Erythrocyte–Platelet Interaction in Uncomplicated Pregnancy

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Abstract: Maternal and fetal requirements during uncomplicated pregnancy are associated with changes in the hematopoietic system. Platelets and erythrocytes [red blood cells (RBCs)], and especially their membranes, are involved in coagulation, and their interactions may provide reasons for the changed hematopoietic system during uncomplicated pregnancy. We review literature regarding RBC and platelet membrane structure and interactions during hypercoagulability and hormonal changes. We then study interactions between RBCs and platelets in uncomplicated pregnancy, as their interactions may be one of the reasons for increased hypercoagulability during uncomplicated pregnancy. Scanning electron microscopy was used to study whole blood smears from 90 pregnant females in different phases of pregnancy. Pregnancy-specific interaction was seen between RBCs and platelets. Typically, one or more platelets interacted through platelet spreading and pseudopodia formation with a single RBC. However, multiple interactions with RBCs were also shown for a single platelet. Specific RBC–platelet interaction seen during uncomplicated pregnancy may be caused by increased estrogen and/or increased fibrinogen concentrations. This interaction may contribute to the hypercoagulable state associated with healthy and uncomplicated pregnancy and may also play a fundamental role in gestational thrombocytopenia.

Key words: uncomplicated pregnancy, erythrocytes, platelets, scanning electron microscopy

Introduction

Pregnancy induces a number of physiological changes that affect hematologic indices, either directly or indirectly (Townsley, 2013), and is a well-established risk factor for venous thromboembolism and a state of hypercoagulability (Comeglio et al., 1996; Rosenkranz et al., 2008; Joly et al., 2013; Wang et al., 2013). Deep vein thrombosis and pulmonary embolism are collectively termed venous thromboembolism, which is triggered by inflammation and blood stasis leading to the formation of thrombi rich in fibrin and red blood cells (RBCs) (Aleman et al., 2014). Although there are many papers referring to pathological complications of hypercoagulability during pregnancy (Chan et al., 2014; Galambosi et al., 2014; McLintock, 2014; Virkus et al., 2014), a general, albeit subtle, change in the hemostatic mechanism during uncomplicated pregnancy is also well known. Changes include increased levels of coagulation factors, enhanced thrombin generation, and suppression of fibrinolysis commonly found in women with uncomplicated pregnancy (Comeglio et al., 1996). It is also well known that platelets play an important role in pathophysiology during pregnancy as seen in, e.g., uteroplacental disease, where platelet reactivity may be an important marker of uteroplacental disease activity (Burke et al., 2013). However, increased platelet activity and reactivity are also present in (Kilby et al., 1993; Burke et al., 2013) and form part of the subtle hypercoagulability in uncomplicated pregnancy. Hormonal changes, especially estrogen, in pregnancy have a fundamental effect on hypercoagulability (Kemkes-Matthes, 2000; Hellgren, 2003; Uchikova & Ledjev, 2005; Swanepoel et al., 2014). Similar hemostatic alterations have been associated with the use of oral contraceptives (Beller, 1994; Knijff & Goorissen, 2000), and elevated levels of estrogen increases the risk of thromboembolism (Speroff & Fritz, 2005).

Owing to the known presence of general hypercoagulability in uncomplicated pregnancy, the current research aims to determine if this hypercoagulability can be visualized in whole blood of healthy pregnant women by looking at the ultrastructure of platelets and RBCs. Figure 1 presents the rationale for these studies.

Literature Review

Platelet secretion, activation, adhesion, and aggregation are influenced by physical and chemical properties of RBCs (Turitto & Weiss, 1980). To understand platelet and RBC morphology, we need to first look at RBC and platelet membrane structure and what happens during platelet activation. The next paragraphs will briefly review RBC membrane structure.

RBC membranes consist of ~10% carbohydrates, 40% lipids, and 50% proteins. The external negatively charged carbohydrate-rich layer or the glycocalyx facilitates the gliding of RBCs through blood vessels (Oberleithner, 2013) and reacts very sensitively to environmental influences

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(Halbhuber et al., 1986). Phospholipids of RBCs are found as bilayer with choline, containing phosphatidylcholine and sphingomyelin in the outer layer, and amine-containing phospholipids like phosphatidylethanolamine and phosphatidylserine in the inner layer (Suwalsky et al., 2013; Sathi et al., 2014). The phospholipid classes of sphingomyelins and phosphatidylcholines constitute >50% of membrane phospholipids (van Meer & Holthuis, 2000; Ohvo-Rekila et al., 2002). Sphingomyelin is the most abundant sphingolipid (Carquin et al., 2014). The sphingolipids constitute a class of structural lipids with ceramide as the hydrophobic backbone (van Meer et al., 2008). The sphingolipids as well as the sterols are found at a higher density than glycerolipids, and resist mechanical stress (van Meer et al., 2008). Membrane cholesterol is unesterified and lies between the two layers of the lipid bilayer. The concentration of cholesterol in the membrane is important for membrane surface area and fluidity. The phospholipid bilayer is embedded with transmembrane proteins, and the maintenance of the asymmetric distribution of phospholipids across the plasma membrane is a prerequisite for the survival of RBCs (van Zwieten et al., 2012).

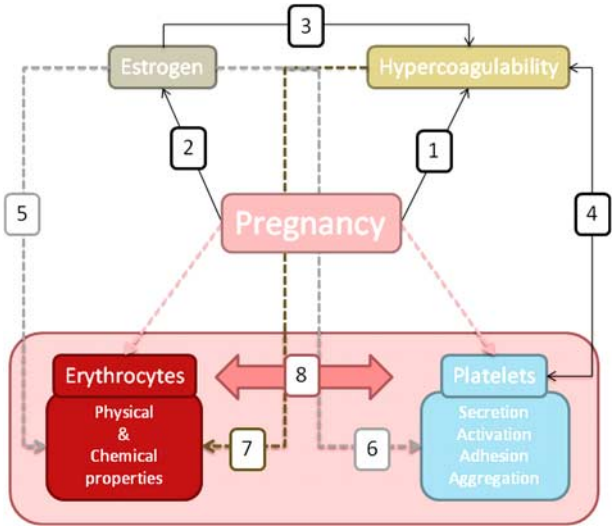

Figure 1. Overview diagram to show the rationale for the research. It is established that pregnancy has an effect on the coagulation system (1). Increased estrogen during pregnancy (2) has been implicated in the hypercoagulable state associated with pregnancy (3). Hypercoagulability occurs with platelet activation (4), including degranulation, adhesion, and aggregation resulting in platelet spreading. We now ask: does elevated estrogen during pregnancy influence red blood cell and platelet morphology (5 and 6, respectively), does the hypercoagulable state associated with pregnancy influence the physical and chemical properties of erythrocytes (7), and will these changes influence the interaction between these two types of blood cells (8).

The four peripheral proteins, spectrin, actin, protein 4.1, and ankyrin, play a key role in the structure of the RBC cytoskeleton (Thevenin & Low, 1990; Davis et al., 1991; Burton & Bruce, 2011; Grey et al., 2012). A mesh-like spectrin–actin cytoskeleton network is anchored to the phospholipid bilayer and ankyrin proteins (Kim et al., 2012). The underlying spectrin–actin cytoskeletal complex supports the phospholipid bilayer, and it forms a simple hexagonal geometric matrix (Fig. 2). Spectrins are cytoskeletal proteins that line the intracellular side of the plasma membrane that form flexible rods with actin-binding sites at each end (Bennett & Healy, 2009). Spectrin bands 1 and 2 are the most abundant membrane proteins and consist of two chains, α and β, wound around each other that are linked at the tail end to actin and interacting with the short actin filaments that act as junctional complexes. Globular actin protein (band 5) is formed by filaments that bind weakly to the tail end of both α and β spectrins. The principal proteins at the

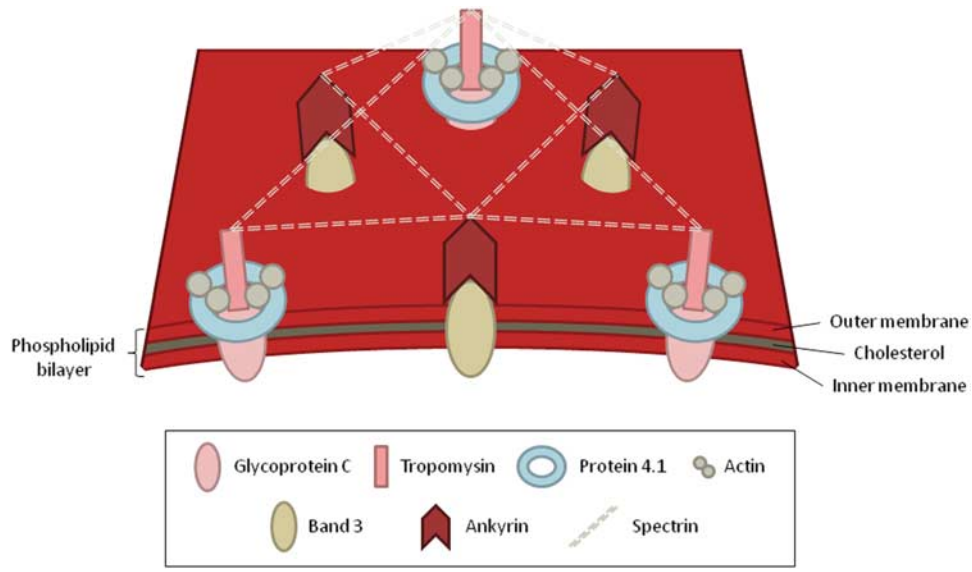

Figure 2. The spectrin–actin cytoskeletal complex supporting the phospholipid bilayer, forming a simple hexagonal geometric matrix.

spectrin–actin junction are protein band 4.1, adducin, tropomyosin, tropomodulin, and dematin. Spectrin is coupled to the inner surface of the RBC membrane, primarily through association with ankyrin, and the transmembrane protein bands 3 and 4.1 (Girasole et al., 2012; Kozlova et al., 2012). The globular protein band 4.1 interacts with spectrin and short actin filaments to form the RBC membrane skeleton and regulates membrane physical properties of mechanical stability and deformability by stabilizing spectrin–actin interaction. The interactions of band 4.1 with the spectrin–actin form the bulk of the membrane skeleton (Baines et al., 2009). Band 4.1 also binds directly to glycophorins A and C and band 3. At the head end, the β-spectrin chains attach to ankyrin, which connects to band 3. Band 3 is an abundant RBC integral membrane protein, which has a membrane-spanning domain and a cytoplasmic domain, and it regulates the structure and function of the RBCs (Straat et al., 2012). The cytoplasmic domain of band 3 serves as a center of RBC membrane organization (Ferru et al., 2011). Band 3 accounts for 25% of total protein content of the RBC membrane. It facilitates anion transport via the RBC membrane and is an important binding site for cytoskeletal and other RBC proteins. Band 3 is a transport protein that mediates the exchange of chloride for bicarbonate across the membrane (van den Akker et al., 2010).

Ankyrin (bands 2.1–2.3) anchors assembled spectrin molecules to the lipid bilayer, by binding simultaneously to the spectrin tetramers and to the interior domain of band 3. Ankyrin proteins are adaptor proteins that mediate the attachment of the spectrin mesh to the integral membrane proteins. The interaction of ankyrin and spectrin provides the major anchor between the membrane skeleton and the lipid bilayer and is important for RBC deformability and stability (Cunha & Mohler, 2009; Czogalla & Sikorski, 2010). β-spectrin contributes stability to the ankyrin protein network (Cunha & Mohler, 2009; Grey et al., 2012).

Owing to the complex structure of the membrane, the question now arises whether RBC membrane structure changes during hypercoagulability or in the presence of hormones, as is the case in uncomplicated pregnancy. The following paragraphs discuss the RBC membrane during hypercoagulability and how hormonal changes affect the membrane structure.

RBC Membranes During Hypercoagulability and Hormonal Changes

Blood hemorheology (Mandelli et al., 1984) and erythrocyte membrane deformability (Lukačín et al., 1996) undergo changes during pregnancy. Erythrocyte membrane deformability progressively decreases during pregnancy and only increases after delivery. Pregnancy-associated hormones have been implicated as the cause for these changes (Lukačín et al., 1996). Steroid hormones are lipophilic, suggesting they intercalate into the bilayer of membranes, potentially altering the fluidity (Whiting et al., 1995; Perez & Wolfe, 1988; Marra et al., 1998) and function of the membrane. Whiting et al. (2000) reported that progesterone decreased the lipid fluidity, and 17β-estradiol increased lipid mobility. Estradiol E20 modulates the structure and function of integral membrane proteins (Golden et al., 1998). There is also evidence that oral contraceptive users have lower concentrations of erythrocyte phospholipids and that this may affect membrane function and prostaglandin synthesis. This may relate to their increased risk of thrombosis (Fehily et al., 1982).

Platelets

Platelets are essential for normal hemostasis by forming a primary plug or thrombus after vascular injury, and play a fundamental role in thrombotic disease, abnormal clotting, and inflammation (Eyre & Gamlin, 2010). They have a plethora of membrane receptors, including receptors for thrombin, thromboxane, ADP, ATP, prostaglandins, von Willebrand factor, collagen, CLEC-2 ligand, fibrinogen, and laminin. Platelets rapidly adhere via receptor–ligand interactions, become activated involving intracellular signaling, secrete contents from the dense and α-granules, and aggregate to form thrombi (Berndt et al., 2014; Swieringa et al., 2014*b*). Activated platelets also express surface phospholipids that promote localized coagulation (Versteeg et al., 2013) leading to thrombin generation and fibrin formation.

Several articles have described the main receptors found on the platelet membrane (Kahn et al., 1999; Soulet et al., 2005; Mackman, 2008; Angiolillo et al., 2009; O'Brien et al., 2012; Swieringa et al., 2014*a*). Figure 3 provides a summary of these receptors.

In the following paragraphs we discuss platelet receptors, the structure of platelet membranes, and what happens when resting platelets are activated. This is important, as we know that there is an increase in platelet activity during uncomplicated pregnancy (Kilby et al., 1993). Complex molecular processes support platelet activation, which result in platelet shape changes, degranulation, and ultimately platelet aggregation (Valera et al., 2010). It is specifically in the third trimester of pregnancy that platelet aggregation induced by various physiological agonists becomes more pronounced (Morrison et al., 1985; Louden et al., 1990; Janes & Goodall, 1994; Sheu et al., 2002). In order to understand how platelets change from resting to activated, we need to briefly look at the structure of the platelet membrane.

Platelets have a highly organized cytoskeleton, which includes a peripheral microtubule coil and cross-linked actin filaments that fill the cytoplasmic space and connect to a membrane skeleton composed of spectrin and its associated proteins. Platelets maintain plasma membrane phospholipid asymmetry in normal blood circulation via lipid transporters, which control transbilayer movement (Lhermusier et al., 2011). The membrane skeleton of platelets is a planar network assembled primarily from spectrin molecules (Barkalow et al., 2003). Spectrin and protein 4.1 are involved in mediating the

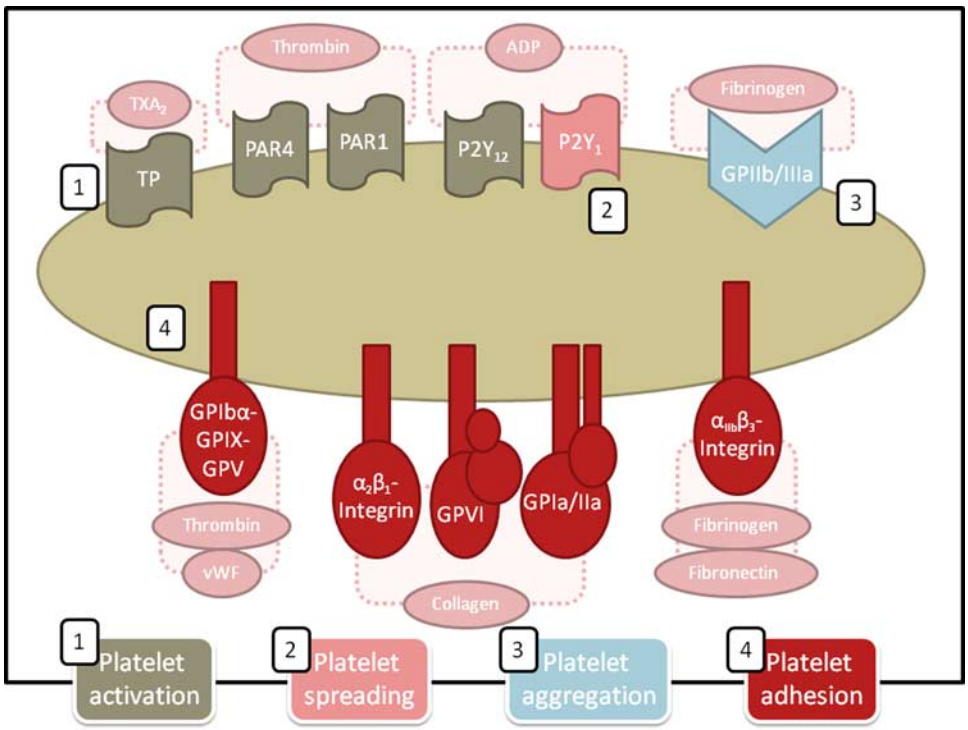

Figure 3. Platelet membrane proteins and their functions.

physiological response of the platelet (Matsuoka et al., 1994). Platelet membranes also contain high concentrations of integrins that are involved in the platelet adhesion to the extracellular matrix. Two platelet integrins, integrin αIIbβ3 (GPIIb/IIIa) and integrin αIIb1 (GPIa/IIa), are major components of the platelet membrane proteins, and are known to contribute to platelet adhesion to fibrin(ogen) and collagen surfaces (Moroi & Jung, 1998). Platelet–platelet interaction (aggregation) and platelet interaction with plasma coagulation factors is facilitated through αIIbβ3 (Jurk & Kehrel, 2005). Filamin is a major component of the membrane skeleton and plays a prominent role in regulating platelet size (Kanaji et al., 2012). Filamin plays a complex role in regulating platelet activation, and, in addition to binding GPIb-IX-V, filamin also binds various other macromolecules, including engaging the β-chain of β-3 integrin (Dyson et al., 2003). These integrins bind soluble ligands (fibrinogen or collagen) after platelets are activated, but only have low affinity toward these ligands when platelets are in the resting state.

Platelet membrane glycoproteins play a key role in hemostasis and thrombosis (Diz-Kucukkaya & Lopez, 2013). Platelet glycoprotein (GP)Ibα of the GPIb-IX-V complex and GPVI initiate platelet aggregation and thrombus formation by primary interactions with von Willebrand factor and collagen (Berndt et al., 2014). Invaginations of the platelet surface membrane form the open canalicular system, a closed-channel network of residual endoplasmic reticulum that form the dense tubular system, a spectrin-based membrane skeleton, an actin-based cytoskeletal network, and a peripheral band of microtubules (Thon & Italiano, 2012). Several novel and important platelet membrane proteins, including CLEC-2, CD148, G6b-B, G6f, and Hsp47, have been identified using proteomics-based approaches (Senis & Garcia, 2012).

Platelet morphology can be categorized into different morphological stages (Allen et al., 1979; Rosenstein et al., 1981; Cenni et al., 2000; Kraus et al., 2010):

- Stage 1: initial discoid shape.
- Stage 2: a spheroidal stage.
- Stage 3: an early spheroidal stage with few, short pseudopods present.
- Stage 4: a late pseudopodial stage with more numerous, longer pseudopods.
- Stage 5: a stage of fluctuation of the hyaline cytoplasm from the central region connecting the pseudopodia.
- Stage 6: a "fried egg" stage with a central granule-containing hillock, the granulomer.
- Stage 7: a final pancake configuration.

The main function of platelets is to participate in primary hemostasis through four distinct biochemical steps: secretion, activation, adhesion, and aggregation (Cimmino & Golino, 2013), resulting in the above-mentioned distinct seven morphological stages. The biochemical changes that platelets undergo in the four steps, will now be discussed.

Binding of biochemical agonists to their receptors on the platelet surface induces a signaling cascade (secretion) resulting in platelet activation, which involves the most numerous platelet integrin αIIbβ3 (Posch et al., 2013). Thereby, a dramatic shape change is required to expose the

relevant receptor (Jurk & Kehrel, 2005). Collagen also interacts directly with platelets via Glycoprotein VI (GPVI) and integrin $\alpha 2\beta 1$, and GPVI is the principal collagen-signaling receptor on platelets (Jarvis et al., 2012). Platelet activation and secretion of granule contents involves the formation of microvesicles by shedding of membranes from the cell surface (Heijnen et al., 1999). Upon platelet activation, the asymmetric orientation of membrane phospholipids is rapidly disrupted, resulting in exposure of phosphatidylserine at the outer platelet surface (Lhermusier et al., 2011). Phosphatidylinositol and phosphoinositides represent essential components of intracellular signaling that regulate diverse cellular processes, including platelet plug formation (Min & Abrams, 2013). GPVI is considered the predominant receptor responsible for collagen-induced platelet activation (Nieswandt & Watson, 2003). Activation and secretion can thus be seen as a shape change, translocation of membrane glycoproteins, exocytosis of granule contents, and the formation of microvesicles (Heijnen et al., 1999). During platelet adhesion, the complex cytoskeletal structure is rearranged, resulting in the formation of F-actin-based filopodia and lamellipodia (Traenka et al., 2009). Stimulatory platelet signaling pathways include binding of integrin αIIb$\beta 3$ to fibrinogen followed by activation of protein tyrosine kinases and phosphorylation of downstream signaling proteins. Platelet adhesion to subendothelial collagen is dependent on the integrin $\alpha 2\beta 1$ and GPVI receptors (Bleijerveld et al., 2013); $\alpha 2\beta 1$ serves mainly as an adhesion receptor (Jarvis et al., 2012). The finger-like platelet filopodial extensions and actin-rich sheets of lamellipodia dramatically increase platelet surface area and stabilize platelet aggregates to form thrombotic plugs (Aslan & McCarty, 2013). Conformational change in αIIb$\beta 3$ increases its affinity for its ligands (e.g., fibrinogen) and an active reorganization of the actin cytoskeleton accommodates shape change and the formation of filopodia (Wei et al., 2009). When platelets adhere to a collagen- or fibrin-coated surface, they become activated and form aggregates (Moroi & Jung, 1998). In spreading platelets, filamin and the GPIb-IX-V complex are localized in distinct cytoskeletal compartments with filamin at the submembraneous cytoskeleton and the GPIb-IX-V receptor centralized to the inner central actin ring (Dyson et al., 2003). The platelet collagen receptors, GPVI and $\alpha 2$, regulate integrin αIIb$\beta 3$-mediated platelet spreading on fibrinogen (Lee et al., 2012).

The various receptor-specific platelet activation-signaling pathways converge into common signaling events that stimulate platelet shape change and granule secretion, and ultimately induce the "inside–out" signaling process leading to activation of the ligand-binding function of integrin αIIb$\beta 3$ (Li et al., 2010). Ligand binding to integrin, αIIb$\beta 3$, mediates platelet adhesion and aggregation and triggers "outside–in" signaling, resulting in platelet spreading, additional granule secretion, and stabilization of platelet adhesion and aggregation, and clot retraction (Li et al., 2010). Agonist-induced platelet activation signals also cross talk with integrin outside–in signals to regulate platelet responses (Li et al., 2010).

Platelet Changes During Hypercoagulability and Hormonal Changes and Interactions with RBCs

Estrogen can facilitate platelet activation (Yamazaki et al., 1979) and also potentiate a proaggregating effect on platelets (Moro et al., 2005). Pregnancy-specific alterations in platelet morphology, including increased and enlarged open canalicular system pores, pseudopodia formation, platelet spreading, and membrane blebbing, are all indicative of platelet secretion and subsequent activation during pregnancy (Swanepoel & Pretorius, 2014).

It has been known that platelet secretion, activation, adhesion, and aggregation are closely linked to the interaction between platelets and the blood vessel wall, and that platelets are greatly influenced by physical and chemical properties of RBCs (Turitto & Weiss, 1980). There is therefore a functional overlap between human vascular systems and cells, linking hemostasis and in pathology where thrombosis and inflammation is involved (Berndt et al., 2014). RBCs have prothrombotic properties (Marcus et al., 1995) by liberating ADP, a platelet-activating agent (Lüthje, 1989). In addition, RBCs may enhance platelet reactivity, by promoting platelet granule release (Santos et al., 1991; Valles et al., 1991) and stimulating additional platelet recruitment into the developing thrombus (Santos et al., 1986, 1991; Valles et al., 1991). Interestingly, RBCs are involved in both αIIb$\beta 3$ activation by platelet releasates and upregulating recruited platelet secretion (Vallés et al., 2002). The interaction of activated platelets with RBCs therefore amplifies the activation and proaggregatory function of platelets (Santos et al., 1991; Valles et al., 1991).

RBC Membranes and Fibrinogen

It is well established that the hypercoagulable state associated with pregnancy is caused by an increase in coagulation factors, including fibrinogen (Hellgren, 2003). Fibrinogen is a plasma protein responsible for the formation of fibrin networks that are fundamental for blood coagulation and wound healing (Standeven et al., 2005; Laurens et al., 2006). Fibrinogen is crucial for hemostasis and has also been deemed an acute-phase protein involved in the process of inflammation, as it displays binding sites for distinctive cellular receptors expressed by cells involved in the inflammatory process (Kamath & Lip, 2003; Adams et al., 2007). As it acts as a "nonspecific glue," it can enhance the adhesion and aggregation of RBCs (Berliner et al., 2000). Low flow rate, which is the cause of deep vein thrombosis, has been shown to induce receptor-mediated RBC adhesion to platelets and/or fibrin (Goel & Diamond, 2002).

Specific binding mechanisms between RBCs and fibrinogen have been reported in normal rats (Lominadze & Dean, 2002). Erythrocyte aggregation has long been attributed to increased plasma adhesion protein levels, specifically fibrinogen (Letcher et al., 1983; Weng et al., 1996). The hyperaggregation of RBCs in the presence of elevated

fibrinogen indicates the involvement of a fibrinogen-binding site to the erythrocyte membrane (Gafarova et al., 2012). This interaction has been proposed to occur through the fibrinogen Aα-chain (Rampling, 1980; Maeda et al., 1987). Receptors on the erythrocyte membrane are an αIIbβ3-related integrin (Carvalho et al., 2010).

What Happens to Platelets and RBCs During Uncomplicated Pregnancy?

During pregnancy, the maternal hematopoietic system undergoes significant changes to facilitate growth and nutrition of the developing fetus (Peck & Arias, 1979). Throughout normal pregnancy, platelet activation contributes to the hypercoagulable state observed on a physiological level (Torres et al., 1996), and platelet activation is said to increase as the pregnancy progresses (Robb et al., 2010). Platelet activation also contributes to the prothrombotic state observed in uncomplicated pregnancy (Torres et al., 1996). Activated platelets interact with intact RBCs to amplify platelet activation and proaggregatory function (Santos et al., 1991; Valles et al., 1991). An increased aggregation of RBCs also occurs during the course of pregnancy (Huisman et al., 1988) and has been associated with elevated plasma fibrinogen levels (Bollini et al., 2005). As thrombus formation is classified as a multicellular event (Marcus & Safier, 1993; Marcus et al., 1995), involving both platelets and RBCs, it is important to investigate possible mechanisms of interaction between these blood components. Therefore, this investigation examines possible platelet/RBC interactions by using scanning electron microscopy (SEM), and to link the observations to the structure and function of the RBC and platelet membranes in healthy pregnancy. The current research excludes forms of pathology-like renal failure, gestational diabetes, hemoglobinopathies, or high-risk pregnancies. No comprehensive literature is available on RBC and platelet structure or their interactions in healthy, uncomplicated pregnancy.

Materials and Methods

Blood Collection

A total of 60 healthy pregnant volunteers were used and two groups were distinguished: the first group included 30 women in the early phase of pregnancy (8–14 weeks) and the second group included 30 women in the late phase of pregnancy (36–40 weeks). The second group also participated in the follow-up phase *postpartum* (6–8 weeks after birth). Women between the ages of 18 and 35 years were employed for the study, and all participant information was handled anonymously. In addition to the pregnant volunteers participated as 30 nonpregnant volunteers, the volunteers were recruited from the Femina Clinic, Pretoria (Ethical clearance number 185/2011). Informed consent was obtained from each participant. The participants were nonsmokers, did not have a history of thrombotic disease, or use any chronic medication known to interfere with coagulation factors and/platelet function. They neither used aspirin or aspirin analogues within 48 h before sampling. A total of 5 mL of blood were drawn by a qualified nurse at Ampath (Drs Du Buisson, Kramer, Swart, Bouwer Inc.) from each women participating in the study. Blood was drawn only once from the 30 women in the first pregnancy group. Women forming part of the second group (late pregnancy) had blood drawn on their last visit to the gynecologist before birth, as well as their first visit again to the gynecologist *postpartum*.

Preparation of Whole Blood for SEM Investigation

A total of 20 μL of the whole blood collected in a citrate tube was used to make a whole blood smear on a glass coverslip. Samples were placed on filter paper dampened with phosphate buffer solution (PBS). This created a humid environment. The samples were placed at 37°C for 5 min. Following incubation the samples were placed in PBS and placed on a plate shaker for 20 min. This washing process assisted in the removal of excess blood cells and plasma.

The whole blood sample was then fixed in a solution of 2.5% gluteraldehyde for 30 min and rinsed three times in 0.075 M sodium potassium PBS with a pH of 7.4 for 5 min. Thereafter, the sample was placed in secondary fixative, 1% osmium tetraoxide solution, for 15 min, rinsed again as previously described, and then dehydrated in 30, 50, 70, 90%, and three changes of 100% ethanol for 5 min in each concentration.

The SEM procedures were completed by critical point drying of the material, mounting and coating the sample with carbon, and examining the fibrin clot with a Zeiss Ultra plus FEG SEM. Photomicrographs (Zeiss, Oberkochen, Baden-Württemberg, Germany) were taken at 1 kV. Coverslips from all donors were systematically examined so as to analyze the whole sample. After this screening, representative micrographs were taken from each participant to ensure that the micrographs chosen were indeed a representative image of the whole slide; at least ten micrographs were taken per individual. The first author did this analysis. The second author systematically viewed these samples blindly to ensure that the micrographs chosen for publication were representative of the particular group.

Results

Estradiol concentrations were determined by a pathology laboratory support service (Ampath South Africa) for both the nonpregnant and pregnant participants. The Beckman method was followed to determine estradiol concentration. Concentrations of estradiol during the menstrual phase is specified as ranging between 99 and 488 pmol/L, while levels during normal pregnancy is specified as ranging between 3,680 and 13,216 pmol/L. An average concentration of 125 and 5,024 pmol/L was representative of the estradiol concentrations of the nonpregnant and pregnant participants, respectively. These concentrations can be considered as normal for each

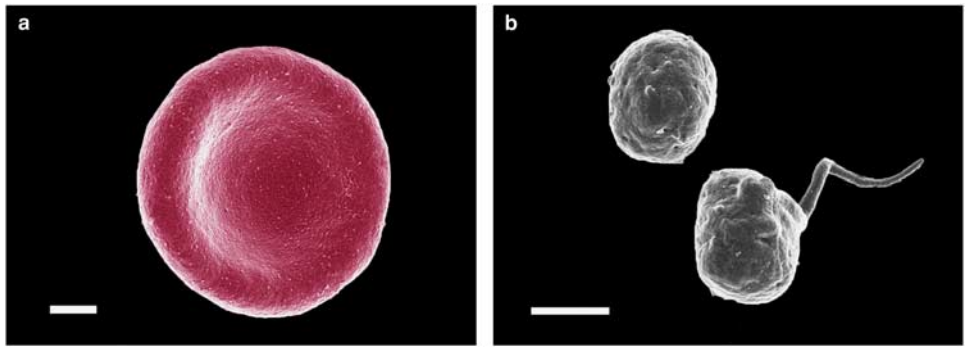

Figure 4. Images from a whole blood smear from a healthy nonpregnant female: (**a**) red blood cell and (**b**) platelet. Scale bar is 1 μm.

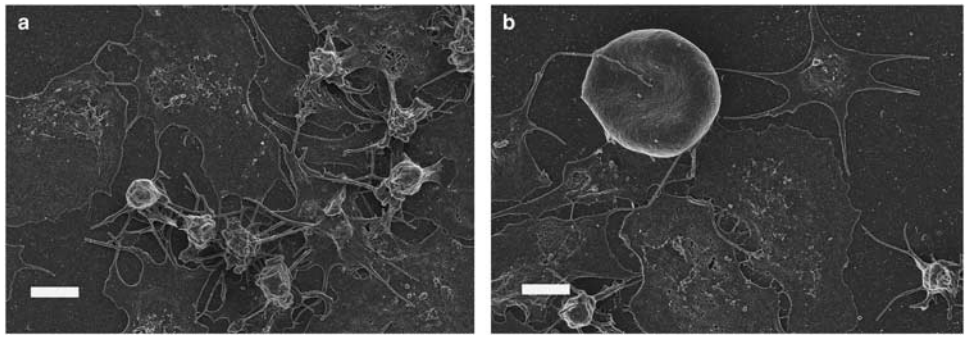

Figure 5. (**a**) Platelet aggregate; (**b**) activated platelets interacting with erythrocyte. Platelet activation, spreading, and interaction with an erythrocyte in uncomplicated early pregnancy. Scale bar is 1 μm.

subset of participants (nonpregnant and pregnant) when the pathology's laboratory ranges are considered.

Figures 4a and 4b show a typical RBC and platelet from a nonpregnant female of child-bearing age. The RBC shows no platelet association, which is a typical occurrence. Figure 5a is a low-magnification micrograph of platelet activation seen during uncomplicated pregnancy. Figure 5b shows platelet activation and spreading along with platelet interaction with an erythrocyte. Platelet activation is visible in both Figures 6 and 7, with characteristic pseudopodia formation, membrane blebbing, and some platelet spreading. It was mainly the platelet pseudopodia that were in close contact with the RBC membrane, though some of the platelet bodies were also positioned on top of the RBCs. The pseudopodia showed characteristic thin endpoints converging with the RBC membrane. Some of the pseudopodia endpoints were flattened and showed a type of spreading on the RBC membrane. Figure 6 shows the interaction of a single platelet with a single erythrocyte. Figure 7a shows two platelets interacting with a single RBC, whereas Figure 7b shows a single platelet interacting with two RBCs. During all three stages of pregnancy, namely early (8–14 weeks) and late pregnancy (36–40 weeks), as well as 6–8 weeks *postpartum*, these same types of interactions were visible. Almost all RBCs in the whole blood smears showed typical discoid morphology with a close association with platelets.

In Figures 8 and 9, the membranes of RBCs and erythrocytes are investigated. Membranes of both RBCs and platelets in nonpregnant females appear smooth (Fig. 8), whereas the membranes of RBCs and platelets from healthy pregnant individuals have a granular appearance (Fig. 9). Interactions between erythrocytes and platelets are seen during pregnancy, and not in nonpregnant females.

Discussion

Platelet adhesion and aggregation is dependent on morphological changes in platelet ultrastructure, especially the formation of pseudopodia (Warren, 1970). These finger-like filopodia processes facilitate platelet–platelet association and fibrin strand formation (Warren & Vales, 1972). The increased pseudopodia formation and visible interaction of these processes with the RBC membrane indicates an integral relationship between these blood components, specifically in pregnancy.

Platelet reactivity is significantly influenced by cell–cell interactions between platelets and RBCs (Santos et al., 1986, 1991; Valles et al., 1991). Cell–cell interaction between activated platelets and RBCs indicates that biochemical communication between RBCs and platelets is initiated upon platelet activation. This is because RBCs cannot promote platelet activation or the recruiting activity of cell-free releasates without a platelet agonist (Santos et al., 1991; Valles et al., 1991).

We suggest that the platelet/RBC interactions during uncomplicated pregnancy, showed in this research, is due to the increased estrogen levels found in (healthy) pregnancy.

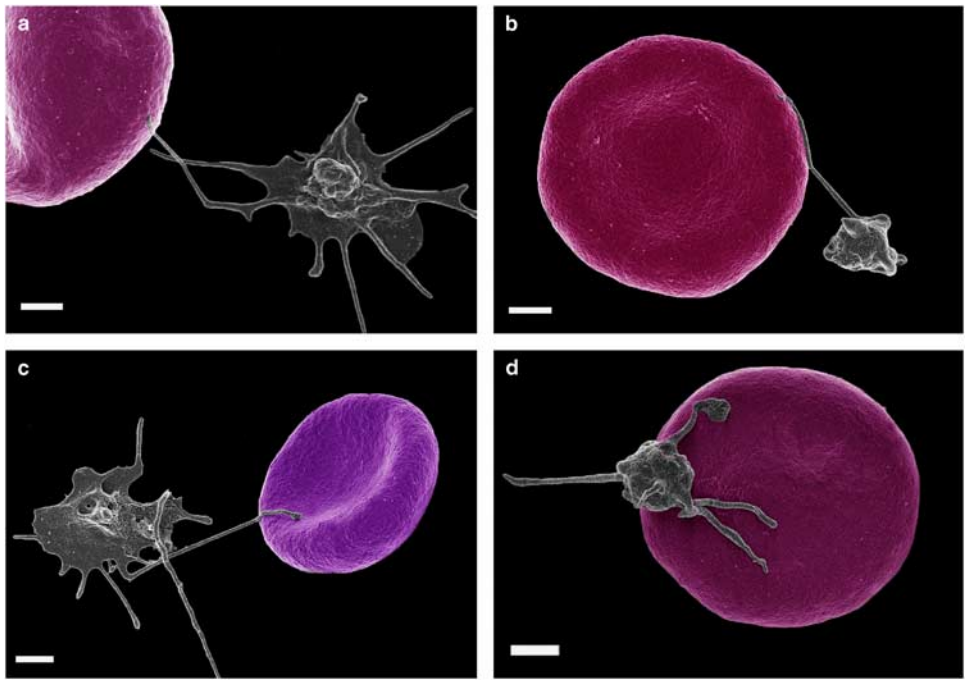

Figure 6. Whole blood smears from healthy pregnant females showing typical RBC–platelet interactions. A single platelet is associated with a single RBC in (**a**–**d**): (**a**) is representative of early pregnancy, (**b**) representative of late pregnancy, and (**c**) and (**d**) representative of the *postpartum* group. Scale bar is 1 μm. RBC, red blood cell.

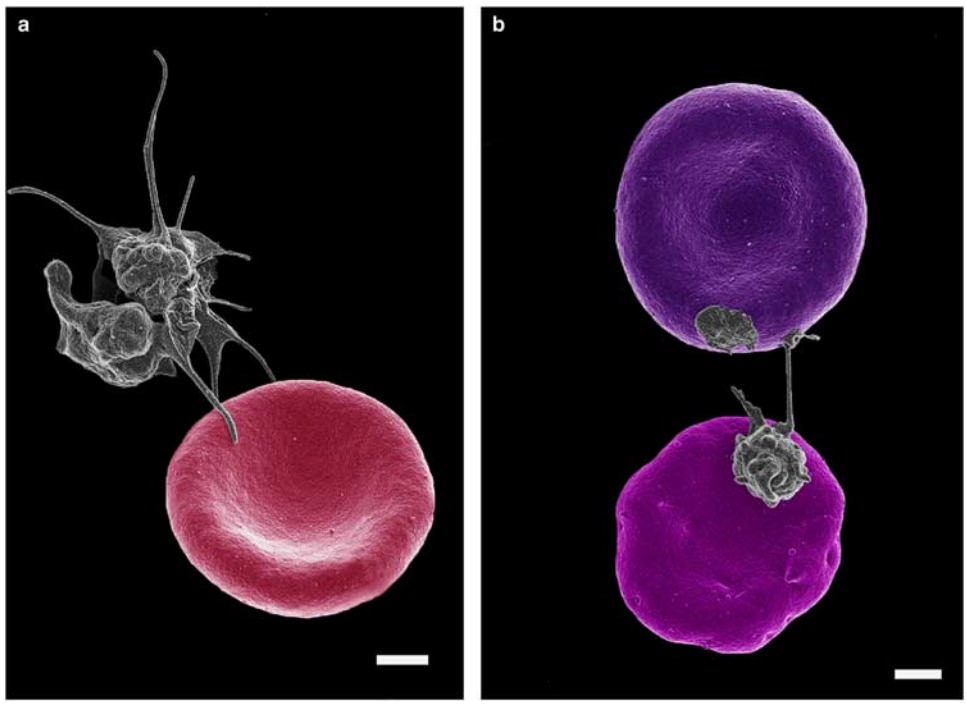

Figure 7. Whole blood smears from healthy pregnant females showing additional platelet–RBC interactions. **a:** More than one platelet interacts with a single RBC in late pregnancy. **b**: Single platelet interacts with multiple RBCs, *postpartum*. Scale bar is 1 μm. RBC, red blood cell.

We have reviewed literature that shows that there is increased hypercoagulability in healthy pregnancy. Fibrinogen acts as an adhesive means to enhance RBC adhesion and aggregation, even to platelets (Goel & Diamond, 2002). As fibrinogen-specific binding sites are present on both the erythrocyte (Carvalho et al., 2010; Gafarova et al., 2012) and platelet (Berndt et al., 2014; Swieringa et al., 2014*b*) membranes, elevated fibrinogen during pregnancy may

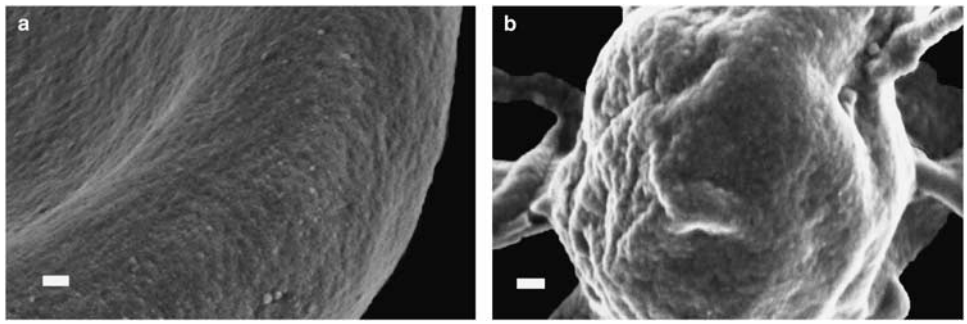

Figure 8. Red blood cell (RBC) and platelet membranes from healthy nonpregnant females: (**a**) smooth RBC membrane and (**b**) smooth platelet membrane. Scale bar is 100 nm.

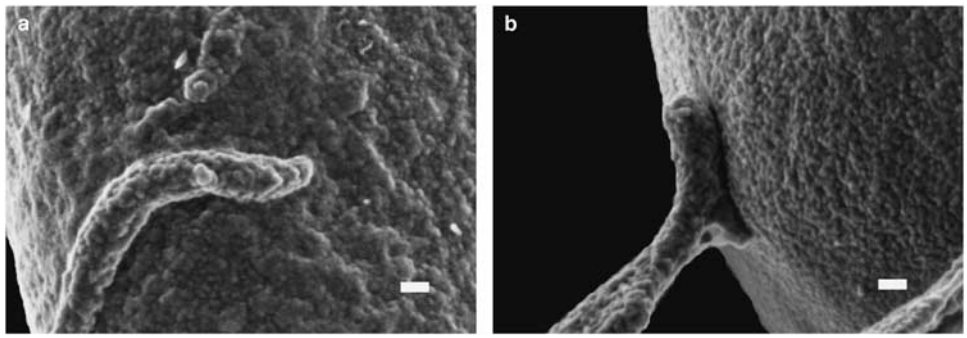

Figure 9. (**a**) Platelet aggregate; (**b**) activated platelets interacting with erythrocyte. Red blood cell and platelet membranes during interaction in uncomplicated late pregnancy. Scale bar is 100 nm.

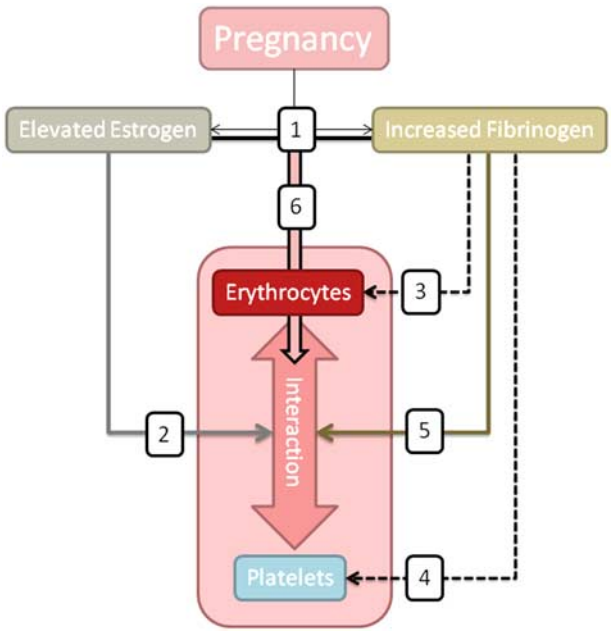

Figure 10. Proposed mechanism responsible for the pregnancy-specific erythrocyte–platelet interaction. Elevated estrogen and increased fibrinogen are both associated with pregnancy (1). We propose increased estrogen levels may be the cause for pregnancy-specific erythrocyte–platelet interaction observed by scanning electron microscopy (2). As fibrinogen can bind to both erythrocytes (3) and platelets (4), it is also implicated as a possible cause for the mentioned interaction (5). It is also possible that it is the combination of the two factors (6) that cause this specific red blood cell–platelet communication.

also explain the mode of the specific erythrocyte–platelet interaction seen during pregnancy. It could also be the combination of elevated estrogen accompanied by the increased fibrinogen levels that brings about this particular morphological phenomenon. Figure 10 illustrates our proposed mechanism responsible for the pregnancy-specific erythrocyte–platelet interaction.

We suggest that the pregnancy-specific changes shown here may be the mechanism through which platelets and RBCs communicate to initiate platelet activation and support aggregation to assist thrombus formation, ultimately resulting in a general hypercoagulable state. The increased interaction will lead to increased platelet activation, degranulation, and aggregation. This will decrease the number of platelets in circulation, resulting in thrombocytopenia typically seen in pregnancy (McCrae et al., 1992; Salnlo et al., 2000; McCrae, 2010).

A limitation of this ultrastructural investigation of RBC–platelet interactions includes the lack of percentages of interactions between RBCs and platelets. Future studies should focus on the statistical implications of this phenomenon. In addition, we only had access to the average estradiol levels for the nonpregnant and pregnant groups. Future studies should include more specified estradiol concentrations.

Conclusion

RBCs provide oxygen to the body and contribute to hemostasis. RBCs have shown proaggregatory properties by liberating ADP, a platelet-activating agent, resulting in a

prothrombotic state (Lüthje, 1989; Marcus et al., 1995). Platelet function is therefore amplified by the interaction of activated platelets with intact RBCs (Santos et al., 1991; Valles et al., 1991). Interactions between RBCs and platelets may be one of the reasons for an increased hypercoagulability during a healthy, uncomplicated pregnancy, and this state continues for a while *postpartum*. Thus, this RBC/platelet interaction could possibly contribute to gestational thrombocytopenia.

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