BIOLOGY OF REPRODUCTION **63,** 1531–1537 (2000)

Seminal Plasma Proteins Revert the Cold-Shock Damage on Ram Sperm Membrane[1]

Beatriz Barrios, Rosaura Pérez-Pé, Margarita Gallego, Agustín Tato,[3] Jesús Osada, Teresa Muiño-Blanco, and José A. Cebrián-Pérez[2]

Department of Biochemistry and Molecular and Cell Biology, School of Veterinary Medicine, University of Zaragoza, 50013 Zaragoza, Spain

ABSTRACT

Ejaculated ram spermatozoa, freed from seminal plasma by a dextran/swim-up procedure and exposed to cold shock, were incubated with ram seminal plasma proteins and analyzed by fluorescence markers and scanning electron microscopy. Seminal plasma proteins bound to the sperm plasma membrane modified the functional characteristics of damaged spermatozoa, reproducing those of live cells. Scanning electron microscopy showed that the dramatic structural damage induced by cooling reverted after incubation with seminal plasma proteins. Assessment of membrane integrity by fluorescence markers also indicated a restoration of intact-membrane cells. This protein adsorption is a concentration-dependent process that induces cell surface restoration in relation to the amount of protein in the incubation medium. Fractionation of ram seminal plasma proteins by exclusion chromatography provided three fractions able to reverse the cold shock effect. Scanning electron microscopy also confirmed the high activity of one fraction, because approximately 50% of cold-shocked sperm plasma membrane surface was restored to its original appearance after incubation. Differences in composition between the three separated fractions mainly resulted from one major band of approximately 20 kDa, which must be responsible for recovering the sperm membrane permeability characteristic of a live cell.

sperm, stress

INTRODUCTION

Mammalian sperm have unusual plasma membranes compared with those of somatic cells. After leaving the testes, sperm ceases plasma membrane lipid and protein synthesis. However, mammalian spermatozoa cannot fertilize oocytes immediately after ejaculation. They acquire this ability only after remaining for a certain period of time in the female tract, through a process called capacitation. Only fully capacitated sperm are competent to undergo the zona-induced acrosome reaction.

Seminal plasma, a physiological secretion from multiple glands of the male reproductive tract, is the natural medium for maturation of the spermatozoa through hormonal, enzymatic, and surface-modifying events. The addition and removal of a variety of proteins during epididymal maturation and at ejaculation play important roles in the capacitation of sperm and fertilization of the egg [1, 2].

Seminal plasma contains a variety of biochemical components, some of which are relatively specific for the regulation of spermatozoa function. Seminal plasma composition has been thoroughly analyzed in several species [3, 4], but its role in sperm function remains largely a matter of speculation. Reports on several species suggest that seminal plasma contains factors that may influence sperm viability. These results are generally based on the observation that ejaculated spermatozoa die if the concentration of seminal plasma in their environment is reduced by dilution or washing [5]. Thus, seminal plasma was reported to be important for maintaining spermatozoa motility in bull [6] and ram [7], for improving ram sperm viability [8, 9], and for increasing the resistance of boar spermatozoa to cold shock damage [10, 11]. Results of other studies have indicated that seminal plasma has little effect on sperm motility or susceptibility to cold shock in mammals [12, 13]. Conversely, detrimental effects of seminal plasma on sperm cell motility [7, 14, 15], viability [5, 16–18], and survival after freezing-thawing [19–21] have also been reported. These contradictory results indicate that seminal plasma is a complex mixture containing a wide variety of components that affect the survival and motility of sperm. Differences in seminal plasma protein profiles from bulls of different fertility have been found [22–24].

The adsorption of large quantities of seminal plasma proteins has been confirmed by Metz et al. [25] in boar spermatozoa, and surface changes of bull, ram, and boar spermatozoa by interactions with seminal plasma proteins have also been described [26]. Likewise, we have already shown that adsorption of seminal plasma proteins by ram spermatozoa previously exposed to detergent reverses the detergent effect on sperm surface [27]. Recently, mammalian spermatozoa have been reported to bind seminal vesicle proteins [28], and mammalian sperm function has been beneficially modified by exposure to a synthetic peptide [29].

Ram spermatozoa are more sensitive to cold shock stress than other species such as bull, rabbit, or human [19, 30, 31], which suggests that species-dependent differences may exist with respect to cold-induced ultrastructural changes of the plasma membrane. Thus, aggregation of particles in the plasma membrane of ram spermatozoa on cooling to 5°C has been shown [30, 32], as has redistribution of intramembranous particles in the plasma membrane of both boar and bull spermatozoa [33]. The reduced longevity and fertilizing ability of cryopreserved mammalian spermatozoa has been suggested to result from their early capacitation state [34–36]. The redistribution of some proteins occurs concomitant with, or only after, capacitation [37]. Some of the proteins function to stabilize the membrane until the appropriate time.

In the present study, we investigate the hypothesis that seminal plasma components could recover membrane in-

[1]Supported by grants DGICYT AGF-97-0919 and DGA-48/99AV.
[2]Correspondence: J.A. Cebrián Pérez, Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, C/Miguel Servet, 177. 50013 Zaragoza, Spain. FAX: 34 976 761612;
e-mail: pcebrián@posta.unizar.es
[3]Current address: Servicio de Soporte a la Investigación Experimental, Universidad de Valencia, 46100 Burjassot, Valencia, Spain.

Received: 31 December 1999.
First decision: 3 February 2000.
Accepted: 5 July 2000.

ISSN: 0006-3363. http://www.biolreprod.org

tegrity of cold-shocked ram spermatozoa. The effect of in vitro exposure to seminal plasma proteins on the cell viability of cold-shocked ram spermatozoa was analyzed using biochemical markers and electron microscopy. In addition, proteins from ram seminal plasma were fractionated, and their ability to recover membrane integrity of cold-shocked sperm was examined.

