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Quantitation of Seminal Factor IX and Factor IXa in Fertile, Nonfertile, and Vasectomy Subjects: A Step Closer Toward Identifying a Functional Clotting System in Human Semen

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ABSTRACT: Coagulation factor (F) IX is a zymogen of the plasma serine proteases, one that plays an essential role in the regulation of normal blood coagulation. Congenital defects of FIX synthesis or function cause hemophilia B (originally called hemophilia C). Factor IX is activated by Tissue Factor (TF):FVII/FVIIa complex and FXIa. Subsequent to its activation, FIXa combines with FVIIIa on the platelet surface and activates FX to FXa. Human semen forms a semisolid gelatinous coagulum, which then liquefies within 5–20 minutes in vitro. In spite of evidence demonstrating the importance of the seminal coagulation and liquefaction process in terms of global fertility and despite the fact that the seminal coagulum is composed of fibrin-like material, it has always been addressed from the perspective of High Molecular Weight Seminal Vesicle (HMW-SV) proteins (Semenogelin I and II) and their cleavage by prostate-specific antigen rather than the conventional hemostatic factors. In this study and as part of our continuing investigation of human seminal clotting factors, we report here on seminal FIX and FIXa in normal, subfertile, and vasectomized subjects. Factors IX and FIXa were studied in a total of 119 semen specimens obtained from subfertile (n = 18), normally fertile (n = 34), and fertile sperm donors (n = 27) and vasectomy subjects (n = 40). Seminal FIX and FIXa levels were also measured in a group defined by normality in several parameters derived from the World Health Organization fertility criteria and termed ''pooled normal semen parameters.'' Both FIX and FIXa were quantifiable in human semen. There was a wide individual variation in FIX and FIXa levels within groups. Despite the group size, statistically significant associations with fertility-related parameters were infrequent. There is a positive correlation between FIX and its activation product, FIXa (n = 36; $r = 0.51$; $P < .05$). Factor IXa elevation in the high sperm-clump group was significant ($P < .05$), and days of abstention correlated with FIXa levels (n = 63; $r = 0.3$; $P < .05$). The key finding of the present study is that both FIX and FIXa are present in concentrations that are not dissimilar to plasma levels and that are apparently functional, as the activated form is also present. This fact, taken with other reports of coagulation factors in semen, raises the likelihood that a functional set of hemostatic coagulation proteins exists in semen, potentially to interact with the HMW-SV proteins and the prostate-specific antigen system.

Key words: Fertility, novel finding.

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Factor (F) IX, also known as Christmas factor and antihemophilic B, circulates in plasma as a single-chain polypeptide with M_r 57 000 Da (DiScipio et al, 1977; Katayama et al, 1979). Its congenital deficiency results in an X-linked bleeding diathesis, hemophilia B or Christmas disease. Factor IX is synthesized by the hepatocytes in a highly tissue-specific manner (Kurachi and Kurachi, 1995) and is subject to posttransitional modifications (Taran, 1997). It is the largest of the vitamin K–dependent plasma proteins and contains 12 τ-carboxyglutamic residues; the first 9 are responsible for the calcium-dependent phospholipid membrane binding (Astermark et al, 1991; Freedman et al, 1995). Factor IXa is composed of N-terminal light chain and a C-terminal heavy chain linked via a disulphide bond (Brandstetter et al, 1995). It is activated to a multidomain protein, FIXa (Yoshitake et al, 1985; Brandstetter et al, 1995) Tissue Factor (TF):FVII complex FVIIa (Østerud and Rapaport, 1977) and FXIa (DiScipio et al, 1978). Factor IXa activates FX to supplement FXa initially generated by TF:FVII/FVIIa complex. This is essential to sustain FX activation, since TF:FVII/FVIIa activity is temporally limited in a FXa-dependent fashion by a stoichiometric inhibitor of blood coagulation, Tissue Factor Pathway Inhibitor (TFPI) (Broze, 1992; Rapaport and Rao, 1992). Independently, FIXa has little activity on its natural substrate FX. However, complex formation with its co-factor FVIIIa (FIXa:FVIIIa) causes a dramatic increase in FX activation. Similarly, FVIIIa alone has no proteolytic effect on FX (Neal and Chavin, 1979). The interaction between FIXa and FVIIIa is calcium mediated but lipid independent. In vivo, however, this reaction occurs on the surface of the endothelial cells or activated platelets (van Dieijen et al, 1981), where

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a suitable membrane phospholipid is provided. Mutations in FIXa can affect FVIIIa binding by at least two mechanisms (Bajaj, 1999).

