Chapter one

1. Introduction and literature review

1.1 Introduction:

Hemostasis is one of number of protective processes that have evolved in order to maintain a stable physiology. It has many feature in common with (and to some extent interact with) other defiance mechanisms in the body, such as the immune system and the inflammatory response. (Hoffbrand *et al.,* (2005))

The high blood pressure generated on the arterial side of vertebrate circulation requires powerful, almost instantaneous but strictly procoagulant response in order to minimize blood loss from sites of vascular injury compromising blood flow generally. Systemic anticoagulant and clot- dissolving components have also evolved to prevent extension of the procoagulant response beyond the vicinity of vascular injury resulting in unwanted thrombus formation in the slow, sometimes intermittent, blood flow in the veins. The resultant haemostatic system is thus complex mosaic of activating or inhibitory feedback or feed-forward pathways, integrating its five major components (blood vessels, blood platelets, coagulation factors, coagulation inhibitors and fibrinolytic elements). Furthermore, links between haemostasis and other elements of the body's overall defiance response, such as the complement and kinin-generating processes and phagocytosis, must be considered. (Hoffbrand *et al*., (2005))

During normal pregnancy the hemostatic balance change in the direction of hypercoagulability, thus decreasing bleeding complication in connection with delivery. Most previous studies suggest a changes representative of hypercoagulability during pregnancy, increased endogenous thrombin generation, acquired activated protein C resistance, slightly decreased activated partial thromboplastin time (APTT) and increased prothrombin time (PT) measured as

international normalized ratio (INR) of less than 0.9 have been reported as well. (Hellgren , (2003)).

Most blood coagulation factors and fibrinogen increase during pregnancy. Factor (F) XI is the only blood coagulation factor that decreases. Blood coagulation inhibitors are mainly unchanged but the level of free protein S decreases markedly and the level of tissue factor pathway inhibitor increases. Thrombomodulin levels increase during pregnancy. Fibrinolytic capacity is diminished during pregnancy, mainly because of markedly increased levels of plasminogen activator inhibitor-1 (PAI-1) from endothelial cells and plasminogen activator inhibitor-2 (PAI-2) from the placenta. Thrombin-activated fibrinolysis inhibitor is reported to be unaffected. The total hemostatic balance has been studied by analyses of prothrombin fragment 1+2, thrombin-antithrombin complex, fibrinopeptide A, soluble fibrin, D-dimer, and plasmin-antiplasmin complex. There is activation of blood coagulation and a simultaneous increase in fibrinolysis without signs of organ dysfunction during normal pregnancy. These changes increase as pregnancy progresses. During delivery, there is consumption of platelets and blood coagulation factors, including fibrinogen. Fibrinolysis improves and increases fast following childbirth and expulsion of the placenta, resulting in increased D-dimer levels. These changes are self-limiting at normal delivery. The hemostatic changes, noted during pregnancy, normalize after delivery within 4 to 6 weeks. Platelet count and free protein S, however, can be abnormal longer. Hemostasis should not be tested earlier than 3 months following delivery and after terminating lactation to rule out influences of pregnancy. (Hellgren , (2003)).

1.2 literature review:

1.2.1 Overview of haemostasis:

In the most simplistic terms, blood coagulation occurs when the enzyme thrombin is generated and proteolyses soluble plasma fibrinogen, forming the insoluble fibrin polymer, or clot; this provides the physical consolidation of vessel wound repair following injury. Hemostasis refers more widely to the process whereby blood coagulation is initiated and terminated in a tightly regulated fashion, together with the removal (or fibrinolysis) of the clot as part of vascular remodeling; as such, hemostasis describes the global process by which vessel integrity and patency are maintained over the whole organism, for its lifetime. (Hoffbrand *et al*., (2005))

1.2.2 Blood vessels structure:

The basic structure of blood vessels can be broken down into three layers; the intima, the media and the adventatia. It the materials that make up these layers and the size of these layers themselves that differentiate arteries from veins, and indeed one artery or vein from another artery or vein. The intima is the innermost layer and the surface is covered with single layer of ECs, which rest on a basement membrane of subendothelial microfibrils that are composed of collagen fibers and some elastin. The media or middle layer contains mainly circularly arranged smooth muscle cells and collagenous fibrils, and is divided from adventitia by the external elastic lamina. The muscle cells contract and relax, whereas the elastin allows vessels to stretch and recoil. The adventitia or outermost layer is composed of collagen fibers and fibroblasts that protect the blood vessels and anchor it to surrounding structures. . (hoffbrand *et al*., (2005))

1.2.2.1 The endothelium:

The endothelium functions in a multitude of physiological processes including the control of cellular trafficking, the regulation vasomotor tone and maintenance of blood fluidity. ECs possess surface receptors for a variety of physiological substances, for example thrombin and angiotensin II, which me influence vascular tone directly or indirectly through various hemostasis-related events. Once activated, ECs express at their surface, and in some cases release into the plasma, a variety of intracellular adhesion molecules (e.g vascular cell adhesion molecule, E-selectin, P-selectin and vWF), which modulate leucocyte and platelet adhesion, inflammation, phagocytosis and vascular permeability. . (hoffbrand *et al.*, (2005))

However, the endothelium should not be regarded as a simple homogenous cell type. It would appear that ECs phenotypes are differentially regulated; at any given point in time, structural and functional phenotypes may vary between segments of the vascular tree and at any given location, the endothelium phenotypes may change from one moment to the next. ECs heterogeneity occurs between different organs, within the vascular loop of given organ, and even between neighbouring ECs of a single vessel. . (Hoffbrand *et al.*, (2005))

1.2.2.2 Endothelium functions:

Endothelial cell activities affecting platelet- vessels wall interaction: intact ECs exert a powerful inhibitory influence on hemostasis by virtue of the factors that they synthesize and release or express on their surface. Two of these, prostaglandin I_2 and nitric oxide, also known as endothelium- derived relaxing factor (EDRF), have powerful vasodilatory activity, acting on smooth muscles in the vessel wall (basal- directed secretion) and hence modulating blood flow. Both substances inhibit aggregation of platelets and leucocytes (luminal- directed

secretion) by raising intraplatelet levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) respectively. . (Hoffbrand *et al.,* (2005))

Endothelial cell anticoagulant activities: ECs express two cellular receptors, tthrombomodulin (TM) and endothelial protein C receptor (EPCR), which regulate the anticoagulant pathway initiated by thrombin (protein C pathway). TM is constitutively expressed on all ECs with the exception of the brain. EPCR is expressed strongly in the ECs of arteries and veins in heart and lung, less intensely in capillaries in the lung and skin, and not at all in the endothelium of small vessels of the liver and kidney. In addition, ECs synthesize and secrete Protein S, the cofactor for APC inactivation of FVa and FVIIIa. . (Hoffbrand *et al*., (2005))

Endothelium and vessel injury: Disruption of the vessel wall following injury leads to exposure of procoagulant stimuli. Cells of the adventitia or epithelial cells of the surrounding tissues TF constitutively on their surfaces. Formation of a complex between TF and FVII present in flowing from the injured vessels initiates coagulation, resulting in fibrin generation. Exposure of collagen within subendothelial layers of vessel wall leads to immobilization of plasma vWF triggering platelet adhesion, aggregation and activation. . (Hoffbrand *et al*., (2005))

Endothelial cell- derived fibrinolytic factors: Three important fibrinolytic factors are detectable in the vessel wall: tissue plasminogen activator (tPA) and plasminogen activator inhibitor type 1 (PAI-1) are synthesized primarily by ECs, whereas urinary plasminogen activator (uPA,Urokinase) is mainly derived from fibroblast- like cells in kidney and gut. . (Hoffbrand *et al.,* (2005))

1.2.3 Platelets:

Platelets are small, anucleate cells that play a critical role in haemostasis and thrombosis. Platelets ordinarily circulate in the blood stream in quiescent state but undergo 'explosive' activation following damage to the vessel wall, leading to rapid formation of platelets aggregate or vascular plug and occlusion of the site of damage. Platelets are therefore enriched in signaling proteins and surface receptors that enable them to achieve a rapid response, whereas major defects in platelets function or platelets number are associated with excessive loss of blood. . (Hoffbrand *et al*., (2005))

Platelets membrane is the site of interaction with the plasma environment and with damaged vessel wall. It consist of phospholipids cholesterol; glycolipids; and at least nine glycoproteins, named GpI-GpX. The membrane phospholipids are asymmetrically distributed, with sphingomyelin and phosphatidylcholine predominating in the outer leaflet and phosphatidy-ethanolamine, - inositol and serine in the inner leaflet. After platelets activation the membrane also expresses binding site for several coagulation protiens such as factor XI and factor VII. The contractile system of the platelets consist of the dense microtubular system and the circumferential microfilaments, which maintain the disc shape. Actin is the main constituent of the contractile system, but myosin and a regulatory calcium binding protein, calmodulin are also present. (lewis *et al*., (2001))

1.2.4 Platelets function in hemostatic process:

The main steps in platelet functions are adhesion, activation with shape change and aggregation. When the vessel wall is damaged, the subendothelial structure including basement membrane, collagen and microfibrils, are exposed. Surface

bound VWF binds to GpIb on circulating platelets, resulting in an initial monolayer of adhering platelets. Binding via GpIb initiates activation of the platelet through a G- protein mechanism. Once activated platelets immediately change shape from a disc to a tiny sphere with numerous projection pseudopods. After adhesion of a single layer of platelets to the exposed subendothelium, platelets stick to each other and to subendothelium, platelets stick to one another to form aggregates. Fibrinogen, fibronectin, and the glycoprotein Ib-IX and IIbIIIa complexes are essential at this stage to increase the cell to cell contact facilitate aggregation. Certain substance (agonists) react with specific platelets membrane receptors to promote platelet aggregation and further activation. The agonists include expsosed collagen fibers, ADP, thrombin, adrenaline, serotonin, and certain arachidonic acid metabolites including TXA_2 . In areas of nonlinear blood flow, such as may occur at the site of any injury, locally damaged red cells release ADP, which further activation platelets.((lewis *et al*., (2001)).

Normal value: The normal value of platelets in adults is 150-400 $\times 10^9$ cell/µ

1.2.5 Coagulation factors:

Almost certainly, all the major soluble protein components of the thrombin-generating pathways, the protein C pathway, the fibrinolytic pathway and their respective inhibitors have now been identified. In addition a number of cell surface receptors relevant to these pathways have also been identified. . (hoffbrand *et al.,* (2005))

1.2.5.1Classification of coagulation factors:

Contact activation system: this system comprises of factor XII, HMWK, prekallikrein/ kalellikrein. They have ability to activate fibrinolytic system and generate vasoactive peptide: in particular bradykinin is released from HMWK by prekallikrein or $FXII_a$ cleavage. Kallikrein and $FXII_a$ function as chemoattractants

for neutrophils. When bound to negatively charged surface in vitro FXII and prekallikrein are able to reciprocally activate one another by limited proteolysis but the initiating event is not clear. Possibly conformational change in factor XII on binding results in limited auto-activation which triggers the process. The contact activation system can activate fibrinolysis by a number of mechanisms; plasminogen cleavage, uPA activation and tissue plasminogen activator release. Most importantly from the laboratory point of view, the contact activation system results in the generation of factor XII_a which is able to activate factor XI, thus initiating the coagulation cascade of the intrinsic pathway. ((lewis *et al*.,(2001)).

Tissue factor**:** tissue factor (TF) is the co factor for the extrinsic pathway and the physiological initiator of coagulation. It is a lipoprotein which is member bound and constitutively present in many tissues outside the vasculature and on the surface of factor VII_a in the presence of calcium ions and this complex is then capable of activating factor X to factor X_a. Small amounts of factor VII_a are present in the circulation but have virtually no enzymic activity unless bound to TF. The factor VII_a-TF complex can activate both factor X and factor IX and therefore two routes to thrombin production are estimated. Factor X_a subsequently binds to tissue factor pathway inhibitor (TFPI) and then to factor VII_a to form quaternary (X_a-TF-VII_a-TFPI) complex. This mechanism therefore functions to shut off the extrinsic pathway after an initial stimulus to coagulation has been provided. ((lewis *et al*., 2001).

The vitamin K-dependent factors: this group comprises coagulation factors II, VII,IX,and X. however, it is important to remember that the anticoagulant protein S,C and Z are also vitamin K dependent. Each of these proteins contains a number of glutamic acid residues at its amino terminus that are γ- carboxylated by a vitamin K-dependent mechanism. This results in a novel amino acid, γ-carboxyglutamic acid, which appears to be important in promoting a

conformational change in the protein which promotes binding of the factor to phospholipid. Because this binding is crucial for coordinating the interaction of the various factors, the proteins produced in the absence of vitamin K (PIVKAs) which are not γ-carboxylated are essentially functionless. The vitamin K- dependent factors are pro enzymes or zymogens which require cleavage sometimes with release of a small peptide (activation peptide) in order to become functional. Measurement of these activation peptides has become a useful test for measuring coagulation activation. ((lewis *et al*., 2001).

Labile factors: factor VIII and V are the two most labile of the coagulation factors and they are rapidly lost from stored blood or heated plasma. They share considerable structural homology and are co-factors for the serine proteases factor IX and factor X respectively, and both require proteolytic activation by factor II_a X_a in order to function. Factor VIII circulates in combination with vWF which is present in the form of large multimers of a basic 200 KD monomer. vWF serves to stabilize factor VIII and protect it from degradation. In the absence of vWF, the survival of factor VIII in the circulation is extremely short. ((lewis *et. al.,* (2001)).

Fibrinogen: fibrinogen is large dimeric protein, each half consisting of three polypeptides named Aα,Bβ and γ held together by 12 disulphide bonds. It is widly recognized as a mediator of plt aggregation and is vital for fibrin formation. It activates plt by binding to plt integrin $\alpha_{IIb}\beta_{III}$. Fibrinogen is precursor of the insoluble fibrin polymer that is formed following proteolytic cleavage by thrombin. ((lewis *et al.,* 2001).

Factor XIII: the initial fibrin clot is held together by non-covalent interactions and can be deformed and resolubilized. Factor XIII which is also activated by thrombin is able to covalently cross-link these fibrin monomers. Factor XIII is a transglutaminase which joins a glutamine residue on one chain to a lysine on an

adjacent chain. This loss of resolubility is the basis of the screening test for factor XIII deficiency. ((lewis *et al.,* 2001).

1.2.6 The coagulation cascade:

Blood coagulation involve biological amplification system in which relatively few initiation substances sequentially activated by proteolysis a cascade of circulating precursor proteins (the coagulation factor enzymes) which culminates in the generation of thrombin. This in turn, converts soluble plasma fibrinogen into fibrin. Fibrin enmeshes platelets aggregates at the site of vascular injury and converts unstable primary platelets plugs to firm, definitive and stable hemostatic plugs. (Christopher, *et al.,* 1999).

Surface- mediated reactions occur on exposed collagen, platelet phospholipids and tissue factor and tissue factor with the exception of fibrinogen, which is the fibrin clot submit, the coagulation factors are either enzyme precursors or cofactors. All the enzymes except factor XIII, are serine protease (i.e their ability to hydrolyze peptide bonds depends upon the amino acid serine at their active center. (Christopher, *et al.,*1999).

1.2.6.1 Initiation:

Coagulation is initiated by the interaction of the membrane bound TF exposed by vascular Injury, with plasma factor VIIa. One to two percent of the total factor VII circulates in the activated form, but does not express any proteolytic activity unless bound to TF. The factor VII_a-tissue factor (extrinsic factor Xase) complex activates both factor IX and factor X. The factor X_a, in the absence of cofactor, it forms small amounts of thrombin from prothrombin. Although insufficient to initiate significant fibrin polymerization, thrombin formed in this initiation stage of coagulation is able to back -activate FV and FVIII by limited proteolysis, in the

amplification phase of coagulation factor $VIII_a$ forms complex with factor IX_a ,The Xase complex ($FVIII_a$-FXI_a) activate sufficient FX_a that in complex with FV_a forms the 'prothrombinase' complex (FV_a-FX_a), resulting in the explosive generation of thrombin that ultimately leads to generation of fibrin clot. (Christopher, *et al*.,1999).

1.2.6.2 Amplification:

The initiation pathway or extrinsic Xase is rapidly inactivated by TFPI which forms quaternary complex of VII_a,TF,X_a and TFPI. The thrombin generation is now dependent on the traditional intrinsic pathway which has been primed by the small amount of thrombin generated during initiation. In the amplification phase the intrinsic Xase formed by IX_a and $VIII_a$ on phospholipid surface, in the presence of Ca^{+2} activate sufficient X_a which then in combination with V_a, phospholipid and Ca^{+2} forms the prothrombinase complex and results in the explosive generation of thrombin which acts on fibrinogen to form the fibrin clot. (Christopher, *et al*.,1999).

In the classic pathway, initiation of pathway required contact reactions between factor XIII, kallikrein and HMWK leading to the activation of factor XI. However the lack of abnormal bleeding in individuals with hereditary deficiency of these contact factors suggests that these reactions are not required for physiological coagulation in vivo. (Christopher, *et al., (*1999).

FXI does not seem to have a role in physiological initiation of coagulation.it has supplementary role in in the activation of factor IX and may be important to major sites of trauma or at operations and potentially causes excess bleeding in factor XI deficient individual. (Christopher, *et al*.,1999).

Thrombin hydrolyses fibrinogen releasing fibrinopeptides A and B to form fibrin monomers. Fibrin monomers link spontaneously by hydrogen bonds to form loose unstable fibrin polymer. Factor XIII is also activated by thrombin together with

Ca^{2+}. Activated factor XIII stabilizes the fibrin polymers with the formation of covalent bond cross link. (Christopher, *et al*.,1999).

1.2.6.3 Coagulation pathways linked the extrinsic, intrinsic and common pathways:

The interaction and intra of the three coagulation pathways was not realized in older theories. Some of these interactions have only been demonstrated in vitro and remains to be confirmed in vivo. The more recently identified pathways are called alternative pathways (cross over) ,The activation of intrinsic pathway by intrinsic pathway (TF:VII_a) complex activate factor IX (may explain lack of bleeding associated with deficiencies of contact factors(XII, HMWK, prekallikrin) and some cases of factor XI deficiency. (Christopher, *et al*.,1999).

1.2.7 Fibrinolysis:

It's widely acknowledged that the principal functions of the fibrinolytic system are to ensure that fibrin deposition in excess of that required to prevent blood loss from damaged vessels is either prevented or rapidly removed (i.e that a localized procoagulant response is achieved without compromising blood circulation generally) and, following re-establishment of haemostasis, an existing fibrin mesh is later removed as part of the process of tissue remodeling. The system of pro- and anti- fibrinolytic factors that has evolved to meet these requirements is closely coupled to that which results in fibrin clot formation. (Hoffbrand, *et al*.,2005).

1.2.7.1Components of fibrinolytic system:

These include plasminogen (PLG) and plasmin, several endogenous (tissue or plasma –derived) or exogenous (e.g bacterial or venom derived) PLG activators, and a number of inhibitors of plasmin or of the PLG activators. (Hoffbrand, *et al*.,2005).

1.2.7.1.1Plasminogen and plasmin:

PLG is a single-chain glycoprotein zymogen of the serine protease plasmin, which carries out the enzymatic degradation of cross-linked fibrin. Plasmin can hydrolyse a variety of substrate including factors V and VIII, but its physiological targets are fibrin and fibrinogen, which are split progressively into a heterogeneous mixture of small soluble peptides known collectively as fibrin degradation products (FDPs). (Hoffbrand, *et al*.,2005).

1.2.7.1.2Plasminogen activators:

Tissue plasminogen activator: tissue plasminogen activator (tPA) is a serine protease secoted by ECs. It is not synthesized by the liver or kidney but is found in most extravascular body fluids, including saliva, milk, bile, cerebrospinal fluid and urine. Intravascular tPA is quickly cleared by the liver or inactivated by the fast-acting tPA inhibitor, the half -life of tPA in plasma being approximately 2min. The resting level of tPA in plasma is around 70 pmol/L, most of which is in an inactive complex with tPA inhibitors. (Hoffbrand, *et al*.,2005).

Urinary plasminogen activator: so called because it was first extracted from urine, urinary plasminogen activator (uPA) is synthesized chiefly by the tubules and collecting ducts in the kidney and by fibroblast-like cells in the gastrointestinal tract. It is a serine protease secreted as an inactive single-chain zymogen (pro-urokinase) that is cleaved by activators in plasma (including kallikrein and plasmin) to produce active, two-chain uPA. (Hoffbrand, *et al*.,2005).

Exogenous plasminogen activator: these are derived from non-human sources, including animals (e.g vampire bat saliva and some snake venoms) and certain plants and micro-organisms. The best known of these is streptokinase (SK), which

is derived from some strains of β-hemolytic streptococci and which has for many years been used, with moderate success, as fibrinolytic agent for the treatment of life-threatening thrombotic states.SK is a non- enzymatic polypeptide, which forms a stable 1:1 complex with PLG as result of which the latter undergoes conformational change, unmasking its serine active center. (Hoffbrand, *et al.*,2005).

1.2.7.1.3 Inhibitors of fibrinolysis:

The plasmin-generating potential of plasma is sufficient to degrade completely all of the fibrinogen in the body in a very short period of time. It is prevented from doing so by the PLG activator inhibitors or PAIs, most of which belong to the serpin family, and by a number of circulating inhibitors of plasmin itself. . (Hoffbrand, *et al*.,2005).

Inhibitors of plasminogen activation: This group include plasminogen activator inhibitor type1 (PAI-1) which is an important, fast-acting serpin inhibitor of tPA, uPA, and to small extent plasmin, which is secreted by ECs. It is also found in platelet α-granules. In plasma it occurs in forms: a functionally active 'free' form (that is stabilized by association with vitronectin) and as an inactive complex with tPA. Basal PAI-1 concentration in plasma is low at 0.5nmol/L, of which at least 80% is in complex with tPA or uPA. It follows a diurnal rhythm, with an early morning peak that is around twice that in afternoon, and its activity is also increased by heparin. There is growing evidence that elevated levels of PAI-1 are associated with increased incidence of venous and arterial thrombosis, and there is suggested association between the early morning peak level of PAI-1 and higher incidence of myocardial infarction at that time, the extent of this diurnal variation being associated with polymorphism in the PAI-1 gene. Another type of plasminogen activator inhibitor is plasminogen activator inhibitor type-2, which is mainly produced by the placenta and may thus contribute to the inhibition of

fibrinolysis which occurs during pregnancy. It is also synthesized in monocytes and epidermal cells, but is not usually found in the plasma of non-pregnant subjects. It is detectable in plasma from about the eight week of pregnancy, rising to peak at around 33 weeks and falling only slowly after delivery, the half -life being around 24h. Paradoxically, levels are often low in pre-eclampsia due to placental insufficiency. (Hoffbrand, *et al*.,2005).

Inhibitors of plasmin: as they do with thrombin and tPA, a number of the broad-spectrum inhibitors contribute to neutralization of plasmin.by the far the most plasmin inhibitor is the serpin α_2-antiplasmin (α_2-AP), single –chain glycoprotein synthesized by the liver, which has half –life about 60h and shows considerable sequence identity with antithrombin and α_1 antitrypsin. Physiological importance is supported by the fact that congenital deficiency is associated with a clinically significant bleeding disorder due to uncontrolled fibrinolytic activity, and that levels are reduced in disseminated intravascular coagulation (DIC) and during thrombolytic therapy. In addition to inactivating preformed plasmin, α_2-AP retards fibrinolysis by reducing PLG activation and by 'masking' the lysine binding sites through which plasmin (ogen) interacts with fibrin. (Hoffbrand, *et al.*,2005).

1.2.8 Coagulation inhibitors:

Soluble plasma proteins act as natural anticoagulants. They prevent the initiation of the clotting cascade. There are two major inhibitors in plasma that keep the activation of coagulation under control.

These inhibitors are:

1. Protease inhibitors: inhibitors of coagulation factors, which include

- Antithrombin
- Heparin cofactor II
- Tissue factor pathway inhibitor

- Alpha-2-antiplasmin
- C1

2. The protein C pathway: inactivation of activated cofactors, which includes

- Protein C and protein S.(Thornton,P.*et al* 2010)).

1.2.8.1 Antithrombin:

AT is a plasma protein made in the liver. AT neutralizes the activities of thrombin (IIa), IXa, Xa, XIa, and XIIa). The inhibitory action of AT against clotting factors is slow; however, its activity is markedly increased when AT binds to heparin.(Thornton,P.*et al* 2010)).

1.2.8.2 Heparin Cofactor II:

Heparin cofactor II is another coagulation inhibitor. It acts against thrombin, and it is heparin dependent. Heparin cofactor deficiency alone is not associated with thrombosis. (Thornton,P.*et al* 2010)).

1.2.8.3 Protein C and Protein S:

Protein C is a vitamin K–dependent protein made in the liver. Protein C circulates in the form of zymogen. Protein C should be activated to a serine protease (activated protein C) in order to exert its inhibitory effects against the clotting factors. Protein C is activated by the action of thrombin-thrombomodulin complex and protein S as cofactor**.** Protein S is a vitamin K–dependent protein made in the liver that is necessary for activation of protein C.

Once protein C is activated, it will deactivate cofactors Va and VIIIa. Deficiencies of proteins C and S are associated with thrombosis.(Thornton.*et al* 2010)).

1.2.9 Physiology of pregnancy:

Pregnancy is associated with profound anatomical, physiological, biochemical and endocrine changes that affect multiple organs and systems. These changes are essential to help the woman to adapt to the pregnant state and to aid fetal growth

and survival. However, such anatomical and physiological changes may cause confusion during clinical examination of a pregnant woman. Similarly, changes in blood biochemistry during pregnancy may create difficulties in interpretation of results. Conversely, clinicians also need to recognize pathological deviations in these normal anatomical and physiological changes during pregnancy to institute appropriate action to improve maternal and fetal outcome. (Chandraharan, *et al.,*2012).

1.2.10 hematological changes during pregnancy:

Cardiovascular and haematological changes begin as early as 4 weeks'gestation and are progressive. During pregnancy the plasma volume increases by 45%. This increase is mediated by a direct action of progesterone and oestrogen on the kidney causing the release of renin and thus an activation of the aldosterone renin-angiotensin mechanism. This leads to renal sodium retention and an increase in total body water. (Heidemann, *et al*., (2003).

Through an increase in renal erythropoietin production, red cell mass increases by 20%. As the increase in red cell mass is relatively smaller than that of plasma volume, the haemoglobin falls from 150 g litre–1 pre-pregnancy to 120g litre–1 during the third trimester (Fig. 1). This is termed the physiological anaemia of pregnancy. At two weeks' postpartum, the blood volume has returned to pre-pregnancy levels. The increased circulating volume offers protection for mother and fetus from the effects of hemorrhage at delivery but it can delay the onset of the classical signs and symptoms of hypovolemia. The white cell count rises throughout pregnancy and peaks after delivery, making diagnosis of infection more difficult. (Heidemann, *et al*., (2003).

1.2.11 Hemostatic change during pregnancy:

Pregnancy is associated with changes in haemostasis, including an increase in the majority of clotting factors, a decrease in the quantity of natural anticoagulants and a reduction in fibrinolytic activity. These changes result in a state of

hypercoagulability are likely due to hormonal changes and increase the risk of thromboembolism. The increase in clotting activity is greatest at the time of delivery with placental expulsion, releasing thromboplastic substances. These substances stimulate clot formation to stop maternal blood loss. As placental blood flow is up to 700ml/min, considerable hemorrhage can occur if clotting fails. Coagulation and fibrinolysis generally return to pre-pregnant levels 3–4weeks postpartum. (Thornton.*et al*.,(2010).

Platelets platelet count decreases in normal pregnancy possibly due to increased destruction and hem-modilution with a maximal decrease in the third trimester. (Thornton,P.*et al* 2010)).

Coagulation factor Factors VIII (FVIII), vonWillebrand factor (vWf), ristocetin cofactor (RCoA) and factors X (FX) and XII (FXII) increase during pregnancy. Levels of factor VII (FVII) increase gradually during pregnancy and reach very high levels (upto1000%) by term. Fibrinogen also increases during pregnancy with levels at term 200% above pre-pregnant levels. (Thornton,*et al*.,2010).

Other factors either remain at non- pregnant levels or decrease during pregnancy. Factor XIII (FXIII), which is responsible for stabilizing fibrin, increases in the first trimester but by term it is 50% of non- pregnant levels. Factor V (FV) concentrations increase in early pregnancy then decrease and stabilize. Factor II (FII, prothrombin) levels may increase or not change in early pregnancy but are normal by term. There is debate about factorXI (FXI) levels with reports indicating increases or decreases. Similarly, FIX levels are reported as increasing, decreasing or remaining stable throughout pregnancy. In one study, 50% of carriers of FIX deficiency had FIX levels _50 IU/dl at term. Protein C levels remain the same or are slightly increased during pregnancy while protein S decreases. AT levels remain normal during pregnancy. (Thornton, *et al* 2010)).

Fibrinolysis: It reduced in pregnancy due to decrease in t-PA activity, which remains low until1-h postpartum when activity returns to normal. This reduction is

due to the gradual, eventually three fold, increase in plasminogen activator inhibitor-1(PAI-1) and the increasing levels of plasminogen activator inhibitor-2(PAI-2). The placenta produces PAI-1and is the primary source of PAI-2.PAI-2levels at term are 25 times that of normal plasma. Postpartum, t-PA levels quickly return to normal as PA-1 levels decrease; however, PA-2 levels remain elevated for a few days. Thrombin-activatable fibrinolysis inhibitor (TAFI) (an antifibrinolytic which cleaves the C-terminal lysine in fibrin to render it resistant to cleavage by plasmin) levels are increased in the third trimester.14 D-Dimer levels increase in pregnancy, but are not thought to indicate intravascular coagulation as fibrinolysis is depressed. These D-Dimers may originate from the uterus. (Thornton, *et al* 2010)).

1.2.12 Disorders that affect coagulation:

1.2.12.1Thrombocytopenia:

Thrombocytopenia affects 6–10% of all pregnancies. 27 A decrease in platelet count is normal in pregnancy although most platelet counts remain within normal limits ($\geq 150\times10^9$). A lower than physiological platelet count may occur in pregnancy for many reasons, ranging from the relatively benign, gestational thrombocytopenia to more sinister conditions, such as HELLP syndrome. Some pre-existing conditions that may cause thrombocytopenia at term include: type 2b von Willebrand disease (vWD), idiopathic thrombocytopenic purpura (ITP), lupus erythematosus and bone marrow disease. Pregnancy-related causes of thrombocytopenia include gestational thrombo-cytopenia, pre-eclampsia including HELLP syndrome, acute fatty liver of pregnancy, DIC and thrombocytopenic purpura. Sever sepsis, some medications (e.g. SH) and viral infections may coincide with pregnancy producing thrombocytopaenia. (Thornton, *et al* 2010).

Gestational thrombocytopenia: is a benign condition that occurs during the third trimester with a platelet count that is generally $\geq 90\times10^9$, but may be as low as 70×10^9. In one study, the incidence of thrombocytopenia was 7.3%, of which 81%

were gestational thrombocytopenia. The diagnosis is made by exclusion of other disorders. Parturients with gestational thrombocytopenia are asymptomatic, have anormal platelet count in early pregnancy with no history of previous thrombocytopenia and no evidence of pre-eclampsia. These patients are not at increased risk of haemorrhage, and there is no contraindication to neuraxial anaesthesia. (Thornton, *et al* 2010)).

1.2.12.2 Essential thrombocythemia:

In pregnancy the commonest complication of ET is first trimester miscarriage which occurs in up to 30% of pregnancies. This is thought to be secondary to micro infarcts and placental insufficiency. The reported increased incidence of antiphospholipid antibodies in ET may contribute to this. Other less frequent complication includes intrauterine death, growth retardation, premature delivery and pre-eclampsia. The risk of maternal thrombosis and hemorrhage is higher than in normal pregnancy, nonetheless a successful outcome (live birth) is achieved in around 60% of cases and no maternal deaths were seen in a recent review of 220 pregnancies. (Hoffbrand, *et al*.,2005).

1.2.12.3 Eclampsia and HELLP syndrome:

The spectrum of hypertensive disorders of pregnancy ranging from pre eclamsia to pre eclamsia and HELLP syndrome to eclamsia may also result in thrombocytopenia, although clotting is more of an issue than is bleeding. There is some debate in the literature as to whether thrombocytopenia can be diagnosed in pre eclamsia without HELLP syndrome; however, data from one large study demonstrate that approximately 15% of cases are complicated by thrombocytopenia.In general the symptoms of pre eclamsia, including hematologic manifestation resolve with delivery, however in small proportion of cases they persist, worsen, even develop immediately postpartum. When symptoms persist postpartum, the differentiation from TTP-HUS becomes more difficult. Some data suggests that maternal recovery from the HELLP syndrome is accelerated by

administration of intravenous dexamethasone; however a recent meta-analysis demonstrated no clear advantage to the use of glucocorticoids in terms of maternal perinatal morbidity or mortality. Observation or treatment of HELLP with steroid alone postpartum should probably not persist beyond the third postpartum day. If the patient is not clearly improving plasma exchange should be initiated as one would do for TTP. (Willims, *et al.,* 2007).

1.2.12.4 Thromboembolic events:

Risk factors: Estimates place the risk of venous thromboembolism (VTE) in pregnant women at two to six times that of non-pregnant women. Factors specific to pregnancy that increase the risk of VTE include; obstruction of venous return by the gravid uterus, acquired prothrombotic changes in hemostatic proteins, and venous atonia caused by hormonal factors. Additional risk factors cesarean section (Especially emergency), obesity and increasing age. Approximately 80% of deep vein thrombosis (DVT) in pregnancy occurs in the iliofemoral veins on the left, probably as a consequence of compression of the left iliac vein by the right iliac and ovarian arteries. Rates of VTE immediately postpartum are difficult to assess as many occur after the patient is discharged; however, some studies suggest that postpartum rates may be even higher than antepartum rates. Inherited thrombophilia plays a role in VTE in pregnancy. The highest rates occur with inherited antithrombin deficiency where it has been estimated that in the absence anticoagulation, 32to44 percent of patients will experience thromboembolism. In a large retrospective study of more than 70,000 pregnancies, the risk of VTE in pregnancy was estimated at about 1 in 437 for carriers of factor V leiden,1 in 113 for protein C deficiency, and 1in 2.8 for type I antithrombin deficiency. Based on result from other studies, the risk for carriers of protein S deficiency appears to be similar to that for protein C deficiency, and risk for carriers of the prothrombin 20210A gene mutation is lower than that of factor V leiden carriers. (Macklon., McColl *et al.,*1997).

1.3 Rationale:

Pregnancy induces hypercoagulable state due to the physiological changes of pregnancy. The number of cases complicated by different types of thrombosis is increased so thromboembolic complication remains a common source of morbidity and mortality associated with pregnancy. This study was done to contribute to present data this topic and to raise the knowledge about the importance of measuring coagulation profile during pregnancy regularly to avoid risk of venous thromboembolism.

1.4 objectives:

1.4.1 General objectives:

To measure some of the coagulation profile among Sudanese pregnant women in khartoum State.

1.4.2 Specific objectives:

1. To determine PT, INR, APTT and PLT count in pregnant women.
2. To compare coagulation profile between pregnant and non-pregnant women
3. To compare coagulation profile according to gestational age.
4. To compare coagulation profile according to pregnant women age.

Chapter two

Material and methods

2.1 Study Design:

This is an analytical case control study which was conducted to explain the effect of normal pregnancy on coagulation system in the period from October 2014 to January 2015.

2.2 Study area:

Study has been conducted in Al-exeer Specialized Medical Center in Khartoum State- east Nile locality.

2.3 study population and sample size:

Study group was (100) women with normal pregnancy as case and (50) non-pregnant women as control group.

2.4 Inclusion criteria:

Pregnant women with normal pregnancy, with no history of medical condition or under treatment that affect the study were included.

2.5 Exclusion criteria:

Pregnant women with complicated pregnancy or drug in take which may affect results.

2.6 Data collection:

Presented data were collected using designed questionnaire and interview to obtain gestational age, family history of coagulation problem and drugs history.

2.7 Sample collection:

Non-probability sampling method was used (only those who accepted study tests were involved in sample.5 ml of venous blood were collected in two containers (EDTA container at concentration 1.5+0.25mg /ml and trisodium

citrate container at concentration 1 to 9. Blood in trisodium citrate container was centrifuged at 2500g for 15 minutes to obtain platelets poor plasma.

2.8 Ethical considerations:

The consent of the selected individuals to the study was taken after being informed with all detailed objectives of the study and its health emphasis in the future.

2.9 Methods:

2.9.1 Platelets count:

Plts count was measured using sysmex KX -300

Principle: The collected blood was diluted and counted by passing the blood through an electronic counter. The instrument was set to count only particles within the proper size range for platelets. The upper and lower levels of the size range were called size exclusion limits. Any objects in the proper size range were counted, however, even if they were not platelets. (Lewis, *et.,al* (2001)).

Reagent and equipment:

1. EDTA blood.
2. Sysmex.
3. Cotton, syringes and alcohol 70%.
4. Mixture.

Procedure: Absence of clot was ensured and sample were put in mixture for 1-2 minutes then introduced to Sysmex device.

2.9.2 Prothrombin time and INR:

PT were analyzed using Coagulometer MC-1

Principle: The test measures the clotting time of plasma in the presence of an optimal concentration of tissue extract (thromboplastin) and indicates the overall efficiency of the extrinsic clotting system. (Lewis, *et.,al*,.2001)).

Reagents and equipment:

1. Patient and control plasma samples.
2. PT reagent is a liquid ready to use rabbit brain thromboplastin, calcium chloride, buffer and 0.05% sodium azide as preservative.
3. Coagulometer.
4. Cuvette.
5. Magnetic ball.
6. Syringe, cotton and 70% alcohol.
7. Centrifuge

Procedure:

1. PT reagent was pre incubated at 37ºC for at least 10 minutes. Suspension of the reagent was maintained by mixing to homogenize the content prior to use. Then magnetic ball was placed on the cuvette followed by 100µl of test/control plasma was pipetted into test cuvette, then incubated at 37ºC for one minute.
2. Rapidly 200µl of the pre-incubated PT reagent were added simultaneously starting the timer.
3. The clotting time in seconds was recorded.
4. The test was duplicated and an average of two results was recorded as prothrombin time

Normal values: Normal range of PT is between 11-16 seconds.

International normalized ratio: The international Committee for Standardization in hematology and the international Committee on thrombosis and hemostasis have agreed on recommendations for the reporting of prothrombin time results as an international normalized ratio (INR). The INR is based on the international sensitivity index (ISI) of thromboplastin reagents. (WHO Expert Committee on Biological standardization (1983)).

The INR is calculated using following formula:

INR= (patient PT/ control PT) ^{ISI}

ISI= lot specific international sensitivity index for the reagent /Instrument system.

2.9.3 Activated partial thromboplastin time:

APTT was analyzed using Coagulometer MC-1

Principle: The capacity of blood to form a fibrin clot by way of the intrinsic hemostatic pathway requires coagulation factors I, II, V, VIII, IX, X, XI and XII, platelets lipids and calcium. The assay is performed by the addition of suspension of rabbit brain cephalin with surface activator. (Owen., *et.,al*., (1975))

Reagents and equipment:

1. Patient and control plasma samples.
2. APPT reagent is a preparation of rabbit brain cephalin and ellagic acid activator with buffer, stabilizer and preservatives.
3. Calcium chloride.
4. Coagulometer.
5. Cuvette.
6. Magnetic ball.
7. Syringe, cotton and 70% alcohol.
8. Centrifuge.

Procedure:

1. Calcium chloride (0.02M) was pre incubated at 37ºC for at least 10 minutes.
2. Magnetic ball in test cuvette was placed.
3. 100µl of test or control plasma was pipetted into a test cuvette. Incubate at 37ºC for 1to 2 minutes.
4. 100µl of the APPT reagent was added to the cuvette containing the plasma.

5. The mixture was incubated at 37ºC for 3 minutes.
6. Rapidly 100µlof the pre-incubated calcium chloride (0.02M) was added and simultaneously start the timer.
7. Clotting time was recorded in seconds.
8. Test was duplicated and the average was obtained as APPTT.

Normal value: Normal range for activated partial thromboplastin time is 20-40 seconds.

Chapter three

3. Results

One hundred samples were collected from pregnant women as case and fifty samples from non-pregnant women as control. Pregnant women were divided into three groups according to the age group (less than 25 years) were 28, (26-30) year age group were 39 from pregnant women and (more than 30) year were 33 table (3-1). According gestational age of pregnancy, pregnant women were divided into three groups 31 of were in the first trimester, 42 in second trimester and 27 of pregnant were in third trimester table (3-2). Mean of coagulation parameters among the study population was as follow: the mean of APTT (31.11 /sec ±13.6), PT (14.49/sec±1.12), INR (1.17±0.12) and platelets count 260 X 10^9cell/µl±69 table (3-3).

Coagulation parameters were compared between pregnant and non-pregnant women and results in case group reveal mean of (PT 14.21/sec±1.13), (INR 1.13±0.11), (APTT 30.67/sec±3.41) and platelet count (227 X 10^9cell/µl±44.60) while in control group results as follow mean of PT (15.04/sec ±0.88), INR (1.23±0.11) APTT (31.98/sec±3.87) and plt count (237 X 10^9cell/µl ±61.25). There is significant difference in PT, APTT and plt count with p.value (0.025),(0.01),(0.00) respectively and no significant difference in INR with p.value (0.949) (Table 3-4) .

coagulation parameters results were compared according to age pregnant women in the study, result as following mean of PT, INR, APTT and plt count in less than 25 year group were (14.14/sec±1.07), (1.12/sec±0.103), (30.21/sec±1.91) and (230 X 10^9cell/µl±42.78) respectively, in second group 26-30 years results show mean of PT (14.23 /sec±1.11), INR (1.13±0.116), APTT (31.05/sec±3.13) and plt count (233 X 10^9cell/µl±46.56), while in third group more than 30 years results obtained PT (14.24/sec±1.22),INR

(1.14±0.118), APTT (30.61/sec±4.57) and plt count (216 X 10^9cell/µl±43.13). There is no significant difference in coagulation parameters when compared according to age pregnant women with p. value (0.934) for PT, INR (0.878), APTT (0.611) and plt count (0.242). (table 3-5)

Then coagulation parameters compared according to gestational age of pregnancy, results mean of PT (14.10/sec±0.98), INR (1.12±0.09), APTT (30.97/sec±3.22) and plt count (249 X 10^9cell/µl±41) in the first trimester of pregnancy while in second trimester mean of PT (14.24/sec±1.24), INR (1.14±0.12), APTT (30.86/sec±3.62) and platelets count (224 X 10^9cell/µl±28), in other hand coagulation profile in third trimester of pregnancy show small difference as follow mean of PT 14.30/sec±1.14), APTT mean (30.04/sec±3.31), INR (1.14±0.12) and platelet count (205 X 10^9cell/µl±56).

Significant differences with P.value (0.01) in plt count mean when compared according to gestational age of pregnancy while no significant difference in other parameters PT, INR and APTT with p. value (0.785), (0.630) and (0.528) respectively (table 3-6).

Table (3-1) Distribution of study population according to age group

Age group/year	Frequencies
Less than 25	28
26-30	39
More than 30	33
Total	100

Table (3-2) Distribution of the study population according to gestational age

Getsational age	Frequencies
First trimester	31
Second trimester	42
Third trimester	27
Total	100

Table (3-3) Variation of APTT, PT, INR and plts count in case and control group

Parameter	Sample	NO	Mean	Std. Deviation	P. value
APTT/sec	Case	100	30.67	3.41	0.01
	Control	50	31.98	3.87	
PT/sec	Case	100	14.21	1.13	0.025
	Control	50	15.04	0.88	
INR	Case	100	1.13	0.11	0.949
	Control	50	1.23	0.11	
Plt $X10^3$µl	Case	100	227	44.60	0.00
	Control	50	327	61.25	

Sig. level at (0.05)

Table (3-4) variation of coagulation parameters APTT, PT, INR and plt count in pregnant women according to age group

Coagulation parameters	Age group	No.	Mean	Std.deviation	P. value
APTT/sec.	Less than 25	28	30.21	1.91	0.611
	26-30	39	31.05	3.13	
	More than 30	33	30.61	4.57	
PT/sec.	Less than 25	28	14.14	1.07	0.934
	26-30	39	14.23	1.11	
	More than 30	33	14.24	1.22	
INR	Less than 25	28	1.12	0.103	0.878
	26-30	39	1.13	0.116	
	More than 30	33	1.14	0.118	
PLT×10^3/µL	Less than 25	28	230	42.78	0.242
	26-30	39	233	46.56	
	More than 30	33	216	43.13	

Sig. level at (0.05)

Table (3-5) Variation of coagulation parameters APTT, PT, INR and plts count according to gestational age

Parameter	Trimester	No.	Mean	Std. Deviation	p. value
APTT/sec	First	31	30.97	3.22	0.528
	Second	42	30.86	3.62	
	Third	27	30.04	3.31	
PT/sec	First	31	14.10	0.98	0.785
	Second	42	14.24	1.24	
	Third	27	14.30	1.14	
INR	First	31	1.12	0.09	0.630
	Second	42	1.14	0.12	
	Third	27	1.14	0.120	
Plt $X10^3$µl	First	31	249	41	0.001
	Second	42	224	28	
	Third	27	205	56	

Sig. level at (0.05)

Chapter four

4. Discussion, Conclusion and Recommendation

4.1 Discussion:

One hundred samples were collected from pregnant women as case and fifty sample from non-pregnant as control. The study revealed decrease in coagulation parameters (PT, INR, APTT and plt count mean) among Sudanese pregnant women in compare with non-pregnant women .This agrees with the study of Mohammed.,*et,al* (2010): (PT 14.69 sec, INR 1.18, APTT 30.73 sec and plt count 227 X 10^9cell/µl)

In the first trimester the mean of PT and INR were decreased when compared with the value of the second and third trimester of pregnancy, APTT show small difference among three trimesters, plt count show minimal decrease in the first trimesters, moderate decrease in second trimester and in the third trimester plt markedly decrease. This finding is agree with Mohammed.,*et.,al*. (2010).

Significant differences between APTT mean of pregnant and non-pregnant women. Sgnificant decrease in PT mean of pregnant in compare to control group were noted. This is study on line with study of Mohammed.,*et.,al* (2010).

This difference may be due to increases in a number of clotting factors, decrease in protein S levels, a significant fall in the activity of activated protein C and inhibition of fibrinolysis. These changes may be important for reducing intrapartum blood loss, but they determine an increased risk of thromboembolism during pregnancy and puerperium..This supported by study of PRISCO, D;(2005).

Significant decrease in platelets count mean during pregnancy in compare with control group. Thrombocytopenia may be due to gestational thrombocytopenia in the late pregnancy, and the spectrum of hypertensive disorders of pregnancy ranging from preeclampsia to sever preeclampsia and HELLP syndrome to eclampsia. This supported by willim *et al* (2007).

There were mild decrease in INR mean during pregnancy compared to control group but not significant (`1P.value 0.949) this agreed with mohammed .,*et.*,*al*.(2010).

4.2 Conclusion

- Significant difference in APTT, PT and PLT count of pregnant women versus non-pregnant were found.
- PLT count, PT, APTT and INR did not vary with the age of pregnant women.
- Coagulation parameters varied with trimester except APTT.
- Platelets varied with the trimester, highest in first trimester and lower in the third one.

4.3 Recommendations:

- Recommend to measure additional parameters to evaluate coagulation profile in pregnant women as fibrinogen, thrombin time, D.dimer.,... etc.
- Make reference values for pregnant women for coagulation profile.
- To measure coagulation profile regularly as routine for pregnant women to avoid thrombosis and ensure safety.

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