MATERIALS AND METHODS

Preparation of Cell Samples

All the experiments were performed using fresh ram spermatozoa. Semen was collected from eight mature *Rasa aragonesa* rams by an artificial vagina. The rams were maintained at the School of Veterinary Medicine under uniform nutritional conditions. Second ejaculates from four rams were pooled and used for each assay to eliminate individual differences. Experiments were performed over time, maintaining sires with an abstinence period of 2 days on the basis of previous results [38].

A seminal plasma-free sperm population obtained by a dextran/swim-up procedure [39] performed using a medium without Ca_2Cl and $NaHCO_3$ [40] was used. For thermal shock, approximately 2×10^9 cells obtained after the swim-up process were incubated for 5 min at 25°C, transferred to 5°C for 10 min, and then replaced at 25°C for a further 5 min. An aliquot of approximately 1×10^6 cells was recovered, diluted to 0.5 ml with the swim-up medium, and then incubated with seminal plasma proteins to assess their effects.

Evaluation of Semen Samples

Sperm concentration was calculated in duplicate using a Neubauer chamber (Marienfeld, Germany).

Cell viability (i.e., membrane integrity) was assessed by fluorescent staining with carboxyfluorescein diacetate and propidium iodide [41]. The cells were then examined under a Nikon fluorescence microscope (Tokyo, Japan), and the numbers of propidium iodide-negative (i.e., membrane-intact) spermatozoa and propidium iodide-positive (i.e., membrane-damaged) spermatozoa per 100 cells were estimated and recorded. At least 200 cells were counted in duplicate for each sample. Results are expressed as the percentage of membrane-intact spermatozoa ± SD.

Obtainment of Seminal Plasma and Proteins

Seminal plasma was obtained by spinning 1 ml of semen at 12 000 × *g* for 5 min in a microfuge at 4°C. The supernatant was centrifuged again, and 400 μl of undiluted seminal plasma were removed and, after filtering through a 0.22-μm membrane, kept at −20°C.

Seminal plasma proteins were obtained by filtering the whole seminal plasma through Microsep microconcentrators (Filtron Tech, Northborough, MA) of a 3-kDa molecular weight cut-off, spinning for 6 h at 3 000 × *g* at 4°C. The obtained sample concentrate was diluted with five volumes of a medium containing 0.25 M sucrose, 0.1 mM EGTA, 4 mM sodium phosphate (pH 7.5), 10% (v/v) of 10× buffer stock Hepes (50 mM glucose, 100 mM Hepes, 20 mM KOH) and then centrifuged again, after which the seminal plasma proteins were recovered and stored at −20°C. Protein concentration was assessed according to the method described by Bradford [42].

Seminal plasma lipoproteins were obtained by NaBr density-gradient ultracentrifugation according to the method described by Kelley and Kruski [43]. After exhaustive dialysis with the same medium described earlier, protein concentration was estimated using the bicinchoninic acid protein assay reagent according to the supplier's instructions (Pierce, Rockford, IL).

Scanning Electron Microscopy

Cell suspensions (2×10^6) were fixed in Karnovsky fixative for 1 h at room temperature and then centrifuged at 600 × *g* for 1 min. The pellet was washed twice in 1.5% sucrose in 0.1 M phosphate buffer (pH 7.4) for 45 min at room temperature. Samples were then placed on Isopore filters (0.2-μm diameter) (Millipore Corp., Bedford, MA) and dehydrated in ascending concentrations of ethanol twice for 5 min in each of 50%, 70%, 96%, and 100% alcohol. Subsequently, samples were critical-point dried (Jeol 50/60 Hz) and coated with gold palladium (100–200 nm, 90 sec, 20 mA). Specimens were observed and photographed using an Hitachi 4100 FE scanning electron microscope (Hitachi Instruments Inc., San Jose, CA). In each instance, approximately 100 cells were observed and scored as membrane-intact or membrane-damaged spermatozoa.

SDS-PAGE Analysis

Analysis with SDS-PAGE was performed in a gradient (4%–22%) of polyacrylamide gel according to the method described by Laemmli [44] using a mini protean II vertical slab gel electrophoresis apparatus (Bio-Rad, Hercules, CA). The samples containing 15 μg of proteins were diluted 4:1 v/v with the sample buffer (25% glycerol, 2% SDS, 0.027 M Tris-HCl [pH 6.8], 5% 2-mercaptoethanol, 0.018% EDTA, and 0.1% bromophenol blue) and heated for 5 min at 95°C. Electrophoresis was performed for 4 h and 30 min at 55 V at room temperature. A mixture of prestained molecular weights ranging from 6.5 to 205 kDa (Sigma, St. Louis, MO) was used as a standard. Gels were stained with 0.025% Coomassie R (Serva, Heidelberg, Germany). Densitometric analysis of the stained gel was performed using Gel Doc 1000 (Bio-Rad) with Molecular Analyst software (Bio-Rad).

Sephacryl-100 HR Chromatography

Forty milligrams of seminal plasma proteins were loaded (0.1 ml/min) on a 1.6- × 90-cm Sephacryl-100 high resolution column (XK 16/100 Pharmacia-Biotech, Uppsala, Sweden) and equilibrated at 4°C in 0.005 M phosphate buffer (pH 8) containing 0.2 M NaCl. The column was then washed at 0.1 ml/min with 120 ml of equilibration buffer. Fractions (1 ml) were collected at the same flow rate. During purification, protein concentration in each fraction was estimated by monitoring the absorbance at 280 nm. Pooled fractions were thoroughly dialyzed against distilled water containing 0.1% sodium azide and kept frozen (dried) until use.

