Introduction and literature review

1.1 General Introduction:

Platelets are anucleate circulating blood particles. They circulate around the body in an inactive state until they come into contact with areas of endothelial damage or activation of the coagulation cascade, Here they adhere to the endothelial defect, change shape, release their granule contents, and stick together to form aggregates. Physiologically these processes help to limit blood loss. [(1)]

A normal platelet count is 150 to 450 x 10^9/L. In this range, an individual will have properly functioning platelets that assist in the coagulation process by creating a platelet plug and stimulating the formation of a solid fibrin clot. A decrease in platelet count will cause bleeding from the mucous membranes. Thrombocytopenia or a decreased platelet count is caused by a number of factors. Decreased production of platelets or increased destruction of platelets usually accounts for the pathophysiology of most quantitative defects in platelets. Additionally, sample related conditions or preanalytic variables may lead to falsely decreased. [(2)]

1.2 Literature review:

1.2.1 Platelet production:

Platelets, also called thrombocytes, are cytoplasmic fragments released from a parent cell known as a megakaryocyte. Megakaryocyte are large cells (80-150μ in diameter), which are found predominantly in the bone marrow and to a smaller degree in the spleen and lungs. Similar to erythrocyte and leukocyte , megakaryocyte develop from a pluripotential stem cell that has been influenced by colony stimulating factor (CSF) produced by macrophages , fibroblast , T lymphocyte , and stimulated endothelial cells. Additional influences, such as interleukin-3 and -6 (IL-3 and IL-6), which appear to be instrumental in differentiation of stem cell into platelet producing Megakaryoblasts, and megakaryocyte CSF (Meg-CSF) and granulocyte CSF (G-CSF) synergistically stimulate production of progenitor cells. Meg-CSF is thought to be generated by bone marrow cells in response to megakaryocytic mass. as the number of megakaryocyte decrease, the amount of Meg-CSF increases.

Thrombopoietin is generated predominantly by the kidney, and to a lesser amount, by the liver and spleen, in response to a demand for platelets. It stimulates megakaryocyte progenitor cell to mature and release platelets, although its chemical nature is still not completely known. The spleen is the part of the regulatory system for platelet production wherein approximately 30% of peripheral blood platelets are sequestered .sudden depletion of platelets, resulting from consumption in clotting or immune and nonimmune destruction, may rapidly empty the splenic pool. In response, Thrombopoietin causes maturation of the Megakaryoblasts to produce a marrow response equal to the loss of platelets. Because the action of Thrombopoietin is similar to that of erythropoietin, any Increases in Thrombopoietin will speed up the maturation of megakaryocyte. This accelerated maturation results in less platelet

production per cell. If the consumption or destruction of platelets continues, the platelet count will fall to a level incapable of maintaining normal vascular and hemostatic integrity and a condition called acute thrombocytopenia.[3]

Megakaryocytes do not undergo complete cellular division but undergo a process called endomitosis or endoreduplication creating a cell with a multilobed nucleus. Each megakaryocyte produces about 2000 platelets.

Platelet development occurs in the following sequence:

- Megakaryoblasts are the most immature cell (10 to 15 μm) with a high nuclear to cytoplasmic ratio and two to six nucleoli
- Promegakaryocyte is a large cell of 80 μm with dense alpha and lysosomal granules.
- Basophilic megakaryocyte shows evidence of cytoplasmic fragments containing membranes, cytotubules, and several glycoprotein receptors.
- The megakaryocyte is composed of cytoplasmic fragments that are released by a process called the budding of platelets. [2]

1.2.2. Platelet structure:

Platelets have several unique features that enable them to efficiently perform their primary function, namely the rapid formation of a vascular plug following vessel injury. Platelets are extremely small and discoid in shape, with dimensions of approximately 3.0 μm by 0.5 μm, and a mean volume of 7–11fL. This shape and small size enables the platelets to be pushed to the edge of the vessel, placing them next to the endothelial cells and in the right place to respond to vascular damage. They are present at a high level in the human circulation, usually between 150 and $400x10^9$ platelets/L. This level of expression appears to represent a considerable degree of redundancy, as individuals with platelet counts as low as $10x10^9$ platelets/L tend to exhibit only occasional, major spontaneous

bleeds, although they are at considerable risk of bleeding during major trauma. [4]

1.2.2.1 The plasma membrane:

The plasma membrane is a trilaminar unit composed of a bilayer of phospholipids in which cholesterol, glycolipids, and glycoproteins are embedded. The plasma membrane is thought to contain the sodium- and calcium-ATPase pumps that control the intracellular ionic environment of the platelet. Approximately 57 percent of platelet phospholipids are contained in the plasma membrane. The phospholipids are asymmetrically organized in the plasma membrane; the negatively charged phospholipids are almost exclusively present in the inner leaflet, whereas the others are more evenly distributed. The negatively charged phospholipids, especially phosphatidylserine, are able to accelerate several steps in the coagulation sequence and so their presence in the inner leaflet of resting platelets, separated from the plasma coagulation factors, is thought to be a control mechanism for preventing inappropriate coagulation. During platelet activation induced by select agonists, the aminophospholipids may become exposed on the platelet surface or on the surface of platelet microparticles. The phospholipids asymmetry in resting platelets may be maintained by an ATP-dependent aminophospholipid translocase that actively pumps phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet. Interactions of negatively charged phospholipids with cytoskeletal or other cytoplasmic elements may also contribute to the asymmetry. The enrichment of selected phospholipids with arachidonic acid is quite striking, furnishing a store of arachidonic acid for release and conversion into thromboxane A2 (TXA2). [5]

