % in non-buffered citrate and increased to a maximum of 3.0 ± 1.9 % 3 hours after blood collection; in buffered sodium citrate SPA was 0.8 ± 0.8 % initially and reached a maximum of 3.4 ± 3.2 % 4 hours after blood collection. In presence of PGE-1 SPA was inhibited and reached a maximum mean value of only 0.8 ± 0.5 % within 4 hours after blood collection.

MRR measurements gave concordant results: SPA increased over time from 1.3 ± 0.3 to 1.7 ± 0.8 AU in non-buffered anticoagulant and 1.0 ± 0.0 to 1.8 ± 0.9 AU in buffered anticoagulant. In presence of PGE-1 SPA was inhibited and reached only 0.7 ± 0.5 AU for non-buffered and 0.6 ± 0.3 AU for buffered citrate (Fig 8).

pH of WB and PRP

As in the earlier part of the study the use of non-buffered versus buffered citrate lowered initial pH in WB to a more physiological value. 45 minutes after blood collection WB pH was 7.72 ± 0.10 and 7.50 ± 0.11 in in non-buffered and buffered citrate respectively; presence of PGE-1 had no effect on pH. pH increased in all anticoagulants over course of experiment to maximum values of 7.85 ± 0.08 and 7.72 ± 0.10 in non-buffered and buffered citrates respectively. **In** PRP buffered anticoagulant resulted in lower pH throughout the experiment bud did not inhibit increase in pH over time, confirming previous data.

Overall changes in pH were smaller in WB than in PRP. The higher resistance of WB to c changes in pH is due to the stronger buffering system of WB compared to PRP (Fig 9).

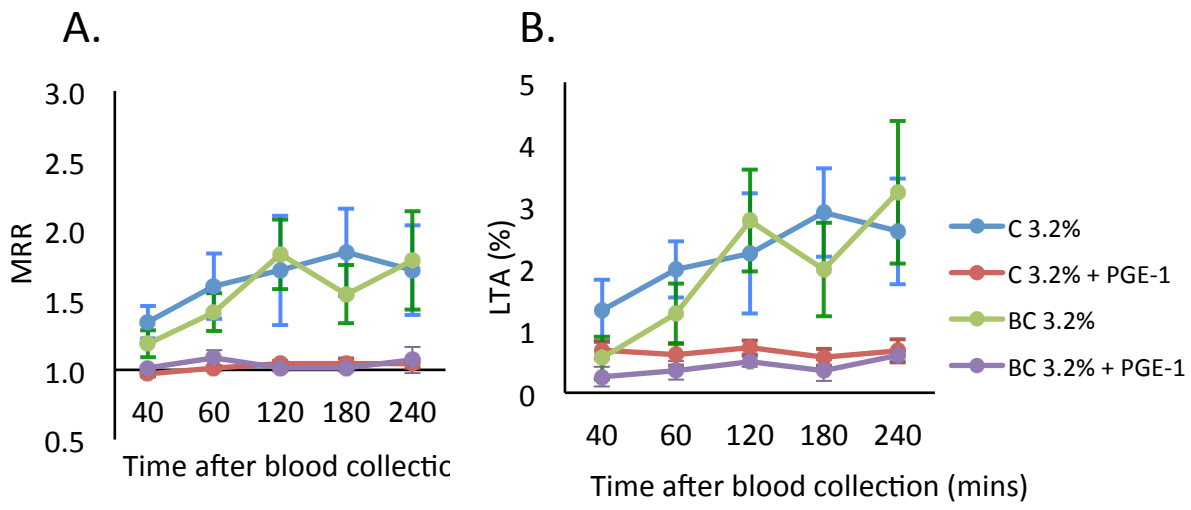
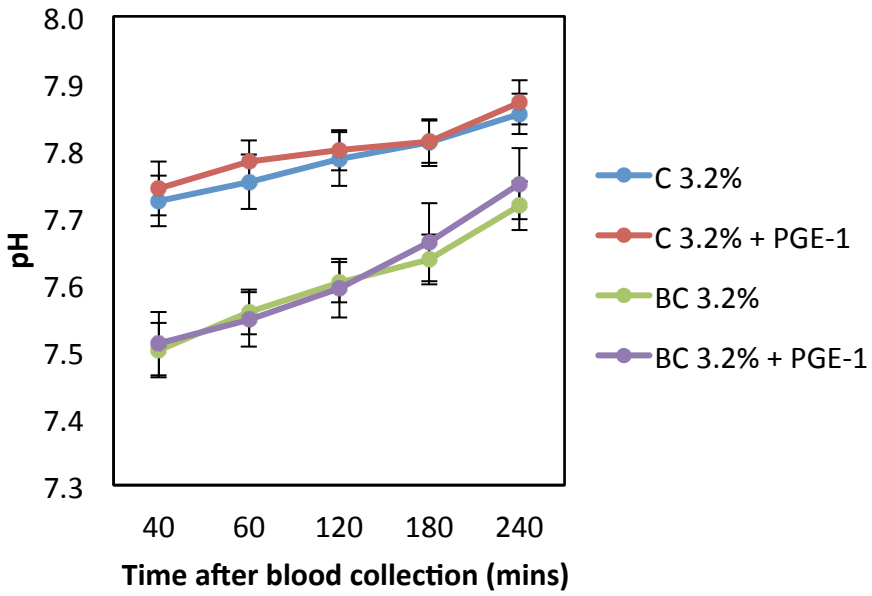