Assessment of Seminal Plasma Protein Effect

Whole seminal plasma proteins and dried fractions isolated from Sephacryl-100 chromatography were diluted with the swim-up medium [40]. Protein concentration was determined according to the method described by Bradford [42]. Different protein amounts between 0.1 to 2.5 mg were added to 1×10^6 cold-shocked spermatozoa and incubated at 20°C for 1 h. Results are expressed as the percentage of reversion of cold shock effect in the sample containing plasma proteins with respect to the control sample (i.e.,

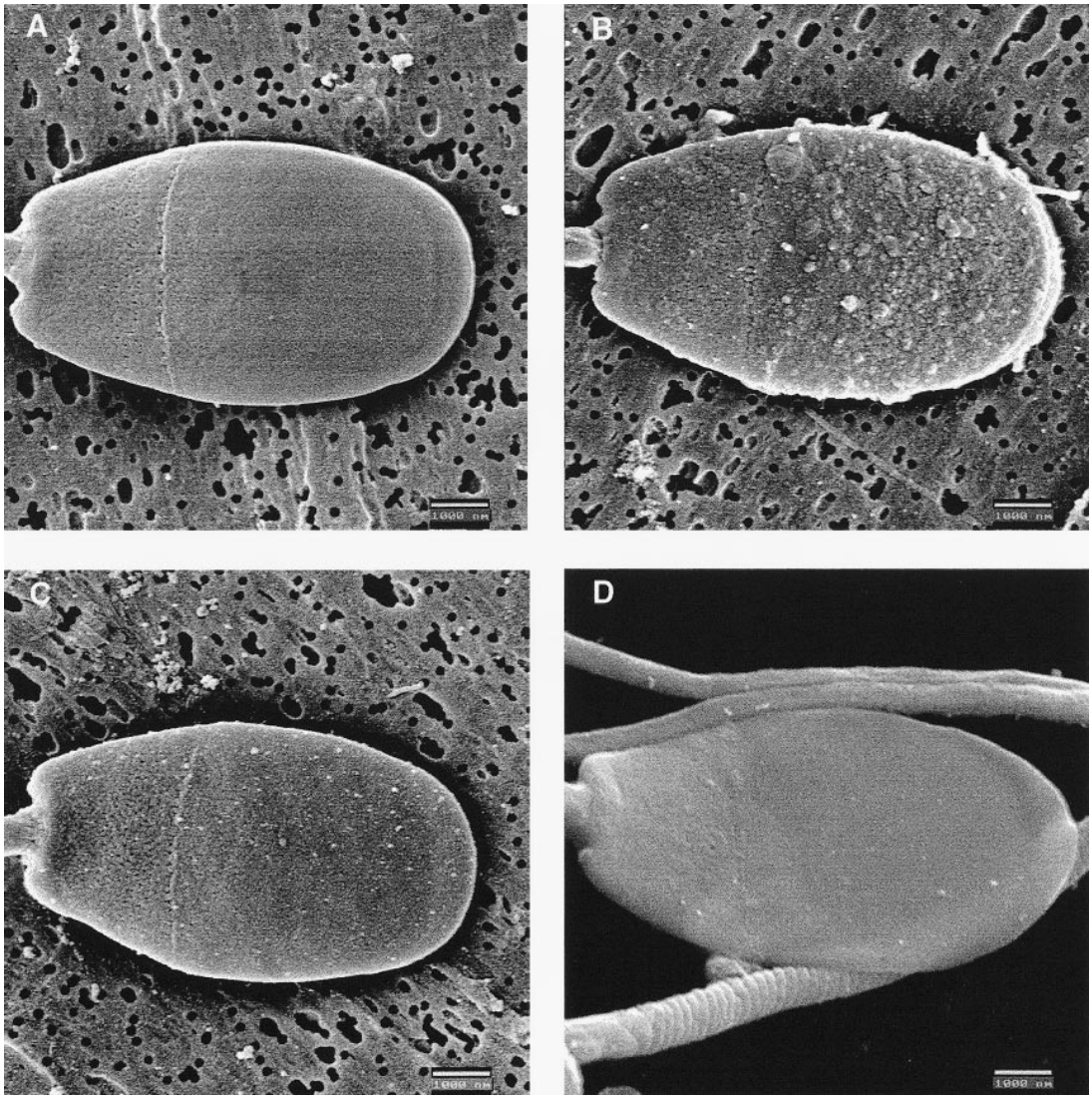

FIG. 1. Scanning electron microscopy of ram spermatozoa. **A**) Obtained by swim-up. **B**) Subjected to cold shock. **C**) Subjected to cold shock and incubated with 0.7 mg of seminal plasma proteins. **D**) Subjected to cold shock and incubated with 0.7 mg of fraction 6 proteins. Magnification ×13 000.

assessing the percentage of membrane-intact spermatozoa [propidium iodide-negative]). The relation between total recovered cells (evaluated as the difference between cell viability after 1 h of incubation with and without proteins) and total theoretically recoverable cell (evaluated as the difference between cell viability in the swim-up obtained sample and the cold-shocked sample after 1 h of incubation) was used to express results, or

$$[(Vp_{60} - Vc_{60})/(Vs - Vc_{60})] \times 100$$

where Vp is the protein-added, cold-shocked sample viability; Vc is the cold-shocked sample viability; Vs is the swim-up sample viability; and 60 indicates the incubation time (min) after addition of the proteins.

