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ScienceDirect
Theriogenology xxx (2009) xxx–xxx
Theriogenology
www.theriojournal.com

Differing sperm ability to penetrate the oocyte in vivo and in vitro as revealed using colloidal preparations

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Received 23 March 2009; received in revised form 10 July 2009; accepted 12 July 2009

Abstract

The penetration ability of boar (Sus scrofa domestica) spermatozoa exposed to viscous preparations under in vivo and in vitro fertilization conditions has been examined. Experiments involving induced ovulation in prepubertal animals and surgical insemination directly into the oviduct isthmus revealed an advantage of colloidal preparations. Based on within-animal comparisons, the incidence of penetration was 100% using both spermatozoa suspended in a viscous preparation of plant extracts and spermatozoa suspended in a control medium. However, percentages of monospermy were 22.2% in 54 oocytes inseminated with the control suspension compared with 62.5% in 48 oocytes inseminated with the colloidal preparation. An in vitro study involving 355 oocytes from slaughterhouse ovaries inseminated with in vitro–capacitated spermatozoa gave similar percentages of penetrated oocytes for both the control and colloidal suspensions. In this case, however, the percentage of monospermy was 32.7% in the control group compared with 10.6% for spermatozoa suspended in the colloidal preparation. Higher mean numbers of sperm inside the oocytes and higher numbers of sperm bound to the zona pellucida were also observed with the colloidal suspensions. In vitro motility and viability for spermatozoa in the colloidal suspensions were enhanced compared with that of the control group. Lower sperm membrane lipid disorder and reactive oxygen species generation were also observed in the viscous solution. These findings suggest that viscous fluids can enhance the ability of sperm to move, bind, and penetrate the oocyte in vitro, although this influence may be masked in vivo due to the already high viscosity in the oviductal fluid close to the time of ovulation.
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Keywords: Fertilization; Glycoprotein; Oocytes; Spermatozoa; Sperm motility; Viscosity

Introduction

Differences between the fluids surrounding the gametes during fertilization in vivo or in vitro include not only chemical but also physical properties. Concerning the former, there have been many studies using different molecules in various in vitro fertilization media attempting to mimic the composition of the oviductal fluid [1–4]. However, information is scarce regarding the physical properties of both physiologic and artificial fluids.

One important physical characteristic of the organic fluids is the viscosity. In the oviduct, the viscous glycoprotein secretions that accumulate in the caudal portion are thought to regulate sperm ascent, contributing critically to formation of a sperm reservoir and thereby to

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doi:10.1016/j.theriogenology.2009.07.011

Please cite this article in press as: Coy P, et al. Differing sperm ability to penetrate the oocyte in vivo and in vitro as revealed using colloidal preparations. Theriogenology (2009), doi:10.1016/j.theriogenology.2009.07.011

a preovulatory sperm gradient along the isthmus. Under the influence of increasing titers of plasma progesterone, such secretions undergo a progressive reduction in extent and viscosity during the postovulatory interval, dissipation being largely completed before passage of embryos into the uterus 46 to 48 h after ovulation [5–7].

Under in vitro conditions, by contrast, the viscosity of the medium is rather consistent, usually close to the viscosity of water and much lower than that of the oviductal fluid. Indeed, fetal calf serum (FCS), bovine serum albumin (BSA), or polyvinyl alcohol (PVA) are frequent components of such media [8–11] providing a source of proteins and/or increasing the viscosity, but never reaching the levels presumed in oviductal fluid, where precise measurements of viscosity are not available.

On the other hand, an influence of the viscosity of the medium on sperm motility has been noted, either as a beneficial or as detrimental factor, depending on the site/status of the spermatozoa. In the oviductal environment, bull spermatozoa in the caudal isthmus develop hyperactive motility with frequent changes in direction that help them detach from the epithelium, progress to the ampulla, encounter the cumulus mass, and more easily penetrate the cumulus matrix and the zona pellucida [12–14]. Such events for bull spermatozoa appear quite different from the hyper-viscoelasticity in the human seminal fluid, which is associated with a lower percentage of motile spermatozoa and infertility [15]. Thus, it could be inferred that a certain degree of viscosity is desirable once spermatozoa have escaped from seminal plasma and are ready to interact with the oocyte, either under in vivo (oviduct) or in vitro (culture medium) conditions.

From the above considerations, we hypothesized that an increase in the viscosity of the fluid in which spermatozoa are suspended just before fertilization might enhance their penetration ability by influencing their motility, viability, membrane stability, or reactive oxygen species (ROS) generation. Experiments to test our hypothesis have been performed either with sperm samples instilled surgically directly into the oviductal isthmus (in vivo) or added to an in vitro fertilization dish with in vitro–matured oocytes. Moreover, measurements of oviductal fluid and in vitro fertilization (IVF) media viscosities were performed.

Materials and methods

In vivo study

The animal experiments described below were carried out in accordance with EC Directive 86/609/EEC.

Animals

Prepubertal animals (Sus scrofa domestica) of the German Landrace breed were obtained from the experimental herd of the Federal Institute for Animal Health (Mariensee, Germany). They were aged 4 to 5 mo and weighed 70 to 85 kg. They were housed indoors under natural lighting and at a temperature of 18 °C and fed a standard commercial diet of concentrates twice daily. There was free access to drinking water.

Gonadotropin treatment

Ovulation was induced using systemic administrations of placental gonadotropins. Treatment consisted of a subcutaneous administration of 2000 IU pregnant mare serum gonadotropin (PMSG; Intergonan; Intervet, Unterschleissheim, Germany) followed 72 h later by an intramuscular administration of 500 IU human chorionic gonadotropin (hCG; Ovogest; Intervet). Ovulation was anticipated approximately 40 h after the hCG administration. The response to treatment was noted at the time of surgery.

Preparation of sperm suspensions

The same boar was used throughout the experiment. Semen was collected into a plastic container within a Dewar flask and brought to the laboratory within 5 min of the end of the procedure. It had been filtered free of the gelatinous fraction during collection, and the Dewar flask was maintained within a warmed and insulated polystyrene box (30 °C). The sperm concentration was estimated using a hemocytometer slide (Neubauer ruling), and dilutions were made to a value of 0.5×10^8 cells per mL. The diluent used on all occasions was Androhep (Minitüb, Tiefenbach, Germany), which had been freshly prepared and prewarmed to 30 °C. Motility was checked under a phase-contrast microscope with heated stage (37 °C) and progressive motility scored subjectively on a scale of 1 to 5. All preparations used had values of 3 or 4 at the time of insemination.

Sperm preparations referred to as control suspensions consisted of the freshly diluted samples in Androhep at a concentration of 0.5×10^8 cells per mL. The experimental preparation was also diluted in Androhep to which a powdered plant extract, E410 (96%) + E327 (4%) (Bindobin; Tartex & Dr Ritter GmbH, Freiburg, Germany) had been added to give a colloidal suspension (5 mg/mL). Referred to as JHB, this was vigorously agitated on a mechanical shaker for 3 min to give a well-dispersed and slightly viscous suspension to which a sperm sample was then added. Once again, the concentration of spermatozoa was 0.5×10^8 cells per mL. These preparatory steps were performed within

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20 min of surgery, and the sperm suspensions were then maintained in an incubator at 30 to 32 °C.

Surgical insemination

Animals had been starved for >12 h before commencement of surgery. Approximately 42 to 44 h after the hCG administration, premedication was administered as an intramuscular administration of Stresnil (Azaperone; Janssen Animal Health, Neuss, Germany) and anesthesia induced and maintained by intravenous administration of thiamylal (Surital; Pharmacia & Upjohn GmbH, Erlangen, Germany). The reproductive tract was exposed though a midventral laparotomy, the presence of recent ovulations or mature Graafian follicles noted with minimum of disturbance to the fimbriated extremity, and was insemination performed via the uterotubal junction. A round-tipped 22-gauge needle attached to a 1-mL prewarmed plastic syringe was passed across the uterine wall and carefully negotiated through the swollen processes of the uterotubal junction and some 0.5 cm further into the caudal isthmus. A sperm suspension (0.5×10^6 spermatozoa in 0.01 mL fluid) was then gently instilled into the tubal lumen. The side of the reproductive tract into which each of the respective sperm suspensions was instilled was noted and routine identification made with a fine silk ligature placed in the uterine wall approximately 10 cm from the junction. The incision was closed in two layers.

Recovery and preparation of oocytes

Animals were euthanized after sedation with Stresnil with an intracardiac administration of T61 (Intervet) within 4 to 7 h of the surgical insemination. The midline incision was promptly opened, the reproductive tract dissected free of its mesenteries, and the ovaries examined for the number of recent ovulations. Working in the neighboring laboratory, the fallopian tubes were freed of adjoining tissue and flushed at least twice with physiologic medium. This was collected in plastic Petri dishes and searched under a dissecting microscope to recover the oocytes. Fixation and staining were performed with acetic-alcohol (1:3) and 1% lacmoid [16], and evaluation of the different parameters was under phase-contrast microscopy at × 400 magnification. Penetration, number of spermatozoa per oocyte, and pronuclear formation were assessed in each oocyte.

In vitro study

In vitro maturation of oocytes

Within 30 min of slaughter, ovaries from Landrace × Large-White gilts were transported to the laboratory in saline containing 100 μg/mL kanamycin sulfate at 38 °C, then washed once in 0.04% cetrimide solution and twice in saline. Cumulus cell–oocyte complexes (COCs) were collected from antral follicles (3 to 6 mm diameter), washed twice with Dulbecco's PBS supplemented with 1 mg/mL PVA and 0.005 mg/mL red phenol, then washed twice more in maturation medium previously equilibrated for a minimum of 3 h at 38.5 °C under 5% CO_2 in air. The maturation medium was NCSU-37 [17] supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 mg/mL insulin, 50 mM β-mercaptoethanol, 10 IU/mL equine chorionic gonadotropin (eCG; Folligon; Intervet International BV, Boxmeer, Holland), 10 IU/mL hCG (Veterin Corion; Divasa Farmavic, Barcelona, Spain), and 10% porcine follicular fluid (vol/vol) as described previously [18]. Only COCs with complete and dense cumulus oophorus were used for the experiments. Groups of 50 COCs were cultured in 500 μL maturation medium for 22 h at 38.5 °C under 5% CO_2 in air. After culture, oocytes were washed twice in fresh maturation medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional 20 to 22 h [19].

In vitro fertilization

Cumulus cell–oocyte complexes cultured for a total of 44 h in maturation medium were washed three times with TALP medium consisting of 114.06 mM NaCl, 3.2 mM KCl, mM Ca-lactate·$5H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 10 mL/L Na-lactate, 1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/mL BSA (Sigma; A-9647, Madrid, Spain), 1 mg/mL PVA, and 0.17 mM kanamycin sulfate. Groups of 45 to 50 oocytes were transferred into each well of a 4-well multidish containing 250 μL TALP medium previously equilibrated at 38.5 °C under 5% CO_2. The sperm-rich fraction of semen from a mature, fertility-tested boar was collected by the gloved hand method and immediately transported to the laboratory and diluted 1:1 in Beltsville thawing solution [20]. Aliquots of the semen samples (0.5 mL) were centrifuged ($700 \times g$, 30 min) through a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient (45% and 90% vol/vol), and the resultant sperm pellets were diluted in TALP medium (without BSA) and centrifuged again for 10 min at $100 \times g$. The sperm concentration was calculated and 1×10^6 spermatozoa in a volume ranging from 5 to 10 μL were diluted with up to 1 mL TALP medium containing or lacking 5 mg/mL JHB. The sperm suspensions were then maintained in an incubator at 30 to 32 °C for 20 min, and at the end of this time, 100 μL of this suspension was added to 150 μL TALP medium. The

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final 250 μL of every suspension was added to the wells containing the oocytes, giving a final concentration of 2×10^5 cells/mL. At 15 min postinsemination (hpi), oocytes were washed twice with fresh TALP by gentle aspiration through a glass pipette and allowed to continue in culture at 38.5 °C under 5% CO_2 for 18 to 20 h. Fixation and staining were performed with 0.5% glutaraldehyde and 1% Hoechst 33342 [18]. Oocytes were examined under epifluorescence microscopy at × 400 magnification. Sperm penetration, number of spermatozoa per oocyte, and pronuclear formation were assessed in each oocyte.

Assessment of sperm motility

Motion parameters were determined using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer; Microptic, Barcelona, Spain). The CASA-derived motility characteristics studied were the percentage of total motile spermatozoa (motil %), percentage of motile progressive spermatozoa (motil prog %), curvilinear velocity (VCL, μm/sec), straight-line velocity (VSL, μm/sec), average path velocity (VAP, μm/sec), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), amplitude of lateral head displacement (ALH, μm), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), and beat cross-frequency (BCF, Hz).

After sperm treatment and the incubation for 20 min in TALP medium containing either BSA or JHB, a 7-μL drop of the sample was placed on a warmed (37 °C) slide and covered with a 24 × 24 mm coverslip. The setting parameters were 25 frames in which spermatozoa had to be present in at least 15 to be counted. Images were obtained at × 200 magnification in a phase- contrast microscope, and spermatozoa with a VAP <20 μm/sec were considered immobile. A minimum of 5 fields per sample were evaluated, counting a minimum of 200 spermatozoa per subsample.

Assessment of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). A 15 mW argon ion laser operating at 488 nm excited the fluorophores. Data from 10,000 events per sample were collected in list mode, and four measures per sample were recorded. Flow cytometric data were analyzed via the program Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

Assessment of sperm capacitation. To detect increase in plasma membrane lipid packing disorder, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro 1 [21]. Stock solutions of M540 (1 mM) and Yo-Pro 1 (25 μM; Molecular Probes, Eugene, OR, USA) in DMSO were prepared. For each 1 mL diluted semen sample (containing 5×10^6 to 10×10^6 cells), 2.7 μL M540 stock solution (final concentration of 2.7 μM) and 1 μL Yo-Pro (25 nM final concentration) were added. M540 fluorescence was collected with a FL2 sensor using a 575-nm band-pass filter and Yo-Pro 1 with a FL1 sensor using a 525-nm band-pass filter. Cells were classified in three categories: low merocyanine fluorescence (viable, uncapacitated), high merocyanine fluorescence (viable, capacitated), or Yo-Pro-1 positive (dead).

Production of ROS. Production of ROS was measured by incubating the spermatozoa in the presence of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; 0.5 μM) during 60 min at 37 °C [21]. This dye is a fluorogenic probe commonly used to detect cellular ROS production. H_2DCFDA is a stable, cell-permeable, nonfluorescent probe. It is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescin upon oxidation. Green 5-IAF fluorescence was collected with a FL1 sensor using a 525-nm band-pass filter. Measurements were expressed as the mean green intensity fluorescence units (mean channel in the FL1), which was used as index of ROS generation.

Assessment of acrosome reaction. Seminal samples were incubated with 2 μL fluorescein-labeled lectin from the peanut