Figure 8. SPA after 6 minutes of stirring (error bars show S.E.M.) for sodium citrate anticoagulant 3.2 % (C3.2) and buffered sodium citrate 3.2 % (BC3.2) in presence and absence of PGE-1 (final concentration 2 μM); (A) Mean relative radius (MRR) of aggregates; (B) LTA.

A.



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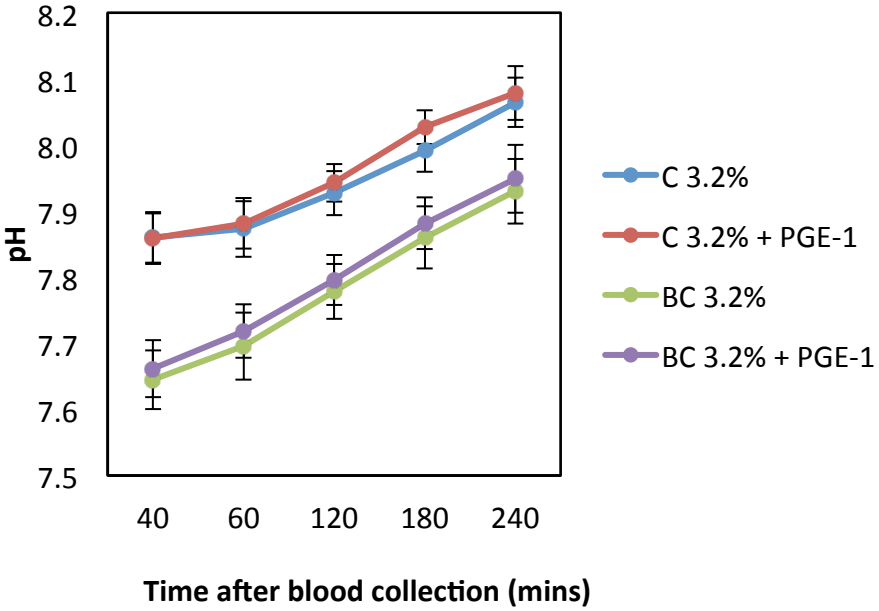

Figure 9. (A) pH in WB anticoagulated with sodium citrate 3.2 or 3.8 % in presence or absence of PGE-1; similarly for PRP (B); mean and S.E.M. values are shown.

4.4 DISCUSSION II:

A decrease in platelet count was observed in WB anticoagulated with sodium citrate over the time-course of 4-hour platelet aggregation study. The loss of platelets in WB was partially inhibited by addition of PGE-1 to freshly collected blood, which raises cyclic AMP levels and thus inhibits platelet aggregation. Platelet loss also occurred in citrated PRP and was completely inhibited by addition of PGE-1, indicating that platelet loss in PRP is due to platelet activation. In WB other mechanisms of platelet removal could be at work, including cell adhesion to leukocytes via P-selectin.

Supporting the role of platelet aggregation in reducing the platelet count in PRP was the presence of SPA measured by LTA and MRR that increased with time (increased light transmission of samples as platelets aggregated and increased mean aggregate radius). SPA was completely inhibited by addition of PGE-1 during blood collection.

Samples anticoagulated with buffered citrate had consistently higher platelet count than the non-buffered samples, and although the difference was small it is in agreement with the fact that lower pH inhibits platelets and thus prevents their activation and aggregation during sample handling in LTA studies. Enhanced platelet activation during PRP preparation and handling leads to removal of the most activated/ reactive platelets.

Together these observations highlight the sensitivity of platelets to pre-analytical and analytical variables during platelet function testing, and the importance of standardising practice, especially when applied to diagnosing platelet function defects or obtaining data on pharmacokinetics of antiplatelet agents. Effects of anticoagulants should be considered when designing experiments to study platelets.

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