RESULTS

Scanning Electron Microscopy-Detected Restoration of Cold-Shocked Sperm Plasma Membrane by Adsorption of Seminal Plasma Proteins

The cold-shock treatment strongly affected the swim-up sperm sample quality, lowering cell viability from 67.2% ± 3.2% to 26% ± 10% (n = 4). Scanning electron microscopy clearly showed the dramatic structural damage induced by the cold shock. Figure 1 is representative of control and cold-shocked ram sperm populations. This damage partially reverted after incubation with whole seminal plasma proteins. When 1×10^6 cells were incubated with 1.4 mg of seminal plasma proteins, approximately 65% of damaged spermatozoa showed the sperm plasma membrane surface restored to its original appearance (Fig. 1C).

These results are in agreement with those for membrane integrity obtained by fluorescence markers, because the 26% ± 2.2% viability value obtained after the cold shock increased to 58.1% ± 7.7% after protein incubation.

The proportion of cells with restored surface characteristics was in relation to the concentration of proteins in the incubation medium (Fig. 2). A recovering percentage of 83% of membrane-intact cells was achieved after incubating 1×10^6 cells with 1.4 mg of seminal plasma proteins. A saturating effect is appreciated between 1.5 and 2.5 mg, because the slope is less pronounced than that in the first part of the curve.

Fractionation of Seminal Plasma Proteins

To establish baseline information about patterns of ram seminal plasma proteins, SDS-PAGE analysis was per-

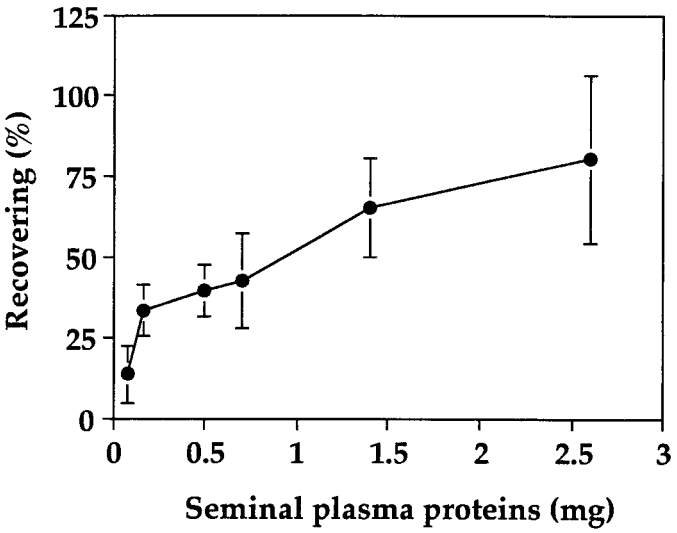

FIG. 2. Survival of cold-shocked ram spermatozoa following addition of seminal plasma proteins after 60 min of incubation. An aliquot of 0.5 ml containing 1×10^6 cells was incubated with different amount of proteins. The recovering percentage of propidium iodide-negative spermatozoa assessed as $(Vp_{60} - Vc_{60})/(Vs - Vc_{60}) \times 100$, where Vs is the swim-up sample viability; Vc is the cold-shocked, control sample viability; Vp is the protein-added, cold-shocked sample viability, and 60 is the incubation time (min) after addition of proteins. Data are shown as mean ± SD of four experiments.

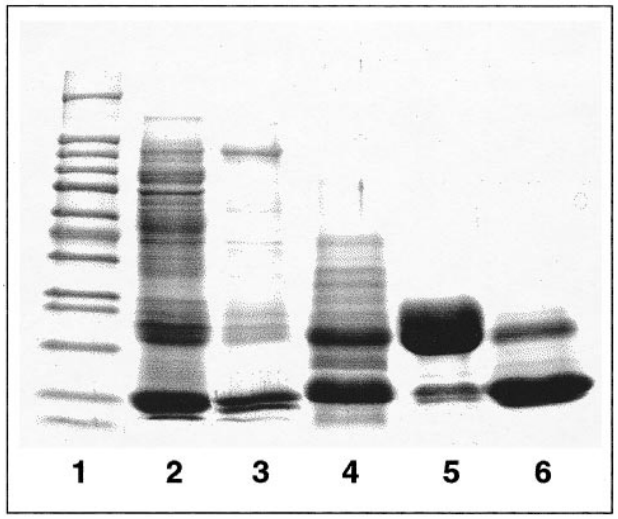

FIG. 3. Coomassie brilliant blue-stained protein bands in ram seminal plasma separated by SDS-PAGE. Each lane was loaded with 15 μg of protein. Lane 1: molecular weight markers; lane 2: whole ram seminal plasma; lane 3: seminal plasma lipoproteins; lanes 4, 5, and 6: fraction 3, 6, and 7, respectively, obtained from chromatography on Sephacryl-100 HR.

formed. Approximately 20 seminal plasma polypeptide fractions were visualized in whole seminal plasma (Fig. 3, lane 2) ranged in molecular weight from less than 12 to nearly 200 kDa. The most prominent bands were those less than 70 kDa.

To investigate which kind of seminal plasma proteins were responsible for the reversion effect, different purification strategies were performed. Seminal plasma lipoproteins are not involved in the recovering effect, because the addition of lipoproteins isolated from ram seminal plasma (Fig. 3, lane 3) were unable to reverse the cold-shock-induced damage (Table 1). Likewise, neither cationic nor anionic exchange chromatography, using fast protein liquid chromatography, was efficient to successfully separate the active proteins (data not shown). Elution profiles of exclusion chromatography on Sephacryl-100 HR revealed that despite seminal plasma polypeptides having a strong tendency to associate between them, eight main fractions were separated (Fig. 4). Fractions 1, 6, 7, and 8 account for 70% of the total seminal plasma proteins.

Cold-Shocked Sperm Recovered Membrane Integrity after Incubation with Protein Fractions

Assessment of membrane integrity by fluorescence markers revealed that among the eight obtained fractions, only three (fractions 3, 6, and 7) could reverse the cold-shock effect on ram spermatozoa (Table 2). Although SDS-PAGE analysis still displayed several polypeptides in the three fractions (Fig. 3, lanes 4, 5, and 6), the prominent band in fraction 6 of approximately 20 kDa is much slighter in fraction 3 and 7.