Men are considered to have normal semen parameters when the ejaculated semen has a volume of 2–6 mL, a sperm concentration of more than 20×10^6/mL, a normal sperm morphology of greater than 15%, above 40% of the total sperm population is motile, and a sperm progression of 2.5 or more, with 4 being the maximum level of progression (World Health Organization [WHO], 2000). Fifteen percent of all married couples with normal semen parameters are involuntarily childless. Thus, not all subjects with high counts are fertile and not all subjects with low counts are infertile in practice. However, infertility could equally be caused by male or female factors (Schwarzstein, 1983). Failure in the liquefaction process of semen has often been seen in male infertility patients having low sperm count and/or motility (Matsuda et al, 1994). The coagulation and subsequent liquefaction of human semen occurs within 5 minutes of ejaculation in vivo (Sobrero and MacLeod, 1962) and is prolonged by up to 30 minutes in vitro at room temperature (Amelar, 1962). The predominant structural proteins of coagulated human semen are those that are secreted by the seminal vesicles—the High Molecular Weight Seminal Vesicle (HMW-SV) proteins (Lilja, 1985). The interaction between the seminal vesicle and prostate components leads to the liquefaction of the seminal coagulum (Mandal and Bhattacharyya, 1986). Human ejaculates vary in their degree of coagulation as well as in their liquefaction time (Mandal and Bhattacharyya, 1986).

Seminal coagulum is also composed of fibrin-like material (Polak and Daunter, 1989). The procoagulant activity of human seminal plasma added to blood plasma was first recognized in 1942 (Huggins and Neal, 1942). Seminal plasma diluted up to 10 000-fold significantly decreased the recalcification clotting time of blood plasma (Huggins and Neal, 1942). However, the molecular basis for this observation remains uncertain. In the present study we report on seminal FIX and FIXa, which might provide further evidence for the presence of a functioning clotting system in human semen.

Materials and Methods

Sample Collection

Ethical committee approval was granted for the study by the Southampton and South West Hants Local Research Ethics Committee (submission number 116/01). Informed consent was sought from each subject on arrival at the Andrology Laboratory, Princess Anne Hospital, and the Pathology Laboratory at Southampton General Hospital NHS Trust.

Stratification of Subjects

A total of 119 subjects, aged 20–64 years were studied. We followed the classification of the fourth edition of the WHO guidelines on semen analysis (World Health Organization, 2000): subfertile subjects have sperm counts of less than 20×10^6/mL (n = 18), normal fertility implies sperm counts that are greater than or equal to 20 and less than 60×10^6/mL (n = 34). Fertile subjects suitable for semen donation (labeled below as ''fertile donors'') in Southampton University Hospitals have sperm counts of greater than or equal to 60×10^6/mL (n = 27). Vasectomy subjects were also studied (n = 40). A further classification was defined with all semen parameters normal according to the WHO criteria (volume of 2–6 mL, a sperm concentration of more than 20×10^6/mL, a normal sperm morphology of more than 15% normal forms, above 40% of the total sperm population motile, and sperm progression of 2.5 or more, 4 being the maximal progression value). This we term ''pooled normal semen parameters.''

Processing of Semen Samples

Seminal plasma was prepared by differential centrifugation. Fresh semen samples were placed in 1.5-mL Eppendorf tubes after liquefaction and spun at $2000 \times g$ for 10 minutes at room temperature in Heraeus Biofuge 28 RS centrifuge. This low-speed supernatant constitutes the plasma fraction. The supernatants from this spin were transferred into new 1.5-mL Eppendorf tubes and were deep-frozen at −72°C for batchwise measurement.

