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Semenogelin, the Main Protein of Semen Coagulum, Inhibits Human Sperm Capacitation by Interfering With the Superoxide Anion Generated During This Process

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ABSTRACT: Semenogelin (Sg), the major protein of the human semen coagulum, is present at high concentrations in seminal vesicle secretions. It is degraded by the prostate-specific antigen (PSA) to generate peptides of various biological activities that were found on and inside spermatozoa. Our aim was to determine the effect of Sg on capacitation, which is the series of transformations that spermatozoa must undergo to become fertile. At concentrations of 0.1 to 1.0 mg/mL (600- to 20-fold lower than those of semen), Sg did not affect sperm motility (%) but completely prevented capacitation induced by fetal cord serum ultrafiltrate; a partial inhibition of capacitation was noted with 0.03 mg Sg/mL. There was also a dose-dependent decrease in the tyrosine phosphorylation of fibrous sheath proteins and in the O_2^{-}•-related chemiluminescence. Ribonuclease (RNase), which has as high an isoelectric point (pI = 9.7) as Sg (pI = 9.5), also prevented sperm capacitation and O_2^{-}•-related chemiluminescence but to a lower extent, suggesting that one mechanism of Sg action on spermatozoa could be related to its positive charge at physiological pH. Sg at 1, but not 0.3 or 0.1 mg/mL, scavenged the O_2^{-}• generated by the mix of xanthine + xanthine oxidase and modified the kinetics of the reaction; RNase did not have such effects. Therefore, Sg is a potential scavenger for O_2^{-}• but probably also affects the sperm oxidase. Spermatozoa rapidly processed Sg; a high proportion of Sg was degraded after 15 minutes of incubation. The resulting polypeptide patterns were reminiscent of those obtained with PSA as a proteolytic enzyme. These data suggest that Sg, its degradation products, or both may be natural regulators of sperm capacitation and could prevent this process from occurring prematurely. One mechanism by which Sg acts could involve an interference with the O_2^{-}• that is normally generated during this process.

Key words: Protein tyrosine phosphorylation, prostate-specific antigen, ribonuclease, protein degradation, seminal plasma motility inhibitor, reactive oxygen species.

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Capacitation is defined as the series of transformations (in membranes, enzymatic activities, ion fluxes, etc) that spermatozoa normally undergo during their migration in the female genital tract in order to reach and bind to the zona pellucida, undergo the acrosome reaction, and fertilize the egg (see de Lamirande et al, 1997 for review). Capacitation is also part of an oxidative process in which the superoxide anion (O_2^{-}•) generated by spermatozoa plays an essential role (de Lamirande and Gagnon, 1995; de Lamirande et al, 1997, 1998) and regulates the calcium- and cyclic adenosine monophosphate-dependent tyrosine phosphorylation of proteins (Carrera et al, 1996; Leclerc et al, 1996, 1998; Aitken et al, 1998). The two main tyrosine phosphorylated proteins in capacitating spermatozoa (named p81 and p105 according to their molecular masses) are part of the fibrous sheath (Leclerc et al, 1996) and appear to be antigenetically related to A kinase anchoring proteins (Carrera et al, 1996; Visconti et al, 1997). Some seminal plasma components are known to be decapacitation factors because they prevent too-early onset and regulate sperm capacitation. For example, phosphatidylcholine-binding proteins found in bovine (Desnoyers and Manjunath, 1992) and in human (Leblond et al, 1993) seminal plasma bind to spermatozoa and are released during capacitation. Cholesterol (Cross, 1996) and zinc (Andrews et al, 1994), which are present at high concentrations in seminal plasma, are membrane-stabilizing agents for spermatozoa and are considered as decapacitating agents. Seminal plasma also prevents the tyrosine phosphorylation of sperm proteins that is associated with capacitation (Tomes et al, 1998). Fine-tuning of capacitation is necessary for the achievement of proper fertilization and its premature onset was associated with lower fertilizing ability in bovine cryopreserved spermatozoa (Cormier at al, 1997). In humans, premature sperm hyperactivation (the erratic and whiplash-like movement

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displayed by capacitating spermatozoa) that occurs in whole semen was considered as a possible cause for idiopathic infertility (de Lamirande and Gagnon, 1993a).