Fraction 6 had the greatest reversion capacity, because a recovery of 70% was achieved after incubation with this fraction. In contrast, recoveries of 22% and 33% were achieved after addition of fractions 3 and 7, respectively (Fig. 5).

TABLE 1. Effect of the addition of seminal plasma lipoproteins.[a]

	0.7 mg		1.4 mg	
Incubation	Control	Lipoproteins	Control	Lipoproteins
0 minutes	30.66 ± 4.98	33 ± 6.97	32 ± 3.25	31 ± 4.16
1 hour	27 ± 7.48	26.66 ± 5.24	25 ± 5.20	24 ± 6.31

[a] Mean values ± SD of intact-membrane spermatozoa (%), n = 3.

Scanning Electron Microscopy Detected Restoration of Sperm Plasma Membrane After Incubation with Fraction 6

Scanning electron microscopy also showed that incubation with fraction 6 reversed the structural damage induced by the cold shock (Fig. 1D), restoring the original appearance of the sperm plasma membrane surface. After incubating 1×10^6 cells with 0.7 mg of fraction 6 protein, approximately 50% of damaged spermatozoa showed repaired surface. These results are in agreement with those for membrane integrity obtained by fluorescence markers. Thus, a swim-up-obtained sample viability of 70% ± 0.2% decreased to 17.6% ± 1.2% after the cold shock and increased to 54.3% ± 3.0% (n = 3) after incubation with 0.7 mg of fraction 6 proteins.

DISCUSSION

The present study supports our previous observations [27, 45, 46] that acquisition of seminal plasma proteins by adsorption to the sperm cell surface modifies the functional characteristics of damaged spermatozoa, reproducing that of live cells.

Several studies have provided direct evidence that specific components of seminal plasma are adsorbed onto the surface of ejaculated sperm [25, 29, 33, 47, 48]. It has been suggested that these sperm-coating components have an important function in the process of capacitation and/or sperm transport [49], and that removal of some of these components in the female genital tract is a prerequisite for fertilization [48]. In addition, the need for some of these adsorbed proteins not only to acquire the fertilizing capacity

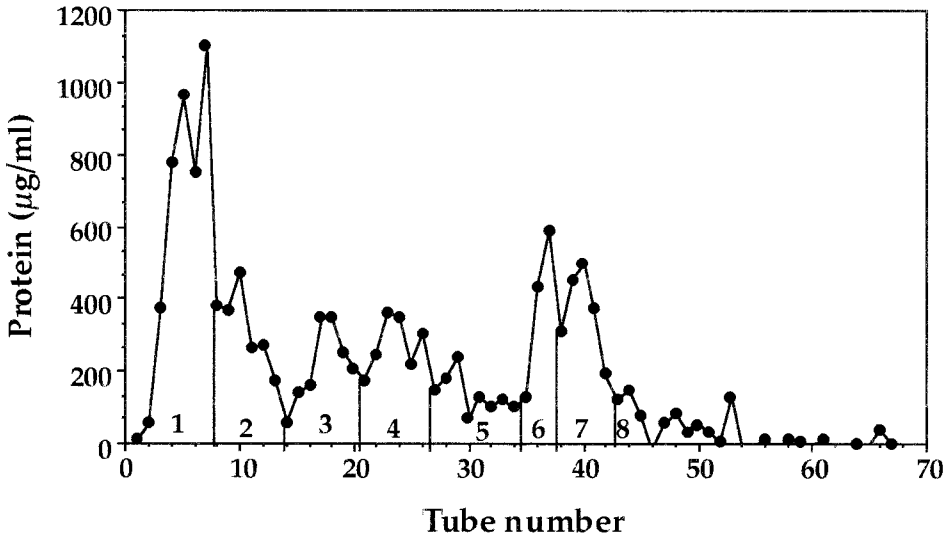

FIG. 4. Elution profile of exclusion chromatography on Sephacryl-100 HR ram seminal plasma proteins. Forty milligrams of proteins were loaded, and fractions of 1 ml were collected. The numbers correspond to the protein fractions depicted in Table 2.

but also to maintain cell viability has already been reported [50]. Some of the proteins function to stabilize the membrane until the appropriate time, whereas others ultimately contribute to membrane loss (i.e., to exocytosis). Thus, it is critically important that capacitation and the acrosome reaction occur in a temporal-spatial framework that is conducive to and facilitative of fertilization.

Results of biochemical studies of cold shock on mammalian spermatozoa have demonstrated that proteins and lipids are released during the treatment and that morphological damage occurs, especially to the acrosome and plasma membrane [19, 51, 52].

Results of the present study indicate that the primary lesion associated with cold-shock damage is the plasma membrane, accounting for considerable morphological alterations. In addition, important changes in membrane permeability also result from the injury. The hypothesis that seminal plasma components could improve the viability (i.e., membrane integrity) of cold-shocked ram spermatozoa was tested. The adsorption of proteins isolated from seminal plasma reverts this damage, as confirmed by the results of electron microscopy and chemical analysis, possibly by occupying sites on the plasma membrane surface. The results obtained with increasing amounts of seminal plasma proteins also support this suggestion. Thus, the increase in the percentage of membrane-intact spermatozoa resulted from the acquisition of greater adsorbed seminal plasma proteins. This protein adsorption would repair damage caused by the cold shock, restoring the impermeability of the plasma membrane to propidium iodide.