Semen Analysis

Conventional seminal fertility parameters were measured according to the WHO guidelines as described in the WHO ''golden'' laboratory manual for the examination of human semen and sperm-cervical mucus interaction (World Health Organization, 2000).

Factor IX and IXa Assays

Factor IX Assay—Factor IX activity was measured by a 1-stage factor assay based on PT/APTT using ACL 300R analyzer (Instrumentation Laboratory, Warrington, United Kingdom). The standard curve was reproducible (n = 4; $r = 0.991$; $P < .001$; Figure 1a). Samples giving values higher than the top standard were diluted, reassayed, and the reading corrected for the dilution factor.

Factor IXa Assay—Factor IXa was measured using spectrozyme fIXa (American Diagnostica Inc, Stamford, Conn) according to the manufacturer's instructions. A standard curve was constructed using recombinant FIXa (American Diagnostica). This was also very reproducible (n = 4; $r = 0.995$; $P < .001$; Figure 1b). Background activation without the substrate spectrozyme fIXa was (0.079 reaction rate) against (0.334 reaction rate) in the presence of spectrozyme fIXa.

Statistical Analysis

Results were entered into a database and analyzed by the STATGRAPHICS™ statistical software system. Summary statistics were expressed as medians and interquartile ranges. Differences

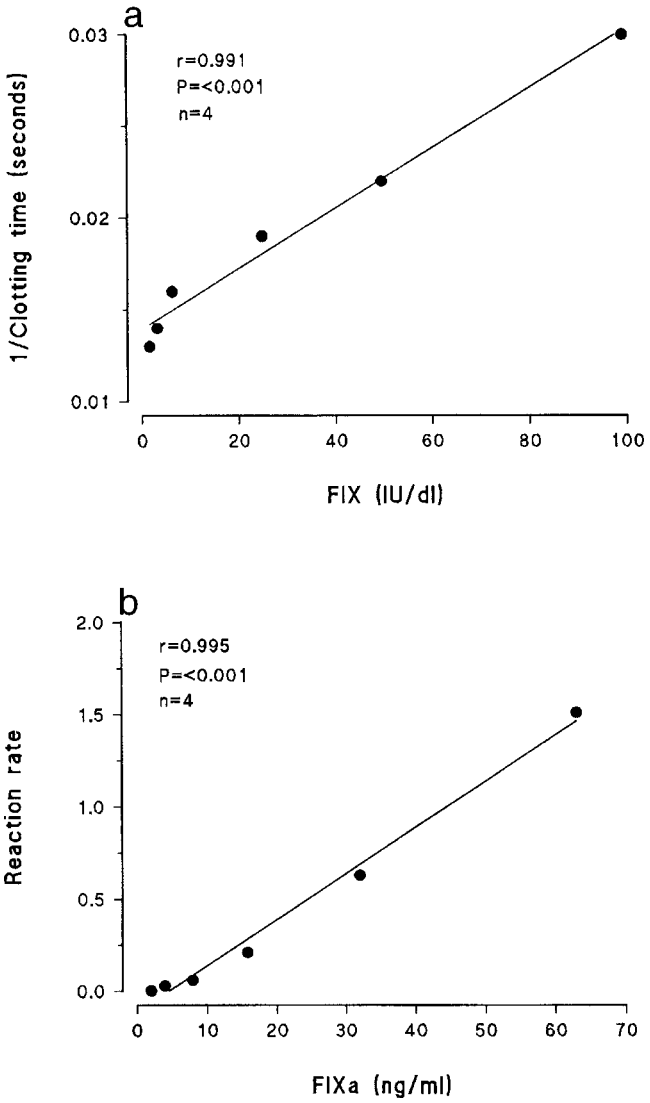

Figure 1. **(a)** A typical standard curve for the factor IX assay. **(b)** A typical standard curve for the factor IXa assay.