Important components of human seminal plasma are those of the coagulum that spontaneously forms after ejaculation. The major proteins of this coagulum are semenogelin I (SgI) and, to a lesser extent, semenogelin II (SgII; see Robert and Gagnon, 1999 for a review). SgI and SgII may represent 20% to 40% of the seminal plasma proteins and share a 78% homology at the amino acid level, the difference in mass between SgI (52 kilodaltons [kd]) and SgII (71 kd) being related to the presence of an extended *C*-terminal in SgII (Lilja and Lundwall, 1992). They are characterized by a basic isoelectric point (pI $>$ 9.5) and a high capacity for zinc binding (Mandal and Bhattacharyya, 1990; Robert et al, 1997; Robert and Gagnon, 1999). SgI and SgII, which will be collectively called Sg in this paper, are rapidly degraded after ejaculation by prostate-specific antigen (PSA), a protease with a chymotrypsin-like activity (Robert et al, 1997; Robert and Gagnon, 1999).

Even though the role of Sg in sperm coagulation is well documented, it may not be the only one. Sg, as well as one of its degradation products, the seminal plasma motility inhibitor (SPMI; 18–22 kd), inhibited the motility of intact human spermatozoa at concentrations similar to those found in seminal plasma (≈20 mg/mL; Robert and Gagnon, 1996; Iwamoto and Gagnon, 1988a,b), suggesting that they could act as physiological motility inhibitors shortly after ejaculation. The dose-dependent inhibition due to Sg was reversible and washing of Sg-treated spermatozoa on a small Percoll gradient allowed recovery of sperm motility (Robert and Gagnon, 1996).

The action of Sg could be much more extensive than a temporary inhibition of motility and could extend to a role in the regulation of capacitation. First, Sg is present at very high concentrations in seminal plasma. Second, immunocytochemistry studies localized Sg antigens to the cell membrane of many compartments—the posterior portion of the head, the midpiece, and the tail of freshly ejaculated spermatozoa (Lilja et al, 1989; Bjartell et al, 1996). Furthermore, the distribution of Sg was not limited to the cell plasma membrane because even after 2 successive Percoll gradients (to maximize removal of seminal plasma proteins loosely associated with the plasma membrane), both the Triton-soluble and Triton-insoluble fractions of human spermatozoa contained polypeptides that are recognized by the SPMI antiserum (Robert and Gagnon, 1999); in addition, a 19-kd polypeptide that appeared to be a proteolytic product from the *N*-terminal domain of Sg was found in human sperm nuclei (Zalensky et al, 1993). Finally, Sg may serve as a precursor molecule that gives rise to multiple polypeptides having various biological functions. Established and potential functions of Sg, its degradation products, or both, include an increase of sperm hyaluronidase activity, a thyrotropin-releasing hormone-like action, Zn^{2+} shuttling, an inhibin-like activity, etc (reviewed by Robert and Gagnon, 1999). The mechanism or mechanisms by which Sg, its degradation products, or both could act on the surface and enter spermatozoa to produce their actions are still to be elucidated.

Considering the amount of Sg in semen, its localization (Lilja et al, 1989; Bjartell et al, 1996) and distribution (Zalensky et al, 1993; Robert and Gagnon, 1999) in spermatozoa, as well as the widespread biological actions of Sg, its degradation products, or both (Robert and Gagnon, 1999), it appeared important to evaluate the potential role of this protein as regulator of sperm capacitation.

The first aim of this study was therefore to determine the effect of Sg on human sperm capacitation. The tyrosine phosphorylation of proteins and the generation of superoxide anion (O_2^{-}•) by spermatozoa were then evaluated because they are important for capacitation (de Lamirande and Gagnon, 1995; de Lamirande et al, 1997) and could help in understanding the mechanism of Sg action. Finally, the potential of spermatozoa to degrade Sg was studied.

Materials and Methods

Materials

Percoll was obtained from Amersham Pharmacia Biotech (Baie d'Urfé, PQ, Canada), the modified *Cypridina luciferin* analogue (MCLA; 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-a] pyrazin-3-one) was obtained from TCI America (Portland, Ore), and xanthine oxidase (from bovine milk) was obtained from Calbiochem (La Jolla, Calif). Superoxide dismutase (SOD; from bovine erythrocytes) was purchased from Roche Diagnostics (Laval, Québec, Canada). Lysophosphatidylcholine (LPC), ribonuclease (RNase), bovine serum albumin (BSA), fluorescein isothiocyanate-conjugated (FITC) *Pisum sativum* agglutinin, and the bicinchoninic protein assay kit were from Sigma Chemical Company (St Louis, Mo). Monoclonal antiphosphotyrosine antibody (clone 4G10; Upstate Technology Inc, Lake Placid, NY), nitrocellulose (0.22-μm pore size; Micron Separations Inc, Westboro, Mass), goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Life Technologies, Burlington, Ontario, Canada), an enhanced chemiluminescence kit (Lumi-Light; Roche Diagnostics) and radiographic films (Fuji, Minami-Ashigara, Japan) were used for immunodetection of phosphotyrosine-containing proteins. All other chemicals were at least of reagent grade.