TABLE 2. Reversion of cold-shock effect on ram spermatozoa.[a]

Fraction[b]	Vs	Vc_0	Vp_0	Vc_{60}	Vp_{60}
3	68.3 ± 2.3	19 ± 6.4	31.6 ± 9.6	18.6 ± 5.5	35.3 ± 8.0
6	70.0 ± 0.2	17.3 ± 1.2	32.3 ± 4.4	17.6 ± 1.2	54.3 ± 3.0
7	70.6 ± 0.4	13.3 ± 3.0	23.0 ± 3.5	18.3 ± 0.4	33.3 ± 4.0

[a] Mean values ± SD (n = 3).

[b] Active fractions of seminal plasma proteins obtained following exclusion chromatography on Sephacryl-100 HR. Subscripts (0 or 60) indicate incubation time after addition of proteins. Vs, Swim-up sample viability; Vc, cold-shocked sample viability; Vp, cold-shocked sample viability after addition of proteins.

The effect of cooling on sperm membrane has been thoroughly studied, and postcooling spermatozoa are suggested to have the features of premature capacitation [8, 35]. Moreover, a high percentage of capacitated sperm has been determined after cooling mouse spermatozoa at 4°C [34]. Capacitation involves the removal of cholesterol and plasma membrane-bound coating proteins, which results in membrane destabilization as well as increased membrane fluidity and permeability, with alterations in the mobility of integral proteins [37, 53, 54]. Seminal plasma can suppress capacitation [55–57] and decapacitate previously capacitated spermatozoa [58]. Cross [57] further established that

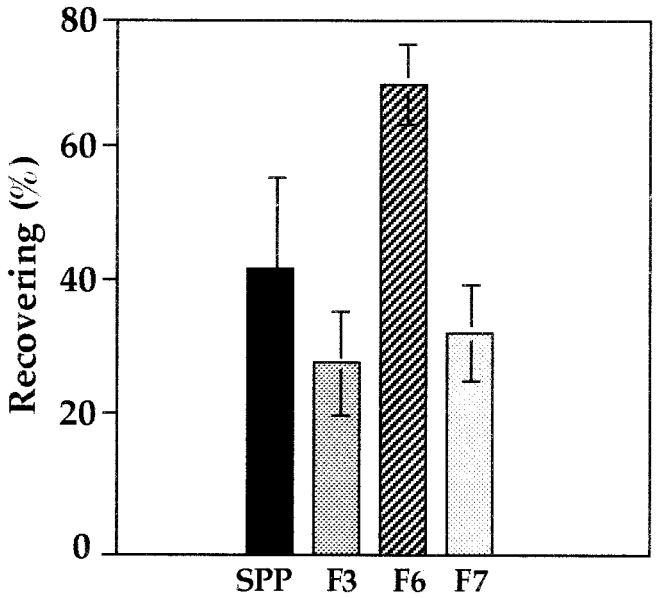

FIG. 5. Recovering percentages of propidium iodide-negative spermatozoa. An aliquot of 0.5 ml, containing 1×10^6 cells, was incubated with 0.7 mg of seminal plasma proteins (SPP) or proteins from fraction 3 (F3), 6 (F6), or 7 (F7) isolated by chromatography on Sephacryl-100 HR. Recovery (%) was assessed as $(Vp_{60} - Vc_{60})/(Vs - Vc_{60}) \times 100$, where Vs is the swim-up sample viability; Vc is the cold-shocked, control sample viability; Vp is the protein-added, cold-shocked sample viability, and 60 is the incubation time (min) after addition of proteins. Data are shown as mean ± SD (n = 3).

seminal plasma can prevent or reverse the development of acrosomal responsiveness in human spermatozoa, and that the main role of seminal plasma may be to maintain the spermatozoa in a decapacitated state. That adsorption of seminal plasma proteins reverts the cooling effect sustains these data. Proteins from seminal plasma adsorbed onto the capacitated spermatozoa surface would revert them to a decapacitated state.

To investigate further which kind of seminal plasma proteins were responsible for the reversion effect, several fractionation processes were performed. Although lipoproteins have been described as being involved in sperm capacitation by interacting with sperm proteins [59–61] and egg yolk lipoproteins are the active substances that can protect sperm membranes from cooling injury [62, 63], our results indicate that lipoproteins isolated from ram seminal plasma cannot reverse the effect of cold shock on ram spermatozoa. Nevertheless, by exclusion chromatography, we separated three seminal plasma protein fractions that could recover the sperm membrane permeability characteristic of a live cell. The results of this study clearly show that a higher proportion of spermatozoa possessed intact membrane after incubation with each of these three fractions. The higher activity of fraction 6 was also confirmed by scanning electron microscopy, which showed approximately 50% of damaged cells with the restored, original appearance of the sperm plasma membrane surface. Differences observed in composition between the three isolated fractions mainly result from one major band of approximately 20 kDa, which accounts for more than 40% of the total proteins in fraction 6; however, this band is much less prominent in the two other fractions. That fraction 6 showed the highest activity suggests this major band must be responsible for the described reversion capacity.

The observation, both in this study and by partition in an aqueous two-phase system [46], that the adsorption of seminal plasma proteins to cold-shocked spermatozoa restores membrane permeability, together with the ultrastructural findings, indicates that membrane integrity is largely recovered. The exact components of seminal plasma that are responsible for the effects observed are yet to be identified, but this work presents convincing evidence that seminal plasma is important in determining the quality of ejaculated spermatozoa. The study of seminal plasma component effects on spermatozoa could help in the formulation of better diluents for preserving spermatozoa during freezing or storage at 4°C, room temperature, or even body temperature. If the protective effect of ram seminal plasma proteins can be promoted at insemination by changing the spermatozoa to a suitable medium, such a procedure may allow storage of spermatozoa for extended periods and improvement of cryopreservation methods for ram semen.

