Review Article

Seminal clotting and fibrinolytic balance: a possible physiological role in the male reproductive system

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Summary

Semen contains enzymes and inhibitors of the haemostatic system as well as the high molecular weight seminal vesicle (HMW-SV) proteins. The former may have roles in seminal clotting and in liquefaction through "fibrinolytic" activity, which may ultimately affect fertility. Although a limited number of studies have addressed the subject, the role of clotting and fibrinolytic factors in semen remains poorly understood. The liquefaction time and the distribution of components vary across split ejaculates. This may have an important bearing on the way clotting/fibrinolytic factors in semen are assessed. Semen contains tissue factor (TF, Thromboplastin, CD142), which originates from the prostate and is associated with prostasomes. The function of TF (and prostasomes) in semen is still a matter for speculation. Recently the presence of minute amounts of factor VII in semen has been demonstrated but its importance is uncertain. Semen also contains a thrombin-like enzyme, prothrombin fragments 1 and 2 (F1+2), D-dimer (DD) and thrombin-antithrombin (TAT) complexes. The presence of several fibrinolytic factors has been demonstrated in semen but few questions about their potential impact on semen quality have been raised. Factors found include tissue plasminogen activator (t-PA), urinary plasminogen activator (u-PA) and plasmin. There are also traces of fibrinogen, plasminogen, plasminogen activator inhibitor-1 (PAI-1), factor VIII coagulant activity (VIII:c) and fibrin monomers. The co-ordinate expression of both TF and PAI-1 by decidual cells of the endometrium is believed to be important in maintaining haemostasis during endovascular trophoblast invasion. Kallikrein-like serine protease inhibitors including prostate specific antigen (PSA) are known to be present in semen at high concentrations. In semen PSA is also found in a complex form with protein C inhibitor (PCI) with mutually inhibitory consequences. A better understanding of the spectrum of coagulating and liquefaction agents in semen to include classical haemostatic processes and the pathogenesis resulting from any imbalances between or within either system may provide the basis for the development of more selective and efficient agents affecting global fertility. Here we review aspects of male reproductive physiology in the light of recent findings concerning conventional clotting/fibrinolytic systems in human semen with a view to stimulating further research.

Keywords

Human seminal plasma, haemostasis, fertilization, implantation, prostasomes, semen quality, review

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Introduction

The procoagulant activity of human seminal plasma added to blood plasma was first recognised in 1942 (1). Seminal plasma diluted up to 10 000-fold significantly decreased the re-calcification clotting time of blood plasma. The molecular basis for this observation remains uncertain. Semen forms a gel-like-coagulum immediately after ejaculation, embracing the sperm.

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Subsequently, semen liquefies spontaneously after 5 to 20 min of *in vitro* emission (2, 3). This process is called coagulation and liquefaction "fibrinolysis", and may play a major role in the so-called capacitation of sperm (4), which is a series of transformations that sperm must undergo to become fertile.

In this review, we will discuss the existence, in semen, of elements of the classical haemostatic system and their coexistence with the HMW-SV (high molecular weight seminal vesicle) protein system mediating coagulum formation and dissolution (Fig. 1). There appears to be a dearth of mechanistic studies in this area. The reader's attention is also directed to the potential, but as yet unresolved clinical relevance of seminal haemostatic and other proteases/protease inhibitors in fertility issues, including involuntary infertility. The possible role of semen-borne clotting/fibrinolytic factors in fertilization and endometrial haemostasis during implantation is highlighted.

The spectrum of coagulum/ liquefaction forming agents in semen

The HMW-SV proteins

The predominant structural proteins of coagulated human semen are those secreted by the seminal vesicle - semenogelins-I and II (Sg-I and Sg-II) and fibronectin. These proteins are stable in the seminal vesicle' secretion for up to twenty hours at 37ºC, but rapidly cleave upon mixing with the proteases of prostatic secretion (5). The interaction between the seminal vesicle and prostate components leads to the liquefaction of the seminal coagulum (3) and progressive release of motile sperm (6) (Fig. 1). Human ejaculates vary in their degree of coagulation as well as liquefaction time (3). The organs of the reproductive tract contribute variously to the overall composition of the ejaculate (7, 8).

Coagulum formation in semen is said to be induced by vasiculase, which originates from the prostate gland (9). Two components are thought to account for seminal clot lysis a neutral protease known as seminin and a metallo-dependent collagenase-like peptidase (7, 10, 11). After liquefaction of the seminal clot the HMW-SV proteins are degraded by the proteases of prostatic fluid to a series of labile proteins which are then further cleaved to peptides of successively decreasing size to give complete liquefaction (12, 13). The proteolytic effect on the HMW-SV proteins could be due to a membrane-bound prostatic peptidase that hydrolyses succinyl $(alanine)_3$-p-nitroanilidea (14) or possibly due to prostate specific antigen (PSA) (13) (Fig. 1). In semen, this proteolytic degradation is reflected by rapid changes among proteins, and a parallel increase of non-