Fetal cord blood, generously provided by the blood bank at the Royal Victoria Hospital (Montréal, Québec), was centrifuged (1000 $\times$ *g* for 10 minutes at 4°C). Sera were pooled and frozen (−20°C) until used. Ultrafiltrates (FCSu) were prepared from 3 pools of 13 to 25 different samples using YM3 membranes (exclusion limit: 3 kd; Amicon, Oakville, Ontario, Canada; de Lamirande and Gagnon, 1993b).

Sg was prepared as described before (Robert and Gagnon, 1996). Briefly, semen samples were collected by healthy volunteers in bottles containing 8 M urea in HEPES-buffered saline (HSS; 25 mM HEPES, 100 mM NaCl pH 8.0). After treatment with 25 mM dithiothreitol [DTT] and 125 mM iodoacetamide, the samples were centrifuged for 15 minutes at 10 000 × *g* and the supernatants were frozen at −80°C until used. After thawing, the protein solution was loaded onto a 1.6 × 21 cm column of SP-Sepharose Fast Flow (Amersham Pharmacia Biotech, Tokyo, Japan). The column was washed with 1 M urea in HSS. Proteins bound to the column were eluted first with 8 M urea in HSS and then a linear gradient of NaCl (0–400 mM) in 8 M urea and HSS. Fractions containing Sg were pooled and loaded onto a Vydac (The Separations Group, Hesperia, Calif) semipreparative C4 column (10 × 250 mm, 10-μm beds, 300 Å pore size) equilibrated in 0.1% trifluoroacetic acid. Proteins were eluted with a linear gradient from 25% to 40% of 80% acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the purified Sg were pooled and lyophilized. Sg preparations contained SgI and SgII. Protein concentration was measured by the bicinchoninic acid assay (Smith et al, 1985) using BSA as a standard.

Sperm Preparations and Treatments

Semen samples from healthy volunteers were washed on 4-layer (95%–65%–40%–20%) Percoll gradients buffered in HEPES-balanced saline (115 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 14 mM fructose, 25 mM HEPES pH 8.0). The samples were centrifuged for 30 minutes at 2300 × *g*, and sperm cells at the 65%–95% Percoll interface and in the 95% Percoll layer were pooled and diluted at 200 × 10^6 cells/mL with the 95% Percoll solution. Only samples in which progressive sperm motility was greater than 70% were used. Spermatozoa were additionally diluted 10-fold in Biggers, Whitten, and Whittingham medium (BWW; pH 8.0; Biggers et al, 1971) devoid of bicarbonate and albumin and containing 1 mM $CaCl_2$.

BWW medium was used without (control) or with FCSu (7.5%, v/v) as a capacitation inducer (de Lamirande and Gagnon, 1993b). The effects of Sg (0.03 to 1 mg/mL) and of RNase (0.3 mg/mL) were tested. RNase was used as a control protein because its isoelectric point (pI = 9.7) is similar to that of Sg (pI = 9.5; Robert and Gagnon, 1999). RNase was used at 0.3 mg/mL because Sg affected sperm function at that concentration. The percentage of sperm motility was not affected by the presence of Sg or RNase at the concentrations used in this study.

Measurement of Sperm Capacitation and O_2^{-}• Generation

Sperm capacitation was measured after a 3.5-hour incubation period at 37°C, by the lysophosphatidylcholine (LPC; 100 μM in BWW containing 3 mg BSA/mL) induction of the acrosome reaction (30 minutes, 37°C) as previously described (de Lamirande et al, 1993, 1997). The acrosomal status of ethanol-fixed spermatozoa was evaluated using the FITC-conjugated *Pisum sativum* agglutinin (Cross et al, 1986). At least 250 spermatozoa were counted for each sample.

Chemiluminescence, which was used to evaluate O_2^{-}• production, was recorded at 5-minute intervals using a computer-driven LKB Wallach 1251 luminometer (Turku, Finland) in the integration mode (5 seconds), with mixing of the measured sample at 37°C. Measurements started immediately after the chemiluminescence amplifier, MCLA (20 μM), was added to spermatozoa (8 × 10^6/mL) exposed to various treatments (FCSu, Sg, RNase; de Lamirande and Gagnon, 1995). MCLA is a very sensitive, cell-impermeant O_2^{-}•-specific probe (Nakano et al, 1986; Nakano, 1990), and its use requires careful selection of controls of blanks. Every sample, spermatozoa and medium, must be run in parallel with a similar one in which SOD is added so that only the SOD-inhibitable signal (the real measure of the presence of O_2^{-}•) is considered and that the contribution of the medium can be subtracted. Calculations were performed as described before (de Lamirande and Gagnon, 1995).