1.2.2.2 Cytoskeletal elements:

The discoid shape of the platelet is formed by the platelet cytoskeleton, which consists of a spectrin-based membrane skeleton; circumferential bands of single microtubule that lies beneath the plasma membrane; and a rigid actin filament network that fills the cytoplasm of the cell. Much of the rigid structure and platelet strength results from the 2 million copies of actin per platelet, of which approximately 40% are assembled into actin polymers. These polymers connect with each other and with the cyosolic tail of the membrane glycoprotein (GP) Ibα via filament in a lattice-like structure. [(4)]

The circumferential band of microtubules present below the plasma membrane probably contributes to the platelet's discoid shape, but it may also be involved in platelet formation from megakaryocytes. On cross-section, approximately 8 to 12 separate coils are observed at the tapered ends of the platelet, but this probably represents a single coil of about 100 μm wound multiple times. Microtubules are 25-nm hollow polymers composed of 13 protofilaments made up of polymers of *M*r 110,000 subunits, each composed of two proteins of *M*r 55,000 (a- and b-tubulin) that associate with several high-molecular-weight proteins (microtubule-associated proteins).Approximately 60 percent of the platelet tubulin is in microtubules, and there is a dynamic equilibrium between the polymerized and free tubulin subunits. Motor proteins of the dynein and kinesin families are also associated with microtubules. *Microfilaments*
The platelet is rich in actin, a protein that can polymerize into microfilamentous bundles. In resting platelets, microfilaments are not prominent, but when platelets change shape, the filopodia they form contain bundles of microfilaments made up of actin and associated proteins. Other proteins that have been found in the membrane skeleton

include talin, vinculin, dystrophin-related protein, molecules implicated in signal transduction, and several isoenzymes of protein kinase C. [5]

1.2.2.3 Organelles:

Peroxisomes: are very small organelles present in platelets. They are thought to contribute to lipid metabolism, especially plasmalogen synthesis, and may participate in the synthesis of platelet-activating factor (PAF).They contain acyl-CoA: dihydroxyacetone phosphate acyltransferase, which catalyzes the first step in the synthesis of ether phospholipids. Deficiencies of this enzymatic activity in platelets have been identified in the cerebro-renal Zellweger syndrome. [5]

Mitochondria: Platelets contain, on average, approximately seven mitochondria of relatively small size that are involved in oxidative energy metabolism. Abnormalities of mitochondrial enzymes, including NADH coenzyme Q reductase (complex I), have been implicated in the pathophysiology of aging and several neurodegenerative disorders including some patients with Parkinson disease. [5]

Coated vesicles: are 70- to 90-nm-diameter platelet organelles, distinguished by their electron-dense bristle coat. The polyhedral surface coat is composed of clathrin, and special staining reveals that the same coat that is in the plasma membranes and Surface-Connected Canalicular System (SCCS) are found on the coated pits and vesicles themselves. Coated pits and vesicles transfer plasma components to platelet granules and the number of coated vesicles in platelets increases after stimulation with ADP. [6]

Glycogen: is found in small particles or in masses of closely associated particles, playing an essential role in platelet metabolism. [6]

1.2.2.4 The granules:

Platelets contain three main types of storage granules: Dense, α-granules, and lysosomes, each of which rapidly release their contents upon

activation. α-Granules are the most numerous, with about 80 per platelet, and contain a rich diversity of proteins and membrane receptors that support many processes in haemostasis and in host defence. Dense granules contain high levels of small molecules that support platelet activation and mediate vasoconstriction. They are present at a 10-fold lower level that for α-granules, with approximately seven per platelet. Lysosomes also release their contents on activation, although the significance of this is unclear. [4]

α-Granules are the storage site of β-thromboglobulin, platelet factor (PF4), platelet-derived growth factor (PDGF), VWF, fibrinogen, factor V, Platelets activation inhibitor (PAI-1), and thrombospondin. Dense bodies contain ADP, ATP, calcium, and serotonin. Dense bodies contain small molecules and ions, such as adenosine diphosphate, adenosine triphosphate, calcium, and serotonin. [7]

Lysosomal granules that contain acid hydrolases, and other enzymes thought to originate from platelet lysosomes are b-glucuronidase, cathepsins, aryl sulfatase, b-hexosaminidase, b-galactosidase, endoglucosidase (heparitinase), b-glycerophosphatase, elastase, and collagenase. When platelets undergo secretion, lysosomal contents are more slowly and incompletely released than are the contents of α granules and dense bodies. [5]

1.2.2.5 Platelet Membranous Systems:

Platelets have features of muscle-related cells in terms of their high content of actin and their contractile response during activation, Similar muscle like qualities are found in the two membranous systems of platelets, the Surface-Connected Canalicular System (SCCS), and the dense tubular system, which resemble transverse tubules and sarcotubules, respectively. The surface-connected canalicular system, also called the open canalicular system, is fenestrated and contiguous

with the surface plasma membrane, weaving through the entire platelet cytoplasm in atortuous fashion. [6]