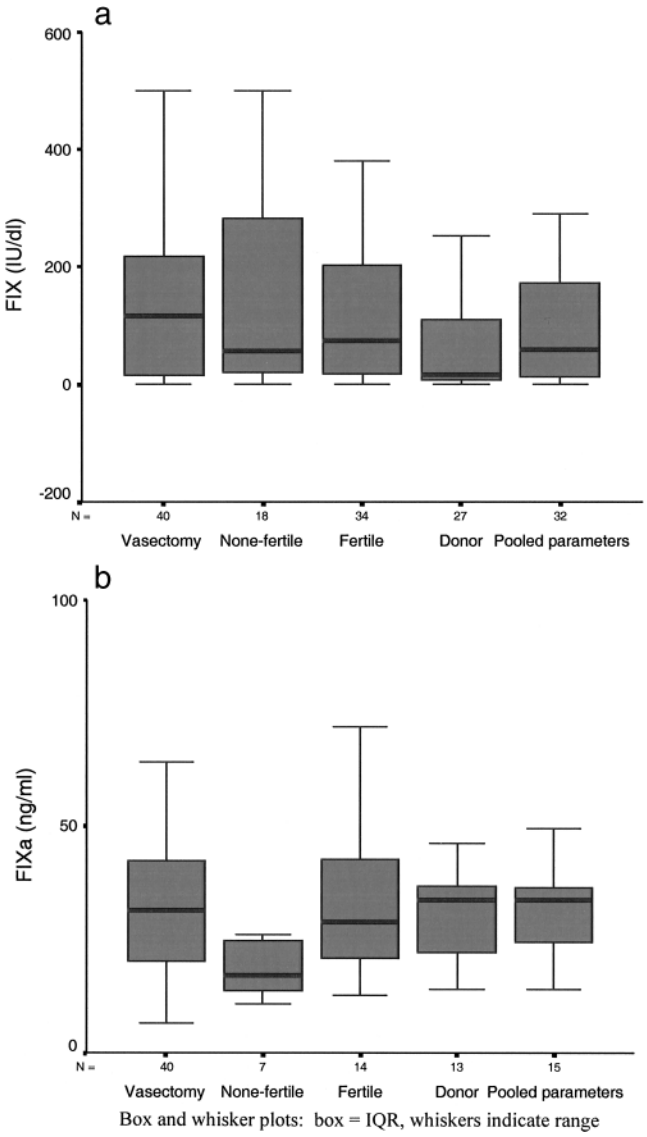

Figure 2. **(a)** Seminal factor (F) IX levels plotted against fertility expressed as sperm count (density). **(b)** Seminal FIXa levels plotted against fertility expressed as sperm count (density).

between two groups were assessed by Mann-Whitney *U* test. Correlations were determined using the Pearson correlation test. Factor IX and FIXa were log-transformed; hence the choice of test. All assay results are expressed per original sample volume for FIX (IU/dL) and FIXa (ng/mL), respectively.

Results

Factor IX and Factor IXa

Both FIX and FIXa were readily quantifiable in seminal plasma from subfertile, normally fertile, and fertile donors and vasectomy subjects. There was a wide variation in FIX and FIXa levels within the studied groups. The fertile donor group showed the lowest median FIX value among the groups, while the vasectomy group showed the highest. For FIXa, the median values are all within a similar range (Figure 2a and b) regardless of group.

Seminal Viscosity and Liquefaction Time

Only FIXa levels were positively and significantly correlated with semen liquefaction time (n = 62; $r = 0.23$; $P < .05$). There was a positive but not significant association between FIX or FIXa levels and semen viscosity (data not shown).

Days of Abstention, Semen Volume, Sperm Count, Motility, and Progression

No significant findings were achieved. The direction of weak trends observed was reversed between FIX and

Table 1. *Factor (F) FIX and FIXa levels and percentages of motile sperm*

Group	n	Median	Interquartile range Lower	Upper
FIX (IU/dL)				
Above 50% motile	46	41	11	103
Below 50% motile	30	56	8	282
FIXa (ng/mL)				
Above 50% motile	25	30	19.3	36.3
Below 50% motile	8	29.6	13.4	42.6

FIXa for motility and progression (Tables 1 and 2) but not for counts, volume, and abstention (data not shown).