Detection of Phosphotyrosine-Containing Proteins

Detection of phosphotyrosine-containing proteins was performed as previously described (Leclerc et al, 1997). Briefly, spermatozoa incubated for 2.5 hours in the absence or presence of FCSu, Sg, or both were solubilized in Laemmli solution (Laemmli, 1970) containing 0.5 mM sodium vanadate, boiled, centrifuged, electrophoresed (10% sodium dodecyl sulphate [SDS]-polyacrylamide gels; Laemmli, 1970), and electrotransferred to nitrocellulose (Towbin et al, 1979). The membranes were incubated with a solution of skim milk (5% w/v) in Tris-buffered saline (20 mM pH 7.8) supplemented with Tween 20 (0.1%; TTBS), then with the antiphosphotyrosine antibody (0.1 μg/mL, 1 hour, 20°C), washed with TTBS, incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (0.2 μg/mL, 45 minutes, 20°C), and washed with TTBS. Positive immunoreactive bands were detected by chemiluminescence using Lumi-Light. The blots were then rinsed and silver-stained (Jackobson and Karsnas, 1990) to ascertain that the amount of proteins loaded in each well was the same.

Degradation of Sg by Spermatozoa

Percoll-washed spermatozoa were resuspended (20 × 10^6 cells/mL) in BWW containing 0.3 mg Sg/mL, and in the absence or presence of FCSu. After 15, 30, 60, and 150 minutes of incubation at 37°C, a 100-μL aliquot of each of the suspensions was collected and centrifuged to remove spermatozoa. A 50-μL portion of the supernatant was harvested, combined with Laemmli solution (Laemmli, 1970), boiled, centrifuged, and electrophoresed (12% SDS-polyacrylamide gels; Laemmli, 1970). A control sample containing spermatozoa in the absence of Sg was also run in order to detect proteins that would be released by these cells over the course of the experiment. Sg was also incubated in BWW in the absence of spermatozoa to ensure that the protein would not spontaneously degrade under these experimental conditions. Gels were stained with Coomassie Brilliant Blue R-250.

O_2^{-}• Scavenging Potential of Sg

Sg potential to scavenge O_2^{-}• was determined by the inhibition of nitroblue tetrazolium reduction due to O_2^{-}• generated by the combination of xanthine + xanthine oxidase as previously described (de Lamirande et al, 1993b). The absorbance of the blue formazan generated was recorded at 570 nm.

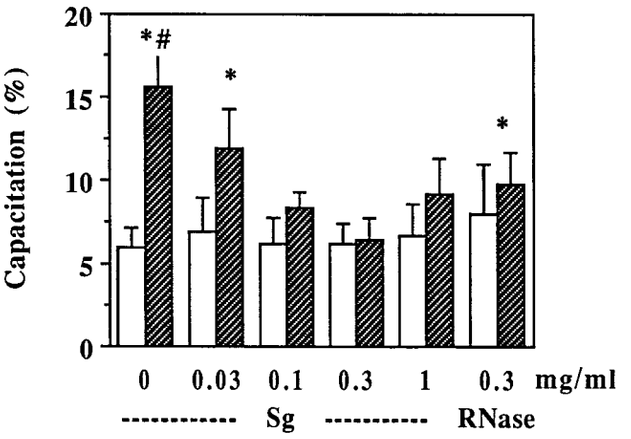

Figure 1. Sg decreases the FCSu-induced capacitation in human spermatozoa. Percoll-washed spermatozoa were incubated for 3.5 hours at 37°C in BWW medium in the absence (white bars) or presence (hatched bars) of FCSu as a capacitation inducer and supplemented or not supplemented with Sg (0.03 to 1 mg/mL) or RNase (0.3 mg/mL). Sperm capacitation was then evaluated by the LPC-induced acrosome reaction as described in ''Materials and Methods.'' Results are means ± SEM of 4 (Sg 0.03 and 1 mg/mL), 8 (Sg 0.1 and 0.3 mg/mL), and 10 (Sg 0 mg/mL, control) values obtained with different sperm samples. *Value different ($P < .05$) from that obtained with spermatozoa incubated in BWW medium alone. #Value higher ($P < .05$) than all the others.