The glycocalyx (is an exterior coat of side chains protruding beyond the membrane surface and connected to the integral glycoproteins) [1] is less prominent in the SCCS, as is one major surface glycoprotein, the GpIb-IX-V complex, whereas the other major receptor, GPIIb-IIIa, is distributed homogeneously through both the surface and the SCCS. The SCCS has several prominent functional roles: first, as an internal reservoir of membrane to facilitate platelet spreading and filopodia formation after adhesion and second, as a storage reservoir for membrane glycoproteins, such as GPIIb-IIIa, that increase on the platelet surface after activation. The system also provides a route for granule release during the secretory phase of platelet activation and serves as a route of ingress and egress for molecules as they translocate between the plasma and the platelet. Unlike the SCCS, the dense tubular system is a closed-channel system consisting of narrow, membrane-limited tubules, approximately 40 to 60 nm in diameter. Characteristically, it contains amorphous, moderately electron-dense material resembling cytoplasm within its Lumina. It is, in fact, residual of smooth endoplasmic reticulum from the megakaryocyte. Adenylatecyclase, and Ca^{2+}- and Mg^{2+}-activated ATPases have been cytochemically demonstrated in the dense tubular system. This channel system is involved in the regulation of intracellular calcium transport because it has been reported to selectively bind, sequester, and release divalent cations after activation. The dense tubular system is also the site of prostaglandin (PG) synthesis in platelets. [6]

1.2.3 Platelet Function and Kinetics:

Platelets play an important role in both the formation of a primary plug as well as the coagulation cascade. The formation of a plug at the site of a cut vessel serves as the initial mechanical barrier. The lumen of the vessel

is lined with endothelial cells; a break in this will initiate a series of reactions. [2]

They circulate around the body in an inactive state until they come into contact with areas of endothelial damage or activation of the coagulation cascade. Here they adhere to the endothelial defect, change shape, release their granule contents, and stick together to form aggregates. Physiologically these processes help to limit blood loss; however, inappropriate or excessive platelet activation results in an acute obstruction of blood flow, as occurs, for example, in an acute myocardial infarction. However, activated platelets also express and release molecules that stimulate a localized inflammatory response through the activation of leukocytes and endothelial cells, and it is now clear that platelet function is not merely limited to the prevention of blood loss. Indeed, platelets have been implicated in many pathological processes including host defense, inflammatory arthritis, adult respiratory distress syndrome, and tumor growth and metastasis. [1]

The formation of the initial platelet plug can be divided into separate steps, which are very closely interrelated in vivo: platelet adhesion, shape change, the release reaction, and platelet aggregation within seconds after endothelial injury. [7]

1.2.3.1 Platelet adhesion:

The initiating event following vascular damage is platelet adhesion to exposed subendothelial matrix proteins. The platelet glycoprotein (GP) receptors which mediate adhesion are dependent on the rate of shear. Under the intermediate to high shear conditions found in arterioles, this event is strictly dependent on von Wille brand factor (vWF) and its receptor, the GpIb–IX–V complex. However, at the lower rates of shear found in the venous circulation and in the static conditions frequently used for experimental purposes, adhesion can occur directly to other

subendothelial matrix proteins such as collagen and fibrinogen, although vWF also supports this event in these vessels. In both cases, adhesion is strengthened considerably through activation of platelet surface integrins, which leads to an increase in affinity for their adhesive ligands. Adhesion applies also to recruitment of circulating platelets into the thrombus. vWF exposed on the surface of the growing thrombus, also plays a fundamental role in this process, most notably at the high rates of shear that exist within arterioles and in diseased vessels. The platelet-bound vWF that supports these events is derived from plasma and via secretion from platelet α-granules. Adhesion to the growing thrombus is supported by binding of fibrinogen to the integrin $\alpha_{IIb}\beta_3$, a process that is more correctly termed aggregation. [4]

1.2.3.2 Platelet activation:

Platelet activation describes the process that converts the smooth, non adherent platelet into a sticky speculated particle that releases and expresses biologically active substances and acquires the ability to bind the plasma protein fibrinogen. Activation occurs rapidly following exposure to chemical stimuli known as agonists. Activation can also occurs a result of the physical stimulus of high fluid shear stress, such as that found at the site of a critical arterial narrowing. Many agonists are generated, expressed, or released at the sites of endothelial injury or activation of the coagulation cascade. Agonists differ in their ability to induce platelet activation; thrombin, collagen, and thromboxane A2 (TXA2) are all strong agonists and can produce aggregation independent of platelet granule secretion. Adenosine diphosphate (ADP) and serotonin are intermediate agonists and require granule secretion for full irreversible aggregation, where as epinephrine is effective only at supra physiological concentrations. Many agonists are generated, expressed, or released at the endothelial injury or activation of the coagulation cascade.

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1.2.3.3 Release granules content (secretion):

The platelet granule contents are released, which recruit and activate other platelets. Phospholipase A2 is activated, generating arachidonic acid from platelet membrane phospholipids. Arachidonic acid is converted to thromboxane A2 (TxA2) by the enzymes cyclooxygenase and thromboxane synthetase. Thromboxane A2 is a potent vasoconstrictor that stimulates platelet aggregation and causes release of platelet granules. Inhibition of cyclooxygenase (by aspirin and other non steroidal anti-inflammatory drugs) blocks the synthesis of thromboxane A2, thus inhibiting platelet aggregation. Inhibition of cyclooxygenase, with subsequent block of TxA2 production, is how aspirin inhibits platelet activation. Note that both prostacyclin (a potent vasodilator and platelet antagonist) and TxA2 (a potent vasoconstrictor and platelet agonist) are derived from arachidonic acid via cyclooxygenase. Endothelial cells predominantly produce prostacyclin, whereas platelets predominantly produceTxA2. [8]