Sperm Morphology

Slight increases of FIX and FIXa were observed with abnormal sperm morphology; however, no significant difference between the normal or abnormal morphology groups was observed for the two factors (data not shown).

Seminal Agglutination

Higher level of FIX and FIXa levels were seen in semen showing many clumps. For FIXa levels, the differences between the high and the low clump group were statistically significant ($P < .05$; Table 3).

Anti-Sperm Antibodies

Subjects with anti-sperm antibodies had high levels of FIX, but statistically, no significant difference was observed between the two groups (Table 4). No such trend was seen for FIXa (Table 4).

Discussion

The identification of coagulation FIX as a substance required for normal blood coagulation activation was first established by Pavlovsky in 1947, who reported that a mixture of blood from two hemophiliacs clotted normally

Table 2. *Factor (F) FIX and FIXa levels and sperm progression*

Group	n	Median	Interquartile Range Lower	Upper
FIX (IU/dL)				
Good	50	44	13	103
Average	10	138.5	17	289
Poor	20	47.5	5	267.5
FIXa (ng/mL)				
Good	8	31	13.8	43.8
Average	11	28	14.9	34.4
Poor	14	30.9	19.3	36.7

Table 3. *Factor (F) FIX and FIXa levels and semen agglutination*

Group	n	Median	Interquartile Range Lower	Upper
FIX (IU/dL)				
Many clumps	3*	463	115	500
Small clumps	8	221.5	59	271
No clumps	35	44	8	177
FIXa (ng/mL)				
Many clumps	3*	46.1	36.8	50.8
Small clumps	8	34.0	23.4	40.4
No clumps	14	24.9	17.0	36.3
Vasectomy	40	117	25.5	252

* Range in place of interquartile range.

(Pavlovsky, 1947). Here, and after almost 6 decades, we report the presence of both FIX and FIXa in human semen. However, we remain short of Pavlovsky's finding in that we are not yet sure what function FIX and/or FIXa have in human semen and, consequently, in the male reproductive system.

Semen is known to contain a potent procoagulant activity that was shown to be dependent on FX and Ca^{++}, indicating the presence of FX activator (Fernández et al, 1997). About 90% of the activity was found in the seminal plasma (Carson and De Jonge, 1998). This procoagulant activity was neutralized by monoclonal antibodies to human TF and FVII (Fernández et al, 1997), indicating the presence of TF (Fareed et al, 1995; Ohta et al, 2002). Indeed, evaluation of seminal TF activity in an infertility context showed 16-fold variation, and no relationship was found between TF and number of days of abstinence before sampling, pH, sperm counts, or sperm motility (Carson and De Jonge, 1998). In the light of this finding and the published reports on seminal FXa (Matsuda et al, 2002) and the current, unpublished data by our group demonstrating quantitative levels of clotting factors V, VII, VIIa, VIII, vWF, IX, IXa, Xa, XI, and XII, as well as TFPI, in human semen, we propose the presence of a complete set of blood coagulation factors in semen, bar thrombin—and even in this case prothrombin degradation products (prothrombin fragment 1.2 [F1.2]), thrombin-

Table 4. *Anti-sperm antibodies and factor (F) FIX and FIXa levels*

Group	n	Median	Interquartile Range Lower	Upper
FIX (IU/dL)				
Positive	14	43	11	72
Negative	16	27.5	10	67.5
FIXa (ng/mL)				
Positive	5	50	49	52.5
Negative	6	46.5	45	53

like enzyme, anti-thrombin III, and thrombin anti-thrombin III complex are present (van Wersch et al, 1992, 1993; Park et al, 1997). Fibrin monomer has also been detected in human semen (van Wersch et al, 1992). In blood plasma, however, when fibrin is formed it is known to provoke fibrinolysis by stimulating tissue plasminogen activator activity, which is present in a rather high concentration in semen (Christ and Binder, 1989). The result is the formation of D-Dimer degradation products, which can only originate from previously formed cross-linked fibrin (covalent). D-Dimer was found in semen in readily measurable concentrations that even permitted the formation of the D-Dimer/thrombin anti-thrombin III complex in seminal plasma (van Wersch et al, 1992, 1993). In addition, semen contains several other fibrinolytic factors, including tissue and urinary plasminogen activators, fibrinogen/fibrinogen-like substance, and plasmin, as well as detectable levels of Plasminogen and Plasminogen Activator Inhibitor-I (PAI-1) (van Dreden et al, 1991; van Wersch et al, 1992; Park et al, 1997). Vitronectin, a regulator of coagulation and fibrinolysis, is also found in semen (Bronson and Preissner, 1997). It protects thrombin from a rapid heparin-dependent inactivation by anti-thrombin III and inhibits the fibrin clot–induced activation of plasminogen by tissue type plasminogen activator. Vitronectin also binds to PAI-1 and stabilizes its inhibitor activity (Schvartz et al, 1999). Taken together, these reports indicate that semen contains a functioning clotting system and that seminal coagulum is at least partly composed of fibrin (van Wersch et al, 1992).

In the present study, however, we discuss our findings with respect to seminal clotting FIX and FIXa and their possible role in male fertility. Concentrations of FIXa were of the same order as the range conventionally accepted for blood plasma (van Hylckama Vlieg et al, 2000). Both FIX and FIXa were measurable at slightly higher concentrations in semen from normal, nonfertile, and vasectomized subjects. We observed a wide variation in FIX and FIXa levels within these groups; as a result, no statistically significant difference could be demonstrated. Wide variation also occurs in peripheral blood. In healthy subjects, individual plasma FIX activity and antigen levels also varied between 50% and 150% of a pooled plasma value (Reiner and Davie, 1994). However, the semen available for assay (from fertility clinics) had inevitably already coagulated and had also subsequently liquefied. This is not directly analogous to plasma, or even serum. Overall, there is substantially more FIXa than FIX in semen. The fertile donor group showed the lowest seminal FIX value, whereas the vasectomy group showed the highest median value (Figure 2a). While the median values for seminal FIXa were lowest in the nonfertile group, all the other medians were similar (Figure 2b). A positive significant correlation was observed between

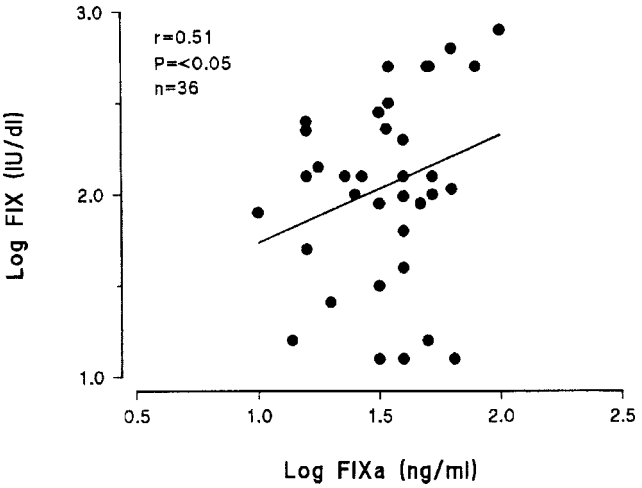

Figure 3. Pearson correlation between Log-factor (F) IX (IU/dL) and Log-FIXa (ng/mL).

seminal FIX and FIXa levels (n = 36; $r = 0.51$; $P < .05$; Figure 3). This is indicative of a functional presence, as both TF and FVII/VIIa, which activates FIX directly, are also found in semen (Carson and De Jonge, 1998). Both FIX and FIXa showed a positive but insignificant correlation with seminal TFPI (n = 119, $r = 0.2$, $P > .05$; n = 68, $r = 0.22$, $P > .05$, respectively, for FIX and FIXa). Attempts to find associations between concentrations of FIX or FIXa and conventional correlates of fertility encountered limited success. The difference between the high and the low sperm-clump groups was only significant for FIXa levels (FIXa in clumped semen is greater than that for nonclumped semen, $P < .05$), and days of abstention correlated with FIXa levels (n = 63; $r = 0.3$; $P < .05$). This paucity of strong correlations may reflect difficulties in obtaining a numerical index for male fertility in practice. Very large studies would be required to determine whether any of the less-than-significant trends observed were in fact true associations and even if so, the distinction between small significant changes and pathophysiologically meaningful differences is another step further removed.

The mere presence of clotting and fibrinolytic factors in semen could reflect transudation from blood plasma. However, in the context of this article, the levels of FIXa obtained in semen, which necessarily must have both coagulated and liquefied before it can be measured, are on the same order of magnitude as those of blood plasma. This is a high proportion for a large molecule, considering that only about 1% of the much smaller albumin and immunoglobulin G molecules, are recovered in seminal plasma after transudation from the prostate (Rümke, 1974). Factor IX product can be localized immunohistochemically in the prostate, but in situ hybridization stud-

ies have not yet been undertaken to assess whether there is mRNA present.

Congenital deficiencies of the coagulation factors are rare disorders, and FIX deficiency in particular is less common. In most cases it is a mild bleeding disorder, autosomally inherited (Bolton-Maggs and Pasi, 2003). Severe hemophilia B may become mild and then may show a normal concentration of FIX activity (Bolton-Maggs and Pasi, 2003). For instance, FIX activity changes significantly with age in individuals with mutations in the promoter sequence of the gene such as ''hemophilia B Leyden'' (Briet et al, 1982; Reitsma et al, 1988; Giannelli, 1997) but not in ''hemophilia B Brandenburg,'' which, in addition to disrupting the binding site of the transcription factor LF-A1/HNF4, also disrupts the androgen responsive elements that overlap the LF-A1/HNF4 site (Crossley et al, 1992). More than 2100 mutations in the FIX gene have been identified (www.kcl.ac.uk/ip/petergreen/haemBdatabase.html). Studies on seminal FIX in subjects with congenital defects in FIX synthesis or function whom are at risk of developing a severe bleeding diathesis would be interesting; in particular, the influence of FIX gene mutations on seminal product levels should be studied.

While we propose that the seminal clotting system may be functional and that it may somehow be involved in seminal coagulum formation, we suggest that one possible way through which this could be achieved is by the interaction with the established HMW-SV proteins and the Prostate Specific Antigen (PSA) system (Lwaleed et al, 2004), which is currently believed to be the sole system responsible for seminal coagulation and liquefaction. For instance, activated Protein C Inhibitor (PCI), a heparin binding serine protease inhibitor (serpins) that has broad protease specificity, acts as an anti-coagulant, anti-anti-coagulant, anti-fibrinolytic, and anti-anti-fibrinolytic factor, complexes with PSA, and partially inhibits its activity (Laurell et al, 1992). PCI is present in semen at a relatively high concentration in both high and low molecular mass form (160 ± 20 μg/mL, mean ± SD). This is more than 30–40 times the concentration of PCI found in blood plasma (5 μg/mL) (España et al, 1991, 1993; Laurell et al, 1992; Christensson and Lilja, 1994). During coagulum dissolution in freshly ejaculated semen, approximately 40% of immunodetected PCI becomes complexed to PSA (Christensson and Lilja, 1994). 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 (Christensson and Lilja, 1994).

In conclusion, studies to date on the seminal coagulation factors are limited to a few reports. An important result of this work is that both FIX and FIXa are quantifiable in human semen. This brings us a step closer toward identifying a functional clotting system in human semen and strengthens the suggestion of a link between the coagulation system in semen and seminal coagulum formation. It is becoming increasingly clear that seminal coagulum formation may be in some measure mediated through the conventional factors of the normal coagulation process. Further studies on the role and functions of seminal clotting factor and its relationship to the HMW-SV proteins in the pathophysiology of male fertility are warranted. Evaluation of the seminal clotting/fibrinolytic proteins could also be useful in assessing the secretory function of the accessory genital glands in fertile and infertile men.

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