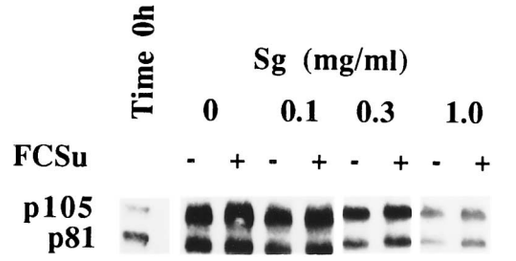

Figure 2. Sg decreases the protein tyrosine phosphorylation of capacitating human spermatozoa. Spermatozoa were incubated for 2.5 hours at 37°C in the absence (−) or presence (+) of FCSu as capacitation inducer and with various concentrations of Sg (0, 0.1, 0.3, and 1 mg/mL). Protein electrophoresis and immunoblotting are described in ''Materials and Methods.'' The position of the 105- and 81-kd phosphotyrosine-containing proteins is indicated on the left. Results of 1 experiment are representative of 3 others performed with sperm samples from different donors.

Statistical Analysis

Analysis of variance (two-tailed; unpaired values) was used to evaluate the differences in the levels of capacitation or O_2^{-}• generation of spermatozoa submitted to the various treatments. A difference was considered significant when $P \leq .05$.

Results

Effect of Sg on Sperm Capacitation and Associated Tyrosine Phosphorylation of Proteins

Sg inhibited FCSu-induced sperm capacitation as measured by the acrosome reaction triggered by LPC in a dose-dependent fashion (Figure 1). This inhibition was complete at Sg concentrations ranging from 0.1 to 1 mg/mL but only partially at 0.03 mg/mL. The effect of RNase was also tested because its isoelectric point (pI = 9.7) is similar to that of Sg (pI = 9.5). RNase (0.3 mg/mL) also prevented sperm capacitation but not entirely; the levels obtained were higher than those observed in spermatozoa incubated with BWW alone or supplemented with 0.3 mg Sg/mL (Figure 1).

Human sperm capacitation is associated with tyrosine phosphorylation of 2 fibrous sheath proteins, called p81/p105, according to their molecular masses (de Lamirande et al, 1997; Leclerc et al, 1997). Dose-dependent inhibition of the tyrosine phosphorylation of p81/p105 was observed in spermatozoa incubated in the presence of FCSu (Figure 2). Even though Sg at 0.1, 0.3, and 1.0 mg/mL completely prevented capacitation, a decrease in tyrosine phosphorylation of p81/p105 could be observed only with the 2 highest concentrations.

Effect of Sg on the O_2^{-}• Generated by Spermatozoa and by the Combination of Xanthine and Xanthine Oxidase

The O_2^{-}• generated by human spermatozoa during capacitation appears essential for this process (de Lamirande and Gagnon, 1995). Therefore, the interference of Sg with O_2^{-}• generated by spermatozoa was tested as a possible mechanism to explain an inhibition of capacitation. Direct measurement of O_2^{-}• generated from live spermatozoa was performed by MCLA-amplified chemiluminescence. As previously observed (de Lamirande and Gagnon, 1995), sperm capacitation triggered by FCSu was associated with an increased generation of O_2^{-}• (27 ± 1 mV/s/10^6 spermatozoa; Figure 3). Sg decreased the FCSu-associated chemiluminescence in a dose-dependent fashion (Figure 3). RNase and Sg, both at 0.3 mg/mL, were tested in parallel in 2 experiments and found to have similar effects.

The decreased chemiluminescence observed in spermatozoa treated with Sg could be due to a reduction of O_2^{-}• generation by spermatozoa or by a scavenging of the O_2^{-}• produced by these cells. The potential of Sg to scavenge O_2^{-}• was therefore evaluated using the combination of xanthine + xanthine oxidase as source of O_2^{-}•. The curves presented in Figure 4 indicate the formation of blue formazan (an increase in absorbance at 570 nm, Abs_{570nm}) by the reaction of O_2^{-}• with nitroblue tetrazolium. When xanthine and xanthine oxidase are used in the absence of supplements, the increase in Abs_{570nm} is linear for 30 minutes and then a plateau is obtained, reflecting the depletion of substrate. There was a concentration-dependent decrease in the Abs_{570nm} measured when Sg was added to the assay (Figure 4A). However, the scavenging