1.2.3.4 Platelet Aggregation:

Platelet aggregation may occur by at least two independent but closely linked pathways. The first pathway involves arachidonic acid metabolism. Activation of phospholipase enzymes (PLA2) releases free arachidonic acid from membrane phospholipids (phosphatidyl choline). About 50% of free arachidonic acid is converted by a lipo-oxygenase enzyme to a series of products including leucotrienes, which are

important chemoattractants of white cells. The remaining 50% of arachidonic acid is converted by the enzyme cyclooxygenase into labile cyclic endoperoxides, most of which are in turn converted by thromboxane synthetase into TXA2. TXA2 has profound biological effects, causing secondary platelet granule release and local vasoconstriction, as well as further local platelet aggregation via the second pathway below. It exerts these effects by raising intracellular cytoplasmic free calcium concentration and binding to specific granule receptors. TXA2 is very labile with a half-life of <1 min before it is degraded into the inactive thromboxane B2(TXB2) and malonyldialdehyde. The second pathway of activation and aggregation can proceed completely independently from the first one: various platelet agonists, including thrombin, TXA2 and collagen, bind to receptors and via a G-protein mechanism, activate phospholipase C. This generates diacylglycerol and inositol triphosphate, which in turn activate protein kinase C and elevate intracellular calcium, respectively. Calcium is released from the dense tubular system to form complexes with calmodulin; this complex and the free calcium act as coenzymes for the release reaction, for the activation of different regulatory proteins and of actin and myosin and the contractile system and also for the liberation of arachidonic acid from membrane phospholipids and the generation of TXA2. The aggregating platelets join together into loose reversible aggregates, but after the release reaction of the platelet granules, larger, firmer aggregates form. Changes in the platelet membrane configuration now occur; ‘flip-flop’ rearrangement of the surface brings the negatively charged phosphatidyl-serine and -inositol on to the outer leaflet, thus generating platelet factor 3 (procoagulant) activity. At the same time specific receptors for various coagulation factors are exposed on the platelet surface and help coordinate the assembly of the enzymatic

complexes of the coagulation system. Local generation of thrombin will then further activate platelets. Platelets are not activated if in contact with healthy endothelial cells. The 'non-thrombogenicity' of the endothelium is the result of a combination of control mechanisms exerted by the endothelial cell: synthesis of prostacyclin, capacity to bind thrombin and activate the PC system, ability to inactivate vasoactive substances and so on. Prostacyclin released locally binds to specific platelet membrane receptors and then activates the membrane-bound adenylate cyclase (producing cyclic adenosine monophosphate or cAMP). cAMP inhibits platelet aggregation by inhibiting arachidonic acid metabolism and the release of free cytoplasmic calcium ions. [9]

1.2.3.5 Procoagulant activity:

A critical function of platelet activation is to provide a negatively charged phospholipid surface for the assembly of two multiprotein complexes that form a vital part of the coagulation cascade, namely the tenase and prothrombinase complexes as discussed in Chapter 46. A complex of FIXaFVIIIa on the negatively charged lipid surface converts factor X to factor Xa (tenase complex) which, in turn, forms a complex with FVa on the same surface to efficiently convert prothrombin to thrombin (prothrombinase complex). In this way, a large amount of thrombin is generated in the vicinity of the platelet surface to convert fibrinogen to fibrin and to further enhance platelet activation. The newly generated thrombin is also able to diffuse to the surface of intact endothelial cells where it binds to thrombomodulin and activates protein C, which itself is bound to the endothelial surface via endothelial cell protein C receptor (EPCR). Once generated, activated protein C (APC) interacts with phosphatidylserine on the surface of activated platelets to prevent assembly of the tenase and prothrombinase complexes through cleavage of FVa and FVIIIa. Thus, the negatively charged platelet surface also

supports the protein C pathway that serves to limit the coagulation cascade. The 'compartmentalization' of reactions to lipid surfaces in this way ensures that thrombin is generated at the place that it is required during haemostasis. The formation of the negatively charged lipid surface on activated platelets is commonly described as aminophospholipid exposure or procoagulant activity. It is formed by the movement of phosphatidylserine from the inner to the outer leaflet of the platelet membrane. The movement of phosphatidylserine can be monitored experimentally by flow cytometry or fluorescent microscopy through the binding of annexin V, factor V or factor VIII, using either fluorescently labeled secondary antibodies or by direct labeling with a fluorescent group such as fluorescein iso thiocyanate (FITC).The molecular basis of the procoagulant response, including the identity of the enzyme (or 'flipase') that promotes the translocation of phosphatidylserine across the membrane, is not established. It is recognized, however, that the response is elicited only by powerful platelet agonists and that it requires Ca^{++}entry across the plasma membrane. Platelets from four patients have been reported to be unable to undergo a procoagulant response. This clinical condition has been termed Scott syndrome and is associated with significant bleeding, although, if managed, does not appear to have an effect on life expectancy. [(4)]