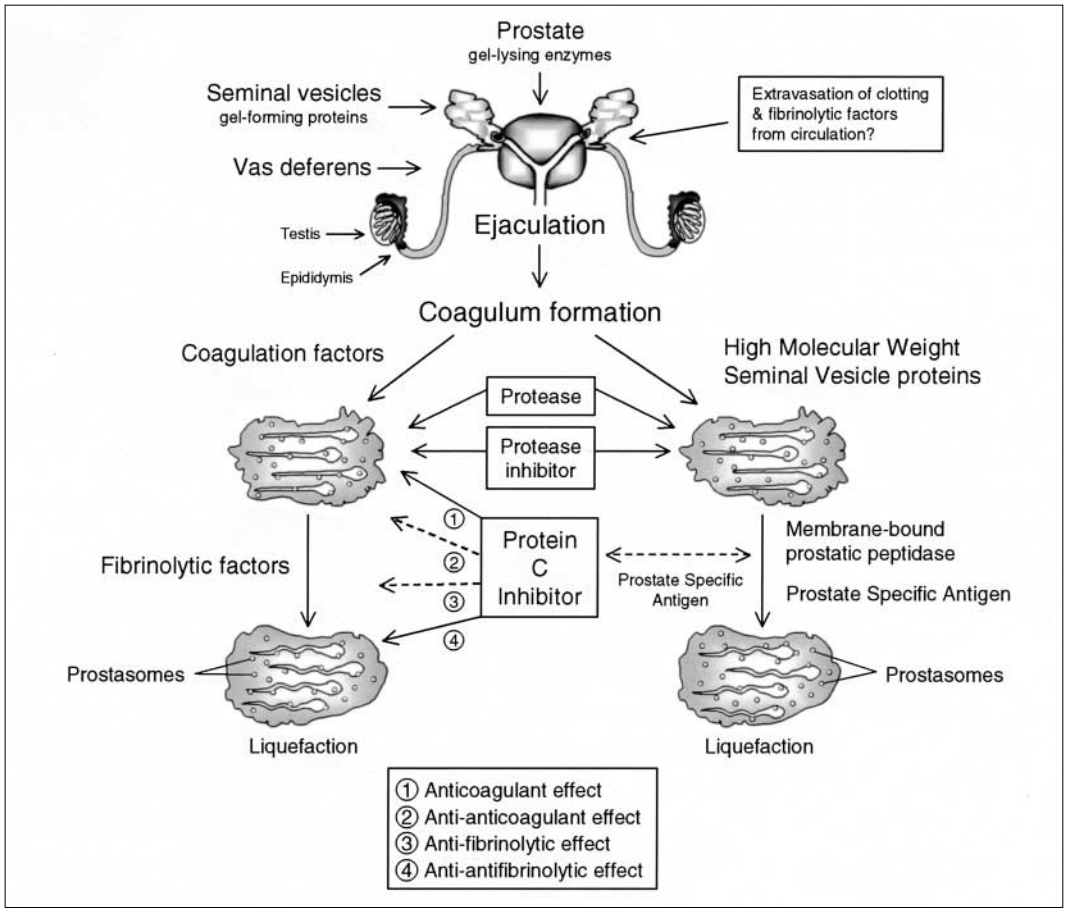

Figure 1: The coagulum/liquefaction forming agents in semen. Coagulum formation might be due to HMW-SV proteins and/or coagulation factors. The liquefaction of the coagulum could be due to proteolytic degradation of HMW-SV proteins by membrane-bound prostatic peptidase or PSA, or through the conventional fibrinolytic system. Other protease or protease inhibitors may act simultaneously on both systems. Protein C inhibitor has a diverse role in the haemostatic system and also interacts with PSA. It may act as a bridge between the HMW-SV proteins and the seminal haemostatic factors.

protein nitrogen in semen (15). This effect, however, could be reversed by the addition of either thiol reagents [serine protease inhibitors or o-phenanthroline] to semen (16, 17).

The classical haemostatic system

Although it is well known that seminal coagulants are made of coagulation proteins (4, 18), and that the seminal coagulum is composed of fibrin-like material (19), the thorough investigation of those components related to the haemostatic system remains rare. The fibrin-like material is considered to be composed of sialo-glycoprotein-metal complexes (19) and of glycerylphosphorylcholine (20). A few studies, however, have reported the presence of individual clotting, and fibrinolytic factors, as well as protease inhibitors in seminal plasma. These include; prothrombin fragments 1 and 2 (F1+2), D-dimer (DD), thrombin-antithrombin (TAT) complexes, plasminogen, tissue plasminogen activator activity, plasminogen activators, plasmin, a kallikrein-like serine protease inhibitor and protein C inhibitor (PCI). Only traces of these proteins, except for PCI (220 mg/l), were found in semen (21-23). On the other hand, the seminal values for tissue plasminogen activator (t-PA) and urinary plasminogen activator (u-PA) were significantly high in comparison to the blood plasma concentrations (24). No correlation between seminal t-PA and u-PA was observed (24). Using zymography assays, it was shown that seminal plasminogen activators are under-active forms. ProUrokinase was not detected (24). Traces of fibrinogen, plasminogen, plasminogen activator inhibitor-1 (PAI-1), factor VIII coagulant activity (VIII:c) and fibrin monomers could be detected but not quantified (24). Although the seminal concentration of antithrombin III is low (600–1800 µg/l) it was high enough for TAT III (TAT-III) complex formation, which was measured in semen (9). Vitronectin is a multifunctional glycoprotein found in blood and in the extracellular matrix. It is also found in seminal plasma and on sperm, where it may be associated with acrosin activation (25). In blood, vitronectin also functions as a regulator of coagulation and fibrinolysis (26). It protects thrombin from a rapid heparin-dependent inactivation by antithrombin III, and inhibits the fibrin clot induced activation of plasminogen by tissue type plasminogen activator. In addition, vitronectin binds to PAI-1 and stabilizes its inhibitor activity (27). The several strands of evidence outlined above suggest potentially the functional presence of the classical haemostatic system in the coagulation and liquefaction process of semen.

The presence of fibrin degradation products, prothrombin fragments and other active clotting factors in seminal plasma is probably due to *in situ* activation of the same clotting factors that are in blood (28). While it has been demonstrated that seminal plasma contains considerable amounts of F1+2, (29) and that thrombin-like enzyme was detected in semen (30), which is indirect evidence for the presence of the prothrombin needed for fibrin production, no one, including our group, has been able to directly measure prothrombin in semen. It is possible that thrombin levels are low in semen due to a short half-life – or high turnover in semen. The degradation products can be detected. Explanations for this discrepancy are transudation from plasma or that the assays are not designed for use with semen and their relative sensitivities might be compromised.

When fibrin is formed in blood plasma it is known to provoke fibrinolysis by stimulating tissue plasminogen activator activity, which is also present in a rather high concentration in semen (31). The result is the formation of DD. D-dimer fragments as degradation products can only originate from previously formed cross-linked fibrin. Evidence for cross-linking activity has been found with the demonstration of transglutaminase secretion through the anterior prostate in guinea pigs (32) and rats (33). While neither transglutaminase activity nor factor XIII was reported to be present in human semen (34), it has subsequently been shown that Sg-I and Sg-II (also known as human seminal plasma motility inhibitor (SPMI) (35), both in seminal vesicle fluid or purified from semen, are substrates for factor XIIIa, the fibrin cross-linking transglutaminase (36). Furthermore, digestion of semenogelin with PSA produced fragments, some of which were cross-linked into complexes by factor XIIIa (36). Semenogelin-I and II and fibronectin interact non-covalently via disulphide bridges to form an instant coagulum upon ejaculation. Semenogelins as well as the semenogelin-related proteins are the major proteins involved in the gelatinous entrapment of ejaculated sperm. Antigenic epitopes common to these proteins have been localised to the locomotive parts of the sperm (37). The sperm become progressively more motile when gel-forming proteins are fragmented by the kallikrein-like protease, PSA, and the gel dissolves (37).