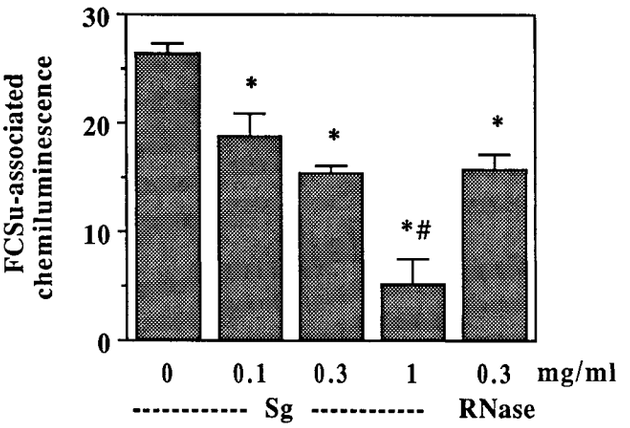

Figure 3. Sg inhibits FCSu-associated chemiluminescence. Spermatozoa were incubated in the absence or presence of Sg at the indicated concentration in BWW medium supplemented or not supplemented with FCSu. MCLA (20 μM) was added and chemiluminescence was recorded. The chemiluminescence of every sample and every incubation medium (± FCSu, ± Sg) was evaluated in the absence and presence of SOD so that only the SOD-inhibitable chemiluminescence (the real measure of the O_2^{-}• generated) is considered and the contribution of the medium can be subtracted. The FCSu-associated chemiluminescence was calculated when the plateau was obtained (after 15–25 minutes of incubation) and corresponds to the chemiluminescence of spermatozoa treated, minus that of not treated, with FCSu. Chemiluminescence is expressed as mV/s/10^6 spermatozoa. Values are mean ± SEM of 4 to 8 values obtained with different sperm preparations. *Value different from that obtained with spermatozoa incubated in the absence of Sg. #Value lower than all the others.

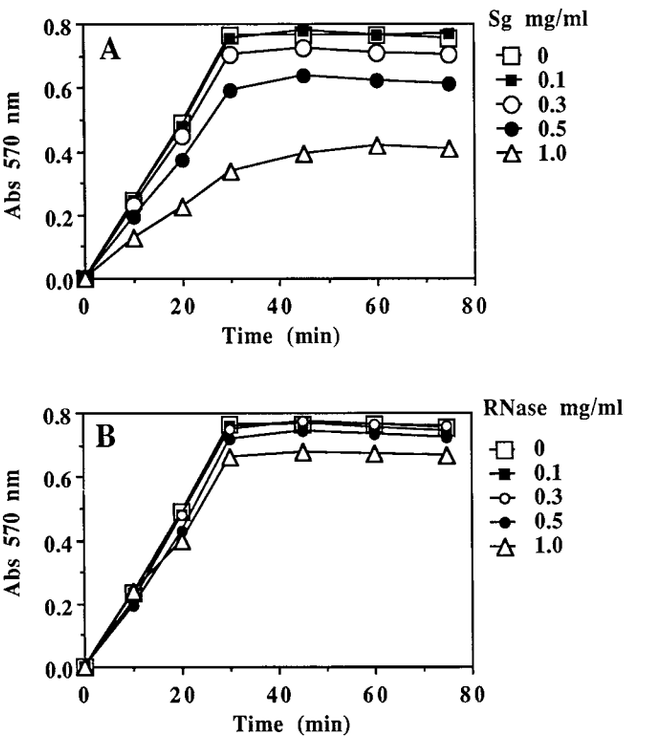

Figure 4 Potential of Sg to scavenge O_2^{-}•. The combination of xanthine (0.2 mM) + xanthine oxidase (0.05 U/mL) was used to generate O_2^{-}• and the reduction of nitroblue tetrazolium (0.4 mM) to blue formazan by O_2^{-}• was measured by the increase in absorbance at 570 nm. The effect of Sg **(A)** and RNase **(B)** at various concentrations was evaluated.

of O_2^{-}• by Sg was absent or very low when this protein was used at 0.1 or 0.3 mg/mL (0% and 6% decreases, respectively). Furthermore, it must be noted that the kinetics of the reaction appeared to be modified when Sg at 1 mg/mL was tested: the Abs_{570nm} linearly increased for 30 minutes but did not plateau immediately after; this could be indicative of an inhibition of xanthine oxidase in addition to O_2^{-}• scavenging. Contrary to what was observed in chemiluminescence experiments, RNase did not have the same effect as Sg in the xanthine + xanthine oxidase assay and there was only a small decrease in Abs_{570nm} (11%) at the highest concentration tested (Figure 4B).

Degradation of Sg by Human Spermatozoa

In whole semen, Sg is rapidly degraded by the protease, PSA, into specific peptides (Robert et al, 1997). Spermatozoa incubated in BWW supplemented or not supplemented with FCSu also degraded Sg (0.3 mg/mL) in a time-dependent fashion and the molecular masses of the resulting peptides (Figure 5) were similar to those of the peptides present in liquefied seminal plasma (Robert and Gagnon, 1995). The electrophoretic pattern of the Sg preparation used for these experiments presents a major band at 52 kd, which is SgI, and a doublet at 71–76 kd, which is SgII (the 76-kd protein is a glycosylated form of SgII; Robert and Gagnon, 1999). Sg was not affected by an incubation in BWW at 37°C for 2.5 hours. However, when spermatozoa (20×10^6 cells/mL) were added, SgII almost completely disappeared after an incubation as short as 15 minutes. SgI was also degraded very rapidly but a small amount remained intact in solution after 15 minutes of incubation and then slowly disappeared over the next 2 hours. Degradation peptides with molecular masses of 38, 35, 33, 28, 25, 20, and 18 kd were present as early as 15 minutes after the beginning of incubation. Furthermore, even the amount of these degradation products decreased with time and the proteins present in the incubation medium after 2.5 hours of incubation were mostly similar to those released by spermatozoa themselves except for one of 15 kd.

Discussion

The results presented here clearly demonstrate that Sg inhibited in a dose-dependent manner human sperm capacitation induced by FCSu (Figure 1), and that this was accompanied by a decrease in tyrosine phosphorylation of the 2 fibrous sheath proteins, p81 and p105 (Figure 2).

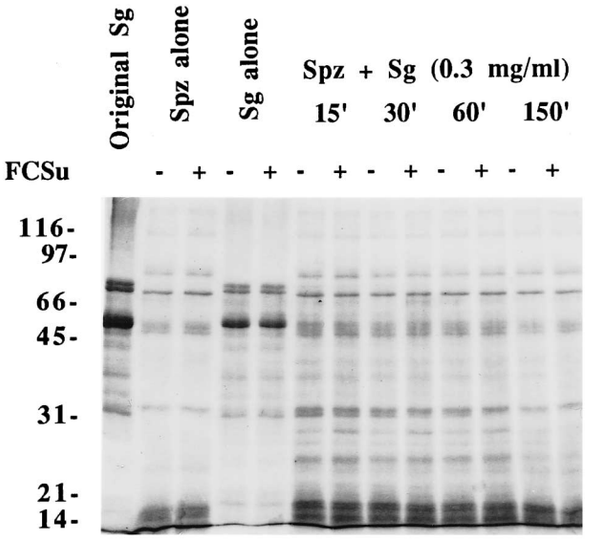

Figure 5. Sg is degraded by spermatozoa. Spermatozoa were incubated with Sg (0.3 mg/mL) at 37°C in the absence (−) or presence (+) of FCSu. An aliquot of each sample was centrifuged after 15, 30, 60, and 150 minutes of incubation and the proteins present in the supernatant were electrophoresed. The electrophoretic pattern of the Sg preparation used in this experiment (original Sg) is also presented. Control tests in which Sg (Sg alone) or spermatozoa (Spz alone) were incubated alone (150 minutes) were also run. The position of molecular mass markers is shown on the left. Results of 1 experiment are representative of 2 others performed with sperm samples from different donors.

These inhibitions could at least partly be explained by the decrease in the amount of $O_2^{-}\bullet$ available for capacitation (Figures 3 and 4). The data also indicate that human spermatozoa rapidly degrade Sg.

Sg completely inhibited FCSu-induced sperm capacitation when used even at 0.1 mg/mL (Figure 1), a concentration that is low if one considers that up to 10–20 mg Sg/mL is present in whole semen (Robert and Gagnon, 1999). However, Sg is rapidly degraded by PSA in semen (Robert et al, 1997) and spermatozoa in vitro (Figure 5), and if one of the physiological roles of Sg is to prevent capacitation, it may be required that it acts at these very low concentrations or that its degradation products remain active. High concentrations of Sg, in the order of 20 mg/mL, immobilize spermatozoa (Robert and Gagnon, 1996) but those used in this study did not affect the percentage of motile cells. However, preliminary experiments suggested that Sg (0.3 mg/mL) decreases sperm hyperactivation (unpublished observations), which is the erratic and whiplash-like movement that spermatozoa display during capacitation (de Lamirande et al, 1997).

The incubation of spermatozoa with Sg caused a dose-dependent decrease in FCSu-associated chemiluminescence (Figure 3). This observation could be explained by an inhibition of the sperm ''oxidase'' (an enzymatic system that still remains elusive), which is responsible for the production of $O_2^{-}\bullet$ or by a direct scavenging by Sg of the $O_2^{-}\bullet$ produced by spermatozoa. Experiments performed with the xanthine + xanthine oxidase assay indicated that Sg is a scavenger for $O_2^{-}\bullet$ but only when used at 1 mg/mL (Figure 4) and that in this case, the kinetics of the enzymatic reaction were modified: the Abs_{570nm} raised linearly for 30 minutes but then did not plateau as for the other assays but rather slowly increased, suggesting that xanthine oxidase activity could be affected by Sg. Because of this, and because Sg even at lower concentrations decreased the FCSu-induced chemiluminescence (Figure 3) without significantly scavenging $O_2^{-}\bullet$ (Figure 4), it is probable that Sg also affects the sperm oxidase. Other observations favor this last hypothesis. First, human spermatozoa treated with FCSu generate $O_2^{-}\bullet$ over several hours (de Lamirande and Gagnon, 1995) even though only a 30-minute exposure to this free radical is needed for the induction of capacitation (de Lamirande et al, 1998). Second, the degradation of Sg by spermatozoa is rapid (Figure 5). Finally, capacitation is progressively increasing with the duration of the incubation with a proper inducer (de Lamirande and Gagnon, 1995; de Lamirande et al, 1997). If Sg action is only to scavenge $O_2^{-}\bullet$ and if this protein in contact with spermatozoa for less than 1 hour, the levels of capacitation measured 2 hours later would be higher than those observed in spermatozoa incubated in BWW alone. It is therefore probable that Sg, its degradation products, or both, act also on the sperm oxidase, as well as by other mechanisms, rather than only scavenging $O_2^{-}\bullet$.

Under our experimental conditions, spermatozoa had a considerable potential to rapidly degrade Sg (Figure 5). It is possible that spermatozoa themselves possess enzymes to process Sg. However, the observation that the Sg degradation pattern is reminiscent of that triggered by PSA (Robert and Gagnon, 1995) would rather indicate that some of this protease from the seminal plasma could also be adsorbed on spermatozoa tightly enough not to be removed by the Percoll wash. Considering the rapid processing of Sg by spermatozoa, it is tempting to speculate that unless Sg itself irreversibly blocks one of the first steps of capacitation, one or a few of the degradation products can also inhibit this process.

The mechanism by which Sg inhibits sperm capacitation could be related, at least in part, to its high content in basic amino acids (pI = 9.5) because another protein with a high isoelectric point, RNase (pI = 9.7), also had a somewhat similar effect. It may be that at the pH of the experiment (8.0), the net positive charge of Sg, and RNase allow a tight binding of these proteins on the sperm membrane, which is negatively charged, in part due to the high content in sialic acid (Kaneko et al, 1984). Even though both Sg and RNase also decreased FCSu-associated chemiluminescence (Figure 3), the mechanism of action of these 2 proteins is not quite the same because only

Sg appeared to have a significant potential to scavenge O_2^{-}• at high concentrations (Figure 4).

Sg has a high affinity for Zn^{2+} (Mandal and Bhattacharyya, 1990; Robert et al, 1997; Robert and Gagnon, 1999), which is recognized as a membrane stabilizing agent for spermatozoa, and is therefore considered as a decapacitating factor (Andrews et al, 1994). Zn^{2+} was reported to prevent sperm hyperactivation in the human (de Lamirande et al, 1997) and capacitation in the human (de Lamirande et al, 1997), the mouse, and the hamster (Aonuma et al, 1981; Andrews et al, 1994). The use of Zn^{2+}-specific fluorochrome allowed the demonstration that Zn^{2+} is removed from hamster spermatozoa in a time-dependent fashion during in vitro capacitation (Andrews et al, 1994). Whether Zn^{2+} chelated on Sg, its degradation products, or both, is involved in the inhibition of human sperm capacitation observed in the present study is still unknown, but such a mechanism could also be conceivable.

In conclusion, we demonstrated that Sg at low concentrations inhibits human sperm capacitation, as well as the associated protein tyrosine phosphorylation and O_2^{-}• availability, and that spermatozoa degrade Sg in a manner reminiscent to that caused by PSA. These data suggest that Sg, its degradation peptides, or both may be natural regulators of sperm capacitation by preventing this important process to occur prematurely. The effects of Sg, its degradation peptides, or both may be aimed at more than 1 target on spermatozoa but one of the mechanisms for Sg action appears to involve an interference with the O_2^{-}• generated during this process.

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