1.2.4 Quantitative disorders of platelets:

A normal platelet count is 150 to 450 x 10^9/L. In this range, an individual will have properly functioning platelets that assist in the coagulation process by creating a platelet plug and stimulating the formation of a solid fibrin clot. A decrease in platelet count will cause bleeding from the mucous membranes such as gum bleeding (gingival bleeding), nose bleeding (epistaxis), extensive bruising (ecchymoses), or petechiae (pinpoint hemorrhages). A patient with a platelet count of 60,000 will

bleed in surgery and a patient with a platelet count of 30,000 may have petechial bleeding at less than 5000 platelets, there is a risk of bleeding into the central nervous system. Laboratory tests that are helpful in the evaluation of platelet function are the evaluation of the peripheral smear for platelet number and morphology, the bleeding time test (or similar platelet function tests), platelet aggregation by one of several methods, or other methods that assess platelet function and aggregation. Thrombocytopenia or a decreased platelet count is caused by a number of factors. Decreased production of platelets or increased destruction of platelets usually accounts for the pathophysiology of most quantitative defects in platelets. Additionally, sample related conditions or preanalytic variables may lead to falsely decreased. [2]

1.2.5 Thrombocytopenia:

Thrombocytopenia is defined as a platelet count below the reference range for a particular laboratory; <150,000/µL can be used as a rough guide. Thrombocytopenia is a far more common clinical problem than thrombocytosis. [8]

Naturally occurring antibodies that cause *ex - vivo* platelet agglutination (i.e after collection of blood into tubes containing anticoagulant) can lead to a false diagnosis of thrombocytopenia because platelets within "clumps" are not counted by the electronic particle counter. This is why inspection of the blood film is crucial before a laboratory reports a first - occurrence of thrombocytopenia in a patient. Pseudothrombocytopenia is clinically insignificant, except when it leads to inappropriate treatment because of wrongly suspected thrombocytopenia, or makes it difficult to obtain an accurate platelet count in an affected patient who also develops true thrombocytopenia. [10]

Causes of pseudothrombocytopenia include platelet clumping, platelet satellitism around neutrophils, hereditary giant platelet syndromes, a

clotted specimen, or an old specimen. The most common cause of pseudothrombocytopenia is platelet clumping. Platelet Clumping: Approximately 1 in 1,000 people have an antibody that induces platelet clumping in blood samples anticoagulated with ethylene diamine tetra acetic acid (EDTA). The clumped platelets are not counted as platelets by hematology analyzers; therefore, the reported platelet count is falsely low. A blood smear from the same anticoagulated specimen will show large clumps of platelets. An accurate platelet count can usually be obtained by drawing another specimen into a citrate anticoagulant tube. Platelet clumping is a purely in vitro phenomenon that is clinically insignificant. Patients with platelet clumping actually have a normal platelet count (unless there is some other reason for them to have an abnormal platelet count) and have no increased risk of bleeding. [8]

This phenomenon may be detected when it gives rise to a ‘flag’ on an automated blood cell counter; it is identifiable on the blood film. It is not associated with any coagulation disturbance and platelet function is normal. When this abnormality is detected it is important that the erroneous platelet count is deleted from the report. If the blood film shows that the platelet count is clearly normal, the statement ‘Platelet count normal’ is sufficient, rather than a repeat sample in an alternative anticoagulant being requested. Occasionally, EDTA inhibits the staining of platelets. Occasionally, platelets may be seen adhering to neutrophils this has been reported in patients who have demonstrable anti platelet autoantibodies, but it is more commonly seen in apparently healthy individuals. It is not seen in films made directly from blood that has not been anticoagulated. Sometimes the platelets are ingested by neutrophils. If a blood film in a patient with platelet satellitism or phagocytosis shows that the automated count is erroneous and the platelet count is in fact

normal, the erroneous count should be deleted from the report and an explanation given.[9]

1.2.6 Thrombocytopenia Related to Sample Integrity/ Preanalytic Variables:

Coagulation samples are drawn into blue top tubes containing sodium citrate. Sodium citrate anticoagulates a specimen by binding calcium in a 1:9 anticoagulant to blood ratio. Sample tubes must be at least 90% full and the phlebotomy must be non traumatic. The blue top tube must be inverted at least three or four times for proper mixing of the anticoagulant. If this does not happen, there is a possibility of small clots being formed on the top of the tube. Platelet satellitism is another condition related to samples that may give a falsely decreased platelet count. First reported in 1963, this condition is an in vitro phenomenon in which the patient's platelets rosette around segmented neutrophils, monocytes, and bands. This phenomenon occurs only in EDTA (ethylene diamine tetra acetic acid) samples and produces a pseudo-thrombocytopenia unrelated to medication or any other disease state. If platelet satellitism is observed on the peripheral smear, the sample should be redrawn in sodium citrate and cycled through the automated hematology counter for a more accurate platelet count. [2]

1.2.7 Anticoagulants:

This substance prevents blood from clotting. The mechanism by which clotting is prevented varies with anticoagulant. [3]

EDTA and sodium citrate remove calcium which is essential for coagulation. Calcium is either precipitated as insoluble oxalate (crystals of which may be seen in oxalated blood) or bound in a non-ionized form. Heparin binds to antithrombin, thus inhibiting the interaction of several clotting factors. EDTA is used for blood counts; sodium citrate is used for coagulation testing and the erythrocyte sedimentation rate. For better

long-term preservation of red cells for certain tests and for transfusion purposes, citrate is used in combination with dextrose in the form of acid-citrate dextrose (ACD), citrate-phosphate-dextrose (CPD) or Alsever's solution. Anticoagulant mixtures are also used to compensate for disadvantages in each and to meet the needs of the analytic process; these include ACD, CPD or heparin combined with EDTA and EDTA, citrate, or heparin combined with sodium fluoride. Any anticoagulant can be used for collecting blood for flowcytometry.[9]