ACKNOWLEDGMENTS

We would like to thank S. Morales for his assistance in the collection of semen samples and J. Medina for technical helping with figures.

REFERENCES

1. Saling PM. Mammalian sperm interaction with extracellular matrices of the egg. In: Milligan SR (ed.), Oxford Reviews of Reproductive Biology. New York: Oxford University Press; 1989: 339–388.
2. Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. Zygote 1994; 3:371–372.
3. Mann T, Lutwak-Mann C. Male Reproductive Function and Semen. Berlin: Springer-Verlag; 1981.
4. Setchell BP, Maddoks S, Brooks DE. Anatomy, vasculature, innervation and fluids of the male reproductive tract. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1994: 1063–1176.
5. Dot HM, Harrison RAP, Foster GCA. The maintenance of motility and the surface properties of epididymal spermatozoa from bull, rabbit and ram in homologous seminal and epididymal plasma. J Reprod Fertil 1979; 62:113–124.
6. Baas JW, Molan PC, Shannon P. Factors in seminal plasma of bulls that affect the viability and motility of spermatozoa. J Reprod Fertil 1983; 68:275–280.
7. Graham JK. Effect of seminal plasma on the motility of the epididymal and ejaculated spermatozoa of the ram and bull during the cryopreservation process. Theriogenology 1994; 41:1151–1162.
8. Ashworth PJC, Harrison RAP, Miller NGA, Plummer JM, Watson PF. Survival of ram spermatozoa at high dilution: protective effect of simple constituents of culture media as compared with seminal plasma. Reprod Fertil Dev 1994; 6:173–180.
9. Maxwell WMC, Welch GR, Johnson LA. Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev 1997; 8:1165–1178.
10. Pursel VG, Johnson LA, Schulman LL. Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. J Anim Sci 1973; 37:528–531.
11. Berger T, Clegg E. Effect of male accessory gland secretions on sensitivity of porcine sperm acrosomes to cold shock. Initiation of motility and loss of cytoplasmic droplets. J Anim Sci 1985; 60:1295–1302.
12. Wales RG, White IG. The susceptibility of spermatozoa to temperature shock. J Endocrinol 1959; 19:211–220.
13. Moore HDM, Hibbit KG. Fertility of boar spermatozoa after freezing in the absence of seminal vesicular proteins. J Reprod Fertil 1977; 50: 349–352.
14. de Lamirande E, Gagnon C. Origin of a motility inhibitor within the male reproductive tract. J Androl 1984; 5:269–276.
15. Iwamoto T, Tanaka H, Osada T, Shinagawa T, Osamura Y, Gagnon C. Origin of a sperm motility inhibitor from boar seminal plasma. Mol Reprod Dev 1993; 36:475–481.
16. García MA, Graham EF. Effects of low-molecular-weight fractions (LMWF) from milk, egg yolk and seminal plasma on freezability of bovine spermatozoa. Cryobiology 1987; 24:429–436.
17. García MA, Graham EF. Factors affecting the removal of low-molecular-weight fractions (LMWF) from egg yolk and seminal plasma in extended semen by dialysis: effect of post-thaw sperm survival. Cryobiology 1987; 24:437–445.
18. García MA, Graham EF. Dialysis of bovine semen and its effects on fresh and freeze-thawed spermatozoa. Cryobiology 1987; 24:446–454.
19. Watson PF. The effects of cold shock on sperm cell membranes. In: Morris GJ, Clarke A (eds.), Effects of Low Temperatures on Biological Membranes. London: Academic Press; 1981: 189–218.
20. Ritar AJ, Salamon S. Effect of seminal plasma and its removal and of egg yolk in the diluent on the survival of fresh and frozen thawed spermatozoa of Angor goat. Aust J Biol Sci 1982; 35:305–312.
21. Schmehl MK, Anderson SP, Vazquez IA, Graham EF. The effect of dialysis of extended ram semen prior to freezing on post-thaw survival and fertility. Cryobiology 1986; 23:406–414.
22. Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in holstein bull seminal plasma. Biol Reprod 1993; 49:1202–1207.
23. McCauley TC, Zhang H, Bellin ME, Ax RL. Purification and characterization of fertility-associated antigen (FAA) in bovine seminal fluid. Mol Reprod Dev 1999; 54:145–153.
24. Parent S, Lefièvre L, Brindle Y, Sullivan R. Bull subfertility is associated with low levels of a sperm membrane antigen. Mol Reprod Dev 1999; 52:57–65.
25. Metz KW, Berger T, Clegg ED. Adsorption of seminal plasma proteins by boar spermatozoa. Theriogenology 1990; 34:691–700.
26. Harrison RAP, Jacques ML, Minguez MLP, Miller NGA. Behaviour of ejaculated spermatozoa from bull, boar and ram during thin-layer countercurrent partition in aqueous 2-phase systems. J Cell Sci 1992; 102:123–132.
27. Ollero M, García-López N, Cebrián-Pérez JA, Muiño-Blanco T. Surface changes of ram spermatozoa by adsorption of homologous and heterologous seminal plasma proteins revealed by partition in an aqueous two-phase system. Reprod Fertil Dev 1997; 9:381–390.
28. Carballada R, Esponda P. Binding of seminal vesicle proteins to the

plasma membrane of rat spermatozoa in vivo and in vitro. Int J Androl 1998; 21:19–28.
29. Amann RP, Hammerstedt RH, Shabanowitz RB. Exposure of human, boar or bull sperm to a synthetic peptide increases binding to a egg-membrane substrate. J Androl 1999; 20:34–41.
30. Holt WV, North RD. Partially irreversible cold-induced lipid phase transitions in mammalian sperm plasma membrane domains: freeze-fracture study. J Exp Zool 1984; 230:473–483.
31. Fiser PS, Fairfull RW. The effect of glycerol-related osmotic changes on post-thaw motility and acrosomal integrity of ram spermatozoa. Cryobiology 1989; 26:64–69.
32. Holt WV, Head MF, North RD. Freeze-induced membrane damage in ram spermatozoa is manifested after thawing—observations with experimental cryomicroscopy. Biol Reprod 1992; 46:1086–1094.
33. Leeuw de FE, Chen HC, Colenbrander B, Verkleij AJ. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology 1990; 27:171–183.
34. Fuller SJ, Wood MJ, Whittingham DG, Watson PF. Cooling mouse sperm to 4°C does not affect fertilization or embryonic development. J Reprod Fertil Abstr Ser 1994; 14:8.
35. Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995; 7:871–891.
36. Perez LJ, Valcarcel A, Delasheras MA, Moses D, Baldassarre H. Evidence that frozen/thawed ram spermatozoa show accelerated capacitation in vitro as assessed by chlortetracycline assay. Theriogenology 1996; 46:131–140.
37. De Jonge CJ. Attribute of fertile spermatozoa: an update. J Androl 1999; 20:463–473.
38. Ollero M, Muiño-Blanco T, López-Pérez M, Cebrián-Pérez JA. Viability of ram spermatozoa in relation to the abstinence period and successive ejaculations. Int J Androl 1996; 19:287–292.
39. García-López N, Ollero M, Muiño-Blanco T, Cebrián-Pérez JA. A dextran swim-up procedure for the separation of highly motile and viable ram spermatozoa from seminal plasma. Theriogenology 1996; 46:141–151.
40. Martí JI, Pérez-Pé R, Grasa P, Báguena O, Muiño-Blanco T, Cebrián-Pérez JA. Estudio de diversos parametros de calidad seminal en semen completo y en muestras seleccionadas mediante un proceso de swim-up/dextrano. In: García Herradón P, Quintela Arias LA, Becerra GonzálezI JJ (eds.), II Congreso Ibérico de Reproducción Animal. Lugo: Breogan; 1999: 326–328.
41. Harrison RAP, Vickers SE. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. J Reprod Fertil 1990; 88: 343–352.
42. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal Biochem 1976; 72:248–254.
43. Kelley JL, Kruski AW. Density gradient ultracentrifugation of serum lipoproteins in a swinging bucket rotor. Methods Enzymol 1986; 128: 170–180.
44. Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 1970; 227:680–685.
45. Pascual ML, Muiño-Blanco T, Cebrián-Pérez JA, López-Pérez M. Acquisition of viable-like surface properties of sperm cells by adsorption of seminal plasma proteins revealed by centrifugal countercurrent distribution. Biol Cell 1994; 82:75–78.
46. García-López N, Ollero M, Cebrián-Pérez JA, Muiño-Blanco T. Reversion of thermic-shock effect on ram spermatozoa by adsorption of seminal plasma proteins revealed by partition in aqueous two-phase systems. J Chromatogr B Biomed Appl 1995; 680:137–143.
47. Johnson MH. The macromolecular organization of membranes and its bearing on events leading up to fertilization. J Reprod Fertil 1975; 44: 167–184.
48. Desnoyers L, Manjunath P. Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. J Biol Chem 1992; 267: 10149–10155.
49. Cross NL. Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. Biol Reprod 1996; 54:138–145.
50. Russell LD, Montag B, Hunt W, Peterson RN. Properties of boar sperm plasma membranes (PM): proteins released by washing and differential solubility in salts, detergents, and sensitivity to surface radiolabelling. Gamete Res 1985; 11:237–252.
51. Mann T. The Biochemistry of Semen and of the Male Reproductive Tract. London: Methuen & Co Ltd.; 1964.
52. Darin-Bennett A, Poulos A, White IG. The effect of cold shock and freeze-thawing on release of phospholipids by ram, and boar spermatozoa. Aust J Biol Sci 1973; 26:1409–1420.
53. Yeagle PL. Modulation of membrane function by cholesterol. Biochimie 1991; 73:1303–1310.
54. Benoff S. Preliminaries to fertilization: the role of cholesterol during capacitation of human spermatozoa. Hum Reprod 1993; 8:2001–2006.
55. Davis B, Niwa K. Inhibition of mammalian fertilization in vitro by membrane vesicles from seminal plasma. Proc Soc Exp Biol Med 1974; 146:11–16.
56. Byrd W, Wolf DP. Acrosomal status in fresh and capacitated human ejaculated sperm. Biol Reprod 1986; 34:859–869.
57. Cross NL. Multiple effects of seminal plasma on the acrosome reaction of human sperm. Mol Reprod Dev 1993; 35:316–323.
58. Chang M. A detrimental effect of seminal plasma on the fertilizing capacity of sperm. Nature 1957; 179:258–259.
59. Manjunath P, Marcel YL, Uma J, Seidah NG, Chrétien M, Chapdelaine A. Apolipoprotein A-I binds to a family of bovine seminal plasma proteins. J Biol Chem 1989; 264:16853–16857.
60. Manjunath P, Chandonnet L, Leblond E, Desnoyers L. Major proteins of bovine seminal vesicles bind to spermatozoa. Biol Reprod 1993; 49:27–37.
61. Huang L-S, Voyiaziakis E, Chen HL, Rubin EM, Gordon JW. A novel functional role for apolipoprotein B in male infertility in heterozygous apolipoprotein B knockout mice. Proc Natl Acad Sci U S A 1996; 93:10903–10907.
62. Quinn PJ, Chow PYW, White IG. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. J Reprod Fertil 1980; 60:403–407.
63. Holt WV, North RD. The role of membrane-active lipids in the protection of ram spermatozoa during cooling and storage. Gamete Res 1988; 19:77–89.