Recently the presence of tissue factor (TF, Thromboplastin, CD142), a cell membrane-associated glycoprotein that serves as the primary physiological initiator of the coagulation process in human semen, has been reported (28, 38, 39). Traces of factor VII may also be present in seminal plasma (28, 39). Elsewhere in the body, the complex of TF and factor VII/VIIa initiates blood coagulation by the proteolytic conversion of factor IX to IXa and factor X to Xa (Fig. 2). Neither factor VII/VIIa or TF alone possesses this activation capability (40). The procoagulant activity in semen was dependent on factor X and Ca^{++}, suggesting the presence of factor X activator (28). This procoagulant activity was neutralised by monoclonal antibodies to human TF and factor VII (28). Immunoblotting and immunoadsorption studies have confirmed that the seminal procoagulant activity is due to 45 kDa TF antigen (28). It could also be argued that platelet activating factor (PAF)-like activity (41), a phospholipid with a wide spectrum of potent biological activities including activation of coagulation, and other cellular microparticle-containing phospholipid and/or cell membrane fragments that promote coagulation activation, are also found in semen. Indeed, TF in urine (uTF) is found in a lipid-associated form,

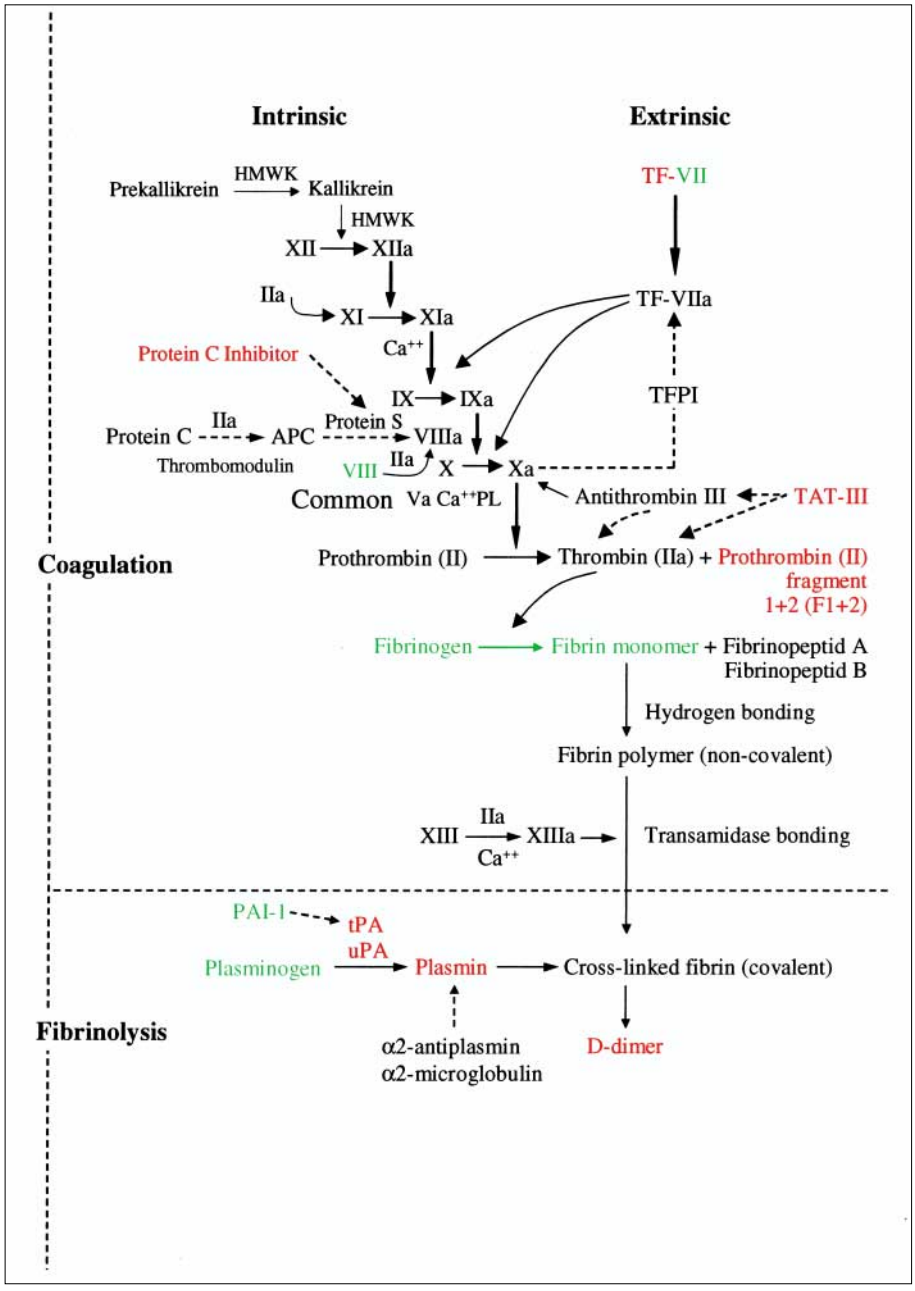

Figure 2: The classic cascade of the coagulation and fibrinolytic system. Dashed arrows indicate pathway inhibition. Factors present in semen are indicated in red. Factors that are detectable in semen but not quantified are shown in green.

probably of kidney origin (42). The labelling of these vesicles with gold-labelled Annexin V [a placental anticoagulant protein-I] (43) showed that they contain not only TF, but also anionic phospholipids (42) which are essential for TF activity (42). Urinary TF activity was inhibited in a dose-dependent manner when it was incubated with different concentrations of recombinant Annexin V (42). Similar results were obtained for monocyte TF activity (44). The procoagulant activity of these cellular elements, however, may not fully explain the TF/factor VII dependent activity found in seminal plasma (28). The classical cascade of clotting and fibrinolytic systems are shown in Figure 2.

The presence of a large concentration of seminal TF is puzzling. About 90% of seminal TF is present in the seminal plasma (39). Centrifugation studies suggested that seminal TF was membrane bound (28) like uTF (42). Furthermore, fractionation of seminal plasma by gel filtration followed by immunoelectron microscopy revealed that TF antigen resides on the surface of prostasome vesicle (28). These are small membrane-surrounded organelles that are secreted by acinar epithelial cells of the prostate gland and are transported in the prostatic fluid to mix with the semen during ejaculation (45, 46). This suggests that seminal TF originates from the prostatic fluid and not from the seminal vesicle secretion. Indeed, Fernandez et al. found no

TF in four seminal vesicle secretions (28). Tissue factor antigen was localised on the glandular epithelial cells of normal but not malignant prostate cells (47). This suggests that prostasome-bound TF in semen is produced by exocytosis of prostasomes from epithelial cells (28).

The functions of seminal prostasomes are not fully understood. In addition to TF, some nucleic acids, proteins, and enzymes have been identified complexed with or on the prostasome membrane. These include ATPase, protein kinase, and zinc-dependent peptidase (46, 48). Prostasomes are also rich in Ca^{++49} and participate in the maintenance of Ca^{++} homeostasis in the environment of sperm (50). The fact that these organelles bear TF may contribute to their role in seminal clotting activity. Furthermore, it is possible that prostasomes promote the progressive forward motility of sperm, through variation in Ca^{++} concentrations (50) and may also protect sperm from phagocytic cells (51). Prostasomes can adhere to and, at least to some extent, fuse with sperm (50, 52) and a hydrophobic interaction between prostasomes and sperm has been suggested (49). Prostasomes can also influence the expression of immunomodulating antigens on the sperm surface (53). Further information on the roles of prostasome in semen can be read elsewhere (28, 54). Details about the structure of prostasomes can be found in the proceedings of the recent first international conference on prostasomes (55).

Other proteases and protease inhibitors

The presence of other seminal serine proteases such as basic arginine amidase (56), acrosin (57, 58), leucine aminopeptidase-like aryl amidases (59), α_1-antitrypsin α_{1x}-antichymotrypsin (34), pancreatic trypsin (34), and PSA (60, 61) have all been demonstrated in human semen. On the other hand, α_2-macroglobulin, inter-α_1-trypsin inhibtor and C_1s-inactivator could not be detected (34) However, some of the proteases that are found in semen may have roles in the seminal clotting and fibrinolytic process. Activated PCI, for instance, complexes with PSA which may partially inhibit its activity (21). The involvement of the relevant seminal protease and protease inhibitors in the context of this review are considered later.

Sources of seminal clotting/ fibrinolytic factors

The seminal coagulum in freshly ejaculated semen is contributed to by the vesicle (50-80%) and the liquid fraction of the prostatic secretion (15-40%) with the testes and epididymes producing (5%) (7, 8). There is no obvious external source of clotting/fibrinolytic factors except perhaps from plasma extravasation, although the presence of fibrin degradation products in readily detectable amounts are considered by some to suggest otherwise (28). Indeed, several elements of the haemostatic system have been localised to the genito-urinary tract in our hands. Prostasomes are derived from the prostate gland and appear freely in semen at a prostasome protein:sperm protein ratio of about 2:1 (45, 62, 63). Moreover, these extracellular organelles are the major TF-bearing structures in semen (28).

Components of split ejaculates

Poorly coagulating samples possessed significantly lower levels of vesicular components and higher concentrations of prostatic products than those of the ejaculates showing normal coagulation (3). Semen contains components that are not evenly distributed across split ejaculates (34). This suggests that the distribution and the quantity of these components throughout the ejaculates may be critical in studying the clotting/fibrinolytic profile in semen. For example, plasminogen activators, immunoglobulins IgG and IgA, albumin and transferrin are at higher concentrations in the first fraction of the split ejaculate which contains the secretion of the prostate and Cowper's glands, as well as most of the sperm (21). Lactoferrin and fructose were high in the last fraction indicating production by the seminal vesicles (34). Protease inhibitors were present in all three seminal fractions, suggesting production by both the prostate gland and seminal vesicles (34). The quantities of these factors, however, vary between split ejaculates. For example, more than 60% of the neutral protease of the total ejaculate activity of human semen is present in the first fraction, which represents about 25% of the total volume of ejaculate. Fraction three, in contrast, contains about 45% of the total volume, which represents only 16% of the total neutral protease activity (34). This is similar to the distribution pattern of plasminogen activators and almost identical to that of sperm (34). Loss of activity after semen ejaculation should also be considered when studying the clotting/fibrinolytic factors in semen. For example, PCI retained only 45% of its activity immediately after ejaculation, and its activity rapidly decreased following incubation of the seminal plasma at 37ºC (64). Complex formation with other proteins, particularly those of prostatic origin, may have been the cause for this lost protein C activity (5, 64).

Assessment of seminal clotting/fibrinolytic factors

There are no assays designed and optimised specifically for the measurements of seminal haemostatic factors. The studies reported in the literature used either kits or protocols intended for plasma clotting/fibrinolytic factors or simple modifications of them. These could either be functional (clotting or chromogenic assays) or immunological (enzyme-linked immunosorbant assays) many of which are commercially available. Other techniques designed to assess clotting/fibrinolytic factors in seminal plasma include the fibrin plate method, zymography assays, and radioimmunoassay. The precision of the commercial

assay kits and the conventional clotting/fibrinolytic methods, however, might be compromised in this application. Some results might be influenced by the nature of the seminal plasma itself or its preparation. For example, the prostasome component comprises particles of varying size, between 50 and 200 nm diameter. Clearing viscous seminal fluid of these depends on the degree of ultracentrifugation. Highly purified plasma is moreover deprived of lipid which is relevant in some assay systems (e.g., TF). Hence values may become dispersed and not give representative values of the real concentrations of these factors. The kits used may also be either diagnostic ones or those labelled "for research purposes". Whether this represents a difference in content or merely a hedge against misuse is unclear. However, some of the results reported in this review include acceptable measures of reproducibility and reliability for the methods used, including intra- and inter assay coefficients of variation.

Seminal clotting/fibrinolytic factors across species

As far as seminal coagulation/fibrinolytic factors are concerned the number of reports is limited and the proteins and mechanism involved are different according to species. The NH2-terminal sequence of human PSA and the canine protease suggest that these proteins are species analogues (65, 66). Carson et al. caution against the possibility of extrapolating results across species (39). Structural differences of the seminal clot between humans and rodents have been reported (67). In mice PCI is only synthesized in the genital tract, where it is needed for spermatogenesis and male fertility (68). Bovine semen was not comparable to human semen with respect to TF activity (67). While rat semen forms an insoluble plug within minutes of expulsion, with transglutaminase-mediated cross-linking, no such transglutaminase-mediated cross-linking has been demonstrated in ejaculated human semen (36).

Comparison of the clotting/ fibrinolytic factors levels in seminal and blood plasma

Tissue factor levels in semen are far greater than those of blood plasma. In fact, it is only the soluble TF "extra cellular domain" form that can be measured in plasma and it averages about 85 pg/ml (69). Seminal TF on the other hand, averages about 21 ng/ml (28). It appears, however, that TF concentration in semen is slightly higher than that of uTF, which averages about 7 ng/ml (70). As yet the structural form of seminal TF is not fully known. Seminal factor VII may present at picomolar concentrations (39), which contrast with the nanomolar concentrations that are normally found in blood plasma (71). The seminal concentrations of TAT-III and t-PA were significantly higher than in blood plasma both before and after vasectomy. The DD values as well as the DD/TAT-III ratios were significantly lower than those in blood plasma. The seminal F1+2 concentrations were in the same range for seminal and blood plasma (29). The seminal values for t-PA were fifty times higher than in blood and fifteen times for u-PA (24). Others have reported an increase of up to 100 fold over blood plasma (72, 73). A very high level of PCI (220 mg/l) was found in seminal plasma as compared to 5.3 mg/l in blood plasma (22, 73). An increase of 30 to 40 fold over those found in blood plasma is recorded (64). The concentration of platelet-activating factor-like activity detected in human semen (41) was similar to that reported for the rabbit (74).

Variations in coagulation/ fibrinolysis and the effect of vasectomy

In ejaculates obtained from selected infertile couples, the extent of coagulation showed a significant positive correlation with liquefaction time, sperm count and motility (74). A significant variation in the amount of coagulum and liquefaction time between fertile and infertile ejaculates has also been reported. This however was not related to the semen volume. It was suggested that a possible relationship between the coagulation and the liquefaction equity of human ejaculates and their semen quality might exist.

Coagulation

The degree of coagulation of ejaculated semen may be correlated with epididymal function (20) and subfertility may be associated with poor coagulation of the ejaculate caused by lower osmolality and buffering capacity and a higher pH than samples showing good coagulation (75). Under scanning electron microscopy both the amount and the structure of the coagulum have been reported to vary in ejaculates exhibiting different states of coagulation (76). Comparison of presumptively fertile and infertile ejaculates also revealed significant variations in their amount of coagulum and liquefaction time (77). The state of coagulation in human semen was postulated in 1985 to be capable of development into an effective vaginal contraceptive (76). These studies addressed the seminal coagulation-liquefaction system independent of the enzyme and inhibitor factors that are commonly associated with the haemostatic system which may, in semen, be equally if not more relevant, especially if the function of seminal haemostatic factors mimic those of blood-coagulation/fibrinolytic enzymes.

Normal blood coagulation activation proceeds through a TF-dependent pathway. Tissue factor also has other functions independent of its role in provoking coagulation activation. It may play a role in controlling the balance of angiogenic and anti-angiogenic factors (78, 79). The relationship between neo-

vascularisation, angiogenesis and angiogenic proteins is not only important in cancer but also in non-neoplastic conditions. Over expression of TF in wounds, modulates wound healing by stimulating neovascularisation into the wounded area (80). Moreover, TF has also been suggested to play an important role as a morphogenic factor during early embryonic development (81, 82).

In semen, it has been shown that the potent clotting activity is due to a very high concentration of functional TF (28, 38, 39). Seminal TF may protect against anti-sperm antibody development and against transmission of infectious agent (28). Tissue factor in semen may serve to limit bleeding and consequent vascular access by semen-born agents as a result of tissue damage during intercourse and may also contribute to the anti-inflammatory properties attributed to prostasomes (28, 48). Tissue factor function in semen itself may not be directly related to its role in blood coagulation, as there is an absence of factor X (the natural substrate for TF and factor VII/VIIa complex). In addition, there might be a limited amount of factor VII or VIIa in semen at picomolar concentrations. It is therefore possible that TF in semen may have entirely different actions, which are as yet to be identified. It is tempting, however, to suggest that the female organ may provide the so-called "missing-seminal clotting factors" (e.g., factor X) in order for the clotting process to proceed. An as yet unpublished study in our department has shown, however, that cervical mucus did not contain factors X and VII or IX activity (n=10). Evaluation of semen TF in patients attending an infertility clinic showed 16-fold variation in TF-VII activity but no relationship was found between TF and number of days of abstinence before sampling, specimen pH, sperm counts or sperm motility (39). It is worth mentioning that these results were based on sixteen observations; a larger study may show an association not evident due to the limited number in this group. Other hypothetical roles for seminal TF include fertilisation and implantation potential. The subject is addressed in detail below.

Fibrinolysis

Failure in the liquefaction process of semen has often been seen in male infertility patients (59). Several seminal enzymes have been suggested to be involved in the liquefaction "fibrinolysis" of the human seminal coagulum (83-86). Of particular interest in this context is the fact that the first fraction of the ejaculated semen enhances the liquefaction of the coagulated third fraction that normally lyses quite slowly. Tauber et al. noticed that fraction three of one donor in their study did not possess neutral protease activity and required a longer time to liquefy (2 hours) (34). The addition of fraction one from a normal donor, which would particularly contain plasminogen activators and the neutral protease, resulted in rapid liquefaction of this same sample. This suggests that the liquefying factor(s) are primarily found in the first fraction (87). Further indirect evidence linking the liquefaction of human seminal plasma and the fibrinolytic and proteolytic enzymes can be drawn from the following studies. A significant correlation has been observed between sialyltransferase activity and the liquefaction time for seminal plasma. Similarly, seminal plasma levels of L-ascorbic acid, total dehydroascorbic acid and glutathione increase; there is a decrease in sperm motility and a decrease in the liquefaction time of the seminal plasma (88). The presence of a stable seminal plasminogen activator has been demonstrated since 1960 (89), the secretary organs probably being the prostate, the Cowper's gland and seminal vesicles (90). Both seminal plasma and sperm were later shown to contain plasminogen activator antigens (91). Plasminogen activators were localized to the plasma membrane and the outer acrosomal membrane of the sperm and it was suggested that they may be involved in the fertilization process (92, 93), i.e., a trigger of the zona reaction. Matsuda et al. showed that a strong plasmin inhibitor, 6-amidino-2-naphty-6-guanidinobenzoate dihydrochloride (Fusan), significantly inhibited the seminal liquefaction process (59). This is strong evidence for the possible role of plasmin in semen liquefaction.

The function of tissue plasminogen activator activity in semen is not fully understood. It has been suggested that it may prevent the occlusion of tubular structure in the urogenital tract (9). It may also participate in the liquefaction of seminal coagulum and play a role in the removal of occasional fibrin deposits in the passageway of semen (2). Indeed, the addition of plasminogen activators increased sperm motility according to Hong et al. (94), however, subsequent work failed to support this finding (91, 95). Interestingly, sperm from infertile men showed significantly high levels of plasminogen activator antigens and plasminogen activator inhibitor activity (96). Seminal plasminogen activator levels were also high in a testosterone enanthate-induced azoospermia or oligozoospermia in both man and experimental animal model. A negative correlation has been reported between plasminogen activator inhibitor activity and sperm motility (91). Whether this is the cause or consequence of impaired semen quality remains to be established.

Coagulation/fibrinolysis balance in vasectomized patients

Vasectomy may predispose to cardiovascular diseases (97), cause antisperm antibody formations (98) and impair the endocrine function of the testis (99). In vasectomized patients there was an alteration in the coagulation/fibrinolysis balance. For instance, the DD concentrations and the DD/TAT-III ratios were significantly lower post-vasectomy as compared to pre-vasectomy patients (29). D-dimer/TAT-III ratios were also low in infertile patients (9). This shift in DD/TAT-III ratios in vasectomized and infertile patients suggests a disturbance of the balance between the coagulation and fibrinolytic system (9). This might

have been caused by a shift towards coagulation activation, by a reduction of fibrinolysis, or both and is independent of the number of sperm previously present (9). No shift after vasectomy was seen in TAT-III, F1+2, and tissue plasminogen activator activity (29). It has been suggested that there is a relationship between the coagulum liquefaction properties of human ejaculates and the semen quality and that there is an association between sub-fertility and/or coagulum formation after ejaculation (20, 34). This is reinforced by the finding that infertile semen, infertile semen after vasectomy and semen from involuntary childless subjects showed rather high TAT-III values with moderate DD values (9). This suggests a retardation of the liquefaction of the coagulum, which may reduce the motility of the sperm. Taken together, these observations suggest a possible relationship between the coagulation-liquefaction property of human ejaculates and their semen characteristic. Differences in the concentrations for clotting/fibrinolytic factors in semen and blood plasma both before and after vasectomy, independent of the time or surgical procedure, have also been reported (19, 29).

Potential effects of coagulation/fibrinolysis factors on fertilization and during implantation

Following semen deposition in the upper vagina, much of the movement of sperm through the uterus to the fallopian tubes results from muscular activity, so the uterus is exposed to diluted semen but what concentration is left at fertilization in the fallopian tubes is unclear. Donation of molecules to sperm e.g., by fusion with prostasomes (50, 51) is a possible mechanism ensuring transport without dilution. These include TF (28), uPA, tPA (91, 96), PAI-1 (100) and PCI (101). It is also known that bovine seminal peptide YY-2 is present and important at fertilization. Clotting factors occur in the testis, but downstream products from the prostate or seminal vesicles will be totally absent. When sperm are derived directly from the testis (even after cryopreservation) and used for intracytoplasmic sperm injection, fertilization and implantation rates are entirely satisfactory (102).

Fertilization

In our laboratory, TF antigen has been detected on sperm using immuno-florescence labelling. When a variety of cells are disturbed or stimulated, the phospholipid asymmetry of the plasma membrane is reversed (44, 103). Sperm capacitation and the acrosome release reaction involve changes in the sperm plasma membrane that would increase binding to coagulation factors (39). Vitamin K-dependent blood coagulation enzymes bind membranes with exposed phosphatidylserine in a calcium-dependent fashion (39). Cellular TF is membrane bound and its expression is related to membrane lipid flipping as detected by Annexin V binding (44). The availability of anionic phospholipid on the outer leaflet of the cell membrane could also regulate the TF/VIIa functional activity. Annexin V has been suggested to be a very sensitive marker in detecting deterioration of sperm membrane function.[104] Decrease in the sperm phospholipid levels was thought characteristic of unexplained infertile patients by Glantz.[105] Interestingly, the acrosome release reaction can be inhibited by benzamidines,[106] protease inhibitors known also to inhibit coagulation enzymes. By analogy with other cell types, changes of sperm TF levels may be related to changes in sperm phospholipid composition. Recently it has been suggested that TF-FVIIa mediates cell signaling by two distinct mechanisms, dependent either on TF cytoplasmic domain or TF-FVIIa protease activity (107). Recent work by our group has demonstrated the presence of both FVII and FVIIa in human semen at quantifiable levels.

Platelet activating factor is a potent phospholipid with a wide range of biological activities in a variety of circumstances including platelet aggregation (108), vascular permeability (109), and embryogenesis (110). Its role in semen is not fully understood. It is also present in human amniotic fluid prior to the onset of labour and may feature in the process of parturition (111). Platelet-activating factor like activity was identified in a lipid extract of sperm and a significant motility enhancement of human sperm upon treatment with synthetic PAF has been reported (41). Moreover, it has been suggested that the amount of PAF in a particular ejaculate may be related to the fertilising potential of sperm (41). It is also involved in murine and rabbit gamete interaction *in vitro* (112, 113). Platelet activating factor-treated human sperm enhances the penetration rates of zona free hamster oocytes (114, 115).

It has been postulated that the fibrinolytic activity of the seminal fluid facilitates the passage of sperm through the cervical mucus (116). However, suppressing this activity by a synthetic inhibitor of fibrinolysis does not impair the ability of sperm to penetrate the mucus (117). Also the effect of the seminal fibrinolytic activity facilitating penetration of the zona pellucida could be derived from an activator of protease occurring in or contaminating the sperm head (117, 118). There remains the possibility; therefore, that inhibition of acrosomal fibrinolytic activity may inhibit fertilization. Activities resident on the sperm head represent the mechanism by which seminal haemostatic factors could impinge on *in vitro* fertilization, dependent on whether they are intrinsic or acquired.

Implantation

Molecular mechanisms in human endometrium during implantation are beginning to be understood. Implantation, which has strong haemostatic associations, follows in the second week of

pregnancy. There can be little remaining from the fertilizing semen in the uterus, but it might have played a part in preparing the uterine wall for possible future implantation. Endometrial changes are essential to allow the establishment of a successful pregnancy (119). They occur in stromal fibroblast-like cells, which undergo a characteristic decidualization reaction (119). The process of implantation and placental development involves tissue remodelling including breaching of endometrial blood vessels by trophoblast cells. Therefore there is a risk of bleeding and the maintenance of endometrial haemostasis is critical to the continuation of vascular stability and prevention of fibrinolysis (120, 121). The latter two functions are opposed by u-PA and t-PA, respectively (121). Immunohistochemical expression of TF and PAI-1 in the decidual cells of luteal-phase or gestational endometrium is enhanced (120, 122). Expression of TF and PAI-1 is induced during progesterone-stimulated decidualization, while progesterone withdrawal reduced TF and PAI-1 and increased tPA and uPA expression (121, 123). *In vitro,* human endometrial cells isolated from first trimester endometrium demonstrate that progestins enhance antigenic and functionally active TF and PAI-1 protein and elevated stromal cells' TF and PAI-1 mRNA expression, suggesting that the decidua, activated by trophoblasts, maintains haemostasis and control trophoblast invasion (124). Sequestration of PAI-1 in the extracellular matrix through vitronectin binding (125) results into conformational change that greatly augments PAI-1 inhibitor activity (120). During human implantation and placentation, plasmin is considered important for both trophoblast migration, invasion and fibrin surveillance (126). uPA mRNA was localized to placental trophoblast, epithelial plaque and endometrial stroma, while tPA mRNA was mainly expressed in glandular cells of endometrium (127), suggesting a fibrinolytic role for the latter during implantation. On the other hand, u-PA binds to trophoblast receptors where it can overcome the conversion of plasminogen to plasmin by trophoblast derived PAI-1 and PAI-2 (119, 127-130). The distribution of uPA protein and its cell surface receptor (uPAR) suggests that they mediate trophoblast adhesion, migration, differentiation, and invasion (perhaps involving transforming growth factor beta) and also play a significant role in angiogenesis (127, 131, 132).

The involvement of other protease/protease-inhibitors

Proteases and protease inhibitors are known to play many significant roles in various biological systems and are important for the reproductive functions of mammals. Prostate-specific antigen degrades the major seminal coagulum forming proteins, the kallikreins stimulate human sperm motility, and PCI modulates the acrosin activity. The following proteins are characteristic of semen but possess known interaction with elements of the haemostatic system.

Prostate-specific antigen

Prostate specific antigen (PSA, also known as P-30 and hK3) was first described in 1979 (133) but some of its characteristics were reported as early as 1971 where it was called γ-seminoprotein (134). It is one of the most abundant prostate-derived proteins in the seminal fluid (133, 135). Seminal PSA concentration is about 0.35 mg/ml compared to 0.6 ng/ml in blood (136). About two thirds of seminal PSA is enzymatically active, the remaining 30-40% is inactive due to internal cleavage(s) (137). In semen, PSA seems partially bound to PCI whereas in serum it is predominantly associated to alpha-1-antichymotrypsin and in a small quantity to alpha-2-macroglobulin (138, 139). During coagulum dissolution in freshly ejaculated semen, approximately 40% of immunodetected PCI becomes complexed to PSA (140). In semen complexes between PCI and PSA are detected at levels that correspond to an inactivation of up to 5% of the PSA activity in the ejaculate (140). Functionally and structurally, PSA manifests great similarities to glandular kallikrein-like proteases (5, 61). Many factors may influence PSA synthesis and production, and among them the most important are androgens, retinoic acid and growth factor stimulation (139). Prostate specific antigen may play a role in the proteolysis of the seminal coagulum (5, 61). It degrades the major "gel forming proteins" of the seminal coagulum, Sg-I and Sg-II, and fibronectin, leading towards semen liquefaction (139). Prostate specific antigen concentrations are indirectly related to semen quality (141). Sperm motility is an important predictor of male fertility and along with sperm concentration, the level of seminal PSA was the most significant and independent parameter in predicting percentage of motile sperm (142).

Prostate-specific human glandular kallikrein 2

Human kallikrein 2 (hK2, also known as hGK-1) is an active trypsin-like protease in human seminal fluid (143). Human kallikrein 2 is one of three serine proteases coded for by the human kallikrein gene family, which includes hK1 (tissue kallikrein) and hK3 (PSA). It has 80% homology to PSA and about one-half of the hK2 is found covalently complex with PCI, PAI-1 and alpha1-anti-chymotrypsin (143, 144). Human kallikrein 2 also formed molecular complexes with alpha 2 -antiplasmin, antithrombin III and alpha 2-macroglobulin but not with alpha 1-antitrypsin (28). Unlike PSA, hK2 activates pro-PSA, pro-hK2 and the zymogen form of urokinase-type plasminogen activator. Human kallikrein 2, immediately after ejaculation, was recovered only in its free form but complex formation with PCI occurred rapidly thereafter and was completed within 10 minutes (145). Protein C inhibitor may regulate its activity in seminal plasma (146). Kinins are important regulatory peptides (especially for vascular permeability), and they may have a role

in enhancing sperm motility (143). High-molecular weight kininogen, which contains the nonapeptide bradykinin, is the substrate for plasma kallikrein (pKa potent kinin-generating enzyme circulating in blood) and for hK1. Although Glandular kallikrein is about 500-fold less active than is plasma kallikrein or tissue kallikrein, they may play a physiologically important role in bradykinin release in seminal fluid (143). Like PSA, free hK2 was shown to hydrolyze semenogelins and fibronectin *in vitro*, though no common cleavage sites were identified for both proteases (145). Unlike semenogelins, fibronectin was hydrolyzed much more efficiently by hK2 than by PSA. This suggests that hK2 is enzymatically active during a short period of time after ejaculation, and that major seminal vesicle proteins can be the target of this proteolytic activity, and also that hK2 and PSA may have different substrate specificities. The absence of seminal hK2:PCI complex is suggestive of obstructive azoospermia (147).

Tissue kallikrein

Tissue kallikrein (tKK, or hK1) is a serine protease with restricted trypsin-like activity that upon cleavage of kininogen precursors liberates bradykinin (148). The primary structure of the molecule displayed about 62% sequence identity with PSA (148). About 28% of the total seminal tKK immunoreactivity is forming complexes with PCI. *In vitro* studies showed that the tKK:PCI complex formation in semen was accomplished in about 1 h and that heparin stimulated both the rate and the extent of complexation of tKK with PCI (149). When semen was collected in the presence of tKK inhibitors it had only about 6% of the tKK complexed to PCI (149). This suggests that PCI is a physiological inhibitor of tKK and provides further evidence of the involvement of PCI in male reproduction.

Protein C inhibitor

Protein C inhibitor (PCI, also called plasminogen activator inhibitor-3) is a heparin binding serine protease inhibitor (serpins) that has broad protease specificity; acting as an anticoagulant, anti-anticoagulant, antifibrinolytic and anti-antifibrinolytic factor. It is also needed for spermatogenesis (150-154). Protein C inhibitor is present in semen at a relatively high concentration in both high and low-molecular-mass form (160 +/- 20 micrograms/ml, mean +/- SD). This is more than 30-40 times the concentration of PCI found in blood plasma (5 micrograms/ml) (21, 23, 140, 155). However, the structure of seminal PCI is identical to that of plasma PCI (23). Protein C inhibitor may protect intact surrounding cells and seminal plasma proteins from proteolytic damage (156). It is a potent inhibitor of acrosin and sperm-egg binding and has been localized on the acrosomal cap of human sperm. Thus it may protect sperm against premature acrosome reaction and degradation, thereby facilitating fusion with the oocyte (150). Some patients under investigation for involuntary barrenness have shown significantly reduced levels of PCI (110 +/- 35 micrograms/ml) (21, 157). Protein C inhibitor levels in seminal plasma of patients with seminal vesicle and/or vasal agenesis was significantly reduced (157). A high amount of PCI is secreted by the seminal vesicles and about 85% of which can remain active, even at autopsy (21). There is, however, conflicting evidence about seminal PCI stability after ejaculation. In one study only 45% activity remained after ejaculation and this loss continued upon incubation at 37°C (21) being almost undetectable after two hours (21). In contrast Kise et al. showed that seminal PCI levels were stable at 48 hours after ejaculation and suggested that it could be used as a marker for agenesis of seminal vesicles and/or the vas deferens (157). Activated PCI complexes with PSA (PCI:PSA) in seminal plasma may be responsible for its partial inactivation. Sperm or other soluble or trapped components may reduce the rate of protein C inactivation following ejaculation. Oligospermia could affect protein C activity in a similar way (21). Protein C inhibitor may also act as a physiologic regulator of urokinase-type plasminogen activator and t-PA in male reproductive tissues (155). The presence of functionally inactive PCI in seminal plasma may be associated with infertility (156). Protein C inhibitor may therefore be involved in human reproduction at several steps, including the fertilization process (158). Recently it has been shown that male PCI knockout mice are infertile (68), suggesting a significant physiological role for PCI in male reproductive system.

Other inhibitors of proteases in semen have roles to play in global fertility but do not appear to impinge directly on the haemostatic process. These, however, are not the subjects of the present review and have been discussed elsewhere (69, 160-166).

The possible impact of genital infections on seminal haemostatic factors

Activation of blood coagulation factors is an important manifestation of systemic inflammatory responses of the host to severe infection (167, 168). Pro-inflammatory cytokines that would be stimulated by infection also affect haemostasis (e.g., IL12, IL18 and INF-gamma) (169). In particular, regulation of TF expression occurs at the transcriptional levels in response to cytokines, growth factor and serum (170-174). IL18 is also critical in the implantation process (175). Semen has antibacterial components of importance (176, 177). Seminal plasmin is among these (178) as are prostasomes (179). These may assume an increased significance, viewing the embryo as a graft, as semen modulates immunity away from cell mediated immunity and natural killer surveillance, through switching the IL10:IL12 balance (180,

181). Esmon et al. describe an inflammatory/coagulation spiral, driven by the protein-C pathway that progresses to septic shock (167). The inhibitor of protein-C is also described in this review as being a critical link between the HMW-SV-proteins and the classical coagulation systems in semen (Fig. 1). Work directly linking seminal haemostatic factors to genital infections remains sparse; however the relationships may be similar to those occurring in blood. The area deserves further investigation.

Conclusion

Haemostatic factors play a significant role in reproductive physiology from the presence of a functional coagulation system in semen through fertilization to implantation, where extensive tissue remodelling relies on maintaining a haemostatic balance. Semen clots and liquefies in a process that has some similarity to that of normal blood. Differences in the constituents and the liquefaction time across semen's split ejaculates have been observed. The fact that many clotting and fibrinolytic factors have been identified in semen reflects a potentially functional presence of the classical coagulation and fibrinolysis systems. Excess of either may compromise fertility i.e., too much clotting activity may cause the sperm to agglutinate and ultimately be destroyed. On the other hand, increased fibrinolytic activity may cause the sperm to stick to other parts of the genital tract and fail to reach the egg. Thus, a proper balance between these two systems should be maintained. Studying the function of clotting and fibrinolytic systems in semen and the interactions between these and the semen specific HMW-SV proteins may have significant clinical ramifications in male reproductive pathophysiology.

Future research directions

The source of many of the haemostatic components detected remains opaque. In particular the alternative possibility of local production or extravasation requires clarification.

- Studies of the distribution and the quantity of the seminal clotting/fibrinolytic activities within split ejaculates are essential in order to understand fully the mechanisms involved in the seminal coagulation and liquefaction processes. Loss of activity and complex formation subsequent to ejaculation should also be considered.
- Clotting and fibrinolytic factors should be assessed in large-scale studies and their levels should be related to conventional fertility markers. The similarity between the DD/TAT-III ratios in involuntary childless patients and in post vasectomy patients is a remarkable finding that warrants further investigation.
- PSA, PCI and their interactions may represent points of functional contact between the HMW-SV proteins system and the haemostatic pathways. This relationship is much under-investigated.
- The ability of haemostatic enzymes to interact with specific seminal proteases and proteases inhibitors to influence the capacity of sperm to fertilize eggs requires assessment.
- Infection control is another area where, by analogy with the situation in the vascular compartment, there is much scope for further evaluation of the role of seminal haemostatic factors.

Abbreviations

TF: Tissue factor; uTF: Urinary tissue factor; VIII:c: Factor VIII coagulant activity; F1+2: Prothrombin fragments 1 and 2; TAT: Thrombin-antithrombin; TAT-III: Thrombin-antithrombin III; DD: D-dimer; PAF: Platelet activating factor; t-PA: Tissue plasminogen activator; u-PA: Urinary plasminogen activator; uPAR: Urinary plasminogen activator receptor; PAI-1: Plasminogen activator inhibitor-1; PAI-2: Plasminogen activator inhibitor-2; PCI: Protein C inhibitor; PSA: Prostate specific antigen; hK2: Human glandular kallikrein 2; pKa: Plasma kallikrein; tKK: Tissue kallikrein; HMW-SV proteins: High molecular weight seminal vesicle proteins; Sg-I and Sg-II: Semenogelins-I and II; SPMI: Seminal plasma motility inhibitor.

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