1.2.7.1 Ethylene diamine tetra-acetic Acid (EDTA):

The sodium and potassium salts of EDTA are powerful anticoagulants and they are especially suitable for routine hematological work. EDTA acts by its chelating effect on the calcium molecules in blood. To achieve this requires a concentration of 1.2 mg of the anhydrous salt per ml of blood (c 4 mmol). The dipotassium salt is very soluble (1650 g/l) and is to be preferred on this account to the disodium salt which is considerably less soluble (108 g/l).Coating the inside surface of the blood collection tube with a thin layer of EDTA improves the speed of its uptake by the blood . The dilithium salt of EDTA is equally effective as an anticoagulant, and its use has the advantage that the same sample of blood can be used for chemical investigation. However, it is less soluble than the dipotassium salt (160 g/l).The tripotassium salt dispensed in liquid form has been recommended in the USA by national committee for clinical laboratory standards (NCCLS).However, blood delivered into this solution will be slightly diluted, and the tripotassium salt produces some shrinkage of red cells which results in a 2–3% decrease in PCV within 4 hours of collection, followed by a gradual increase in mean cell volume (MCV). By contrast, there are negligible changes when the dipotassium salt is used. Accordingly, the International Council for Standardization in Hematology recommends the dipotassium salt at a concentration of 1.50–

2.2 mg/ml of blood; the tripotassium salt may be accepted as an alternative.Na3-EDTA is not recommended because of its high pH. Excess of EDTA, irrespective of which salt, affects both red cells and leucocytes, causing shrinkage and degenerative changes. EDTA in excess of 2 mg/ml of blood may result in a significant decrease in PCV by centrifugation and increase in mean cell hemoglobin concentration (MCHC).The platelets are also affected; excess of EDTA causes them to swell and then disintegrate, causing an artificially high platelet count, as the fragments are large enough to be counted as normal platelets. Care must therefore be taken to ensure that the correct amount of blood is added, and that by repeated inversions of the container the anticoagulant is thoroughly mixed in the blood added to it. Blood films made from EDTA blood may fail to demonstrate basophilic stippling of the red cells in lead poisoning. EDTA has also been shown to cause leuco-agglutination affecting both neutrophils and lymphocytes, and it is responsible for the activity of a naturally occurring antiplatelet auto-antibody which may sometimes cause platelet adherence to neutrophils in blood films. Monocyte activation measured by release of tissue factor and tumour necrosis factor activity has been reported as being lower with EDTA than with citrate and heparin. Similarly, neutrophil activation measured by lipopolysaccharide-induced release of lactoferrin is low with EDTA. EDTA also appears to suppress platelet degranulation. [9]

1.2.7.2 Tri sodium citrate:

For coagulation studies, 9 volumes of blood are added to 1 volume of 109 mmol/l sodium citrate solution (32 g/l of Na3C6H5O7.2H2O).This ratio of anticoagulant to blood is critical as osmotic effects and changes in free calcium ion concentration affect coagulation test results. This ratio of citrate to blood may need to be adjusted for samples with a high haematocrit requiring coagulation studies. For the erythrocyte

sedimentation rate (ESR), four volumes of blood are added to 1 volume of the sodium citrate solution (109mmol/l) and immediately well mixed with it. The mixture is taken up in a Westergren tube.[9]

1.2.8 Platelet indices:

The same techniques that are used to size red cells can be applied to platelets. The mean platelet volume (MPV) is derived from the impedance platelet size distribution curve. The MPV is very dependent on the technique of measurement and on length and conditions of storage prior to testing the blood. When MPV is measured by impedance technology, it has been found to vary inversely with the platelet count in normal subjects. If this curve is extrapolated, it has been found that data fit the extrapolated curve when thrombocytopenia is caused by peripheral platelet destruction; however, the MPV is lower than predicted when thrombocytopenia is caused by megaloblastic anaemia or bone marrow failure. Large platelets are haemostatically more active than smaller platelets and may be more important functionally than smaller platelets. An increase in MPV has been observed in patients at risk of and following myocardial infarction and cerebral infarction. A high MPV can provide important evidence of an inherited macrothrombocytopenia. The MPV is generally greater than predicted in myeloproliferative neoplasms, but differentiating essential thrombocythaemia from reactive thrombocytosis on this basis has not been very successful. Other platelet parameters that can be computed by automated counters include the platelet distribution width (PDW), which is a measure of platelet anisocytosis and the ‘plateletcrit’, which is the product of the MPV and platelet count and, by analogy with the haematocrit, may be seen as indicative of the volume of circulating platelets in a unit volume of blood. The platelet large cell ratio (P-LCR), reported by some instruments is the number of platelets falling above the 12 fl threshold on the platelet size histogram divided by the total number of platelets. A high P-LCR or PDW may indicate peripheral immune destruction of platelets. The PDW has been found to be of some use in distinguishing essential

thrombocythaemia (PDW increased) from reactive thrombocytosis (PDW normal). [9]

1.3Previous studies:

McShine RL, Sibinga S, and Brozovic B said that platelet counts on whole blood samples collected into EDTA showed a statistically significant drop (P less than 0.01) . The decreases in citrate were significant (18-30%, P less than 0.001). The addition of EDTA (1.5 mg/ml) to the citrated samples after the sixth hour count created a significant rise (6-22%, P less than 0.01). their observations show that platelet counts in citrated blood samples are lower than those in EDTA and highlight the necessity to present citrated samples mixed with dried EDTA Analysis of the mean platelet volume (MPV) showed significantly lower values (6-13%, P less than 0.05) in the citrated samples as compared to the same samples in EDTA, and a significant increase (4-6%, P less than 0.01) on the addition of EDTA to the citrated samples after the sixth hour analysis. [11]

M. Żmigrodzka, A. Winnicka, and M. Guzera said that platelet count and platelet haematocrit were significantly lower in citrate blood than in tripotassium ethylene diamine tetra acetic acid (EDTA-K3) blood. The study confirmed the limited usage of sodium citrate in haematology analysis. [12]

1.4 The rational:

Platelet has an important role in haemostatic mechanism, estimation of platelets count or indices should be done properly.

References show that Approximately 1 in 1,000 people have an antibody that induces platelet clumping in blood samples anticoagulated with ethylene diamine tetra acetic acid (EDTA). [8]

Thus using EDTA as anticoagulant for automated blood count will give false result due to pseudothrombocytopenia.TSC anticoagulant has been suggested as substitute to solve this problem. The present study will be useful to distinguish between the two anticoagulants used in the laboratories.

1.5 Objectives:

1.5.1 General objective:

To detect anticoagulants effect on platelet profile among healthy Sudanese people in Khartoum state.

1.5.2 Specific objective:

- To estimate platelet count and platelet indices in healthy Sudanese people.
- To compare platelet count and platelet indices between EDTA anticoagulated samples and TSC anticoagulated samples from same individual.
- To compare platelet count and platelet indices between commercial TSC vacutainer tubes and TSC manually prepared tubes.

Materials and methods

2.1 Study design:

- Cross sectional study.

2.2 Study group:

The study group includes hundred healthy individuals from Khartoum state, blood samples were collected from (November 2013 to Mach 2014) for platelet count, PDW, MPV, and P - LCR.

2.3 Sample:

200 samples of venous blood collected from 100 healthy individuals, 100 samples collected into EDTA blood containers, 50 samples collected into commercial TSC vacuum tubes, and 50 samples collected into TSC manually prepared blood containers.

2.4 Data collection:

Data collected by using questionnaire which include informative data (name, age, gender, residence).

2.5 Materials:

- Cotton.
- Sterile needle.
- 70 % ethyl or methyl alcohol.
- Tourniquet.
- Plastic containers (EDTA and TSC).
- TSC Vacutainer tube.
- Marker.
- Sysmex apparatus.

2.6 Methods:

2.6.1 Sample collection:

The venous puncture site was cleaned by 70% alcohol, and with a sterile disposable syringe, 5ml of blood was collected, then 2.5 ml of blood transferred to EDTA blood container and 2.5ml to TSC blood container.

2.6.2 Analyzing by sysmex:

Particles can be counted and sized either by electrical impeders or by light scattering. Automated instrument have at least two channels. In one channels a diluents is added and red cells are counted and sized. In another cannel a lytic agent is added together with diluents, to reduce red cells to stroma, leaving the white cells intact for counting and also producing a solution in which Hb can be measured. Further channels are required for a differential WBC, which is often dependent on study of cells by a number of modalities, e.g. impedance technology with current of various frequencies, light scattering and light absorbance.

2.6.3 Detection principles:

- Direct current (DC) detection method (WBC, RBC, and PLT), Non-cyanide method (HGB), and cumulative pulse height detection method (HCT).

2.6.4 Procedures:

The reagent needed was checked for availability, validity and completion, the power switch was turned on, self auto rinse, and background check was automatically performed and the vend was appeared, whole blood mode was selected. Sample number inputted by pressing sample number of sample was entered, Enter key was pressed. Sample was mixed sufficiently. The tube was slated to the sample probe, and in that condition the start switch was pressed. When the liquid-crystal display (LCD) screen display analyzing the tube was removed, after that the unit executes automatic analysis and the result was displayed in the LCD screen, the result was printed out. [11]

2.7. Method of preparation and staining of blood films:

2.7.1 Requirements:

- Slides (76 x 26 mm and approximately 1.2 mm thick).
- Spreader (from a glass slide that has smooth end).

- Absolute methanol.
- Romanowsky stains (Leishman's stain).

2.7.2 Procedure:

- Blood films were prepared from the blood with EDTA and TSC anticoagulant.
- A small drop of blood was placed in the center line of slide about 1 cm from one end.
- Without delay a spreader was placed in front of the drop at an angle of about 30 degree to the slide and moves it back to make contact with the drop.
- The drop should soar out quickly along the line of contact. With a steady movement of the hand, the drop of the blood was spread along the slide.
- The films were allowed to dry by natural air and labeled immediately.
- Films were fixed by absolute methanol.
- The film was placed on staining rack, flooded with Leishman's stain, and left for 2 minutes to be fixed and stained.
- Twice buffered distilled water was added.
- Left to stain for 10 minutes.
- Stain was washed off with tap water.
- After that the back of the slide has been wiped clean, set it up right to dry.
- The film was examined macroscopically to assess whether the spreading technique was satisfactory and to judge its staining characteristic and whether there are any abnormal particles present.

Results

Table (3.1) Mean platelets count using EDTA compared to TSC anti coagulant:

Type of anticoagulant	Number of samples	Mean	Std. Deviation	P. value
EDTA	100	272.83	67.6	0.000
TSC	100	190.75	52.2	

Table (3.2) mean platelets distribution width using EDTA compared to TSC anticoagulant:

Type of anticoagulant	Number of samples	Mean	Std. Deviation	P. Value
EDTA	100	12.03	1.7	0.001
TSC	100	11.15	1.7	

Table (3.3) mean platelets volume using EDTA compared to TSC anticoagulant:

Type of anticoagulant	Number of samples	Mean	Std. deviation	P. value
EDTA	100	9.841	.9520	.000
TSC	100	8.996	.9780	

Table (3.4) mean platelets-large cell ratio using EDTA compared to TSC anticoagulant:

Type of anticoagulant	Number of samples	Mean	Std. deviation	P. Value
EDTA	100	23.99	7.0	0.000
TSC	100	19.20	6.5	

Table (3.5) mean platelets count using commercial TSC containers compared to manually prepared TSC containers:

Type of anticoagulant	Number of samples	Mean	Std. deviation	P. Value
TSC (C)	50	195.06	53.1	0.413
TSC (M)	50	186.44	51.5	

Table (3.6) mean platelets distribution width using commercial TSC containers compared to manually prepared TSC containers:

Type of anticoagulant	Number of samples	Mean	Std. Deviation	P. Value
TSC (C)	50	11.44	1.6	0.102
TSC (M)	50	10.86	1.8	

Table (3.7) mean platelets volume using commercial TSC containers compared to manually prepared TSC containers:

Type of anticoagulant	Number of samples	Mean	Std. deviation	P. Value
TSC (C)	50	9.14	0.9	0.131
TSC (M)	50	8.84	0.9	

Table (3.8) mean platelets-large cell ratio using commercial TSC containers compared to manually prepared TSC containers:

Type of anticoagulant	Number of samples	Mean	Std. deviation	P. Value
TSC (C)	50	19.95	6.4	0.258
TSC (M)	50	18.46	6.6	

Discussion, conclusion and recommendations

4.1. Discussion:

This study was done to detect the anticoagulant effect on platelet profile among healthy Sudanese people in Khartoum state. The study group composed of 100 individuals from Khartoum state; they fall within the age range of (17-24) years old. The platelet profile has been estimated by sysmex analyzer. The results were analyzed by statistical package of social science (SPSS) program.

There was significant difference between EDTA platelet count (mean 272.8±SD) and TSC platelet count (mean 190.7±SD) P value is (0.000). Also there was significant difference between EDTA (mean 12.0±SD) And TSC (mean 11.1±SD) in measuring PDW (P value 0.001), significant difference in measuring MPV (P value 0.000) between EDTA (mean 9.8±SD) and TSC (mean 8.9±SD), And significant difference in measuring P-LCR (P value 0.000) between EDTA (mean 23.9±SD) and TSC (mean 19.2±SD).The study reveal that the platelet profile in TSC samples are lower than those in EDTA samples. A similar result has been founded by McShine RL, Sibinga S, and Brozovic B (1990) that, platelet counts in citrated blood samples are lower than those in EDTA and highlights the necessity to present citrated samples mixed with dried EDTA. Also similar result founded by M. Żmigrodzka, A. Winnicka, and M. Guzera (August 2012) that, platelet count and platelet haematocrit were significantly lower in citrate blood than in tripotassium ethylene diamine tetra acetic acid (EDTA-K3) blood.

There was insignificant difference in platelet count (P value 0.413) between commercial TSC tubes (mean 195.0±SD) and manually prepared TSC tubes (mean 186.4±SD). Also there was insignificant difference in PDW (P value 0.102) between commercial TSC tubes (mean11.4±SD)

and manually prepared TSC tubes (mean 10.8±SD), insignificant difference in MPV (P value 0.131) between commercial TSC tubes (mean9.14±SD) and manually prepared TSC tubes (mean 8.84±SD), and insignificant difference in P-LCR (P value 0.258) between commercial TSC tubes (mean19.9±SD) and manually prepared TSC tubes (mean 18.4±SD).The study reveal that there is no difference between using of commercial TSC tubes and using of manually prepared TSC tubes in obtaining platelet profile.

4.2. Conclusion:

The TSC anticoagulant show low results in all platelet profile (platelet count, PDW, MPV, P-LCR) in comparing with EDTA anticoagulant from same individuals.

So TSC anticoagulant cannot be use as substitute to solve the problem of pseudo thrombocytopenia which cause by EDTA.

4.3. Recommendations:

1. Other studies should be done using large samples size.
2. Addition of EDTA to TSC samples for platelet count.
3. Evaluate the using of magnesium salt - anticoagulated blood samples for platelet count as substitute for EDTA.

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Appendix (1): Questionnaire:

Sudan University of Science and Technology

College of Medical Laboratory Science

Department of Hematology

Questionnaire

Name: ……. Number: …….

Gender: …….. Age: ….....

Residence: ……

Time of collection: …… Time of delay: ……

Type of tube: …….

Type of anticoagulant: …….....

Type of sample: …….. Amount of blood: ……

Method of analysis:

Automation (sysmex):

Platelet count: ……..

Platelet indices: …….

……

……

Appendix (2): sysmex Kx21N:

Appendix (3): EDTA blood container:

Appendix (4): TSC vacutainer tube:

Appendix (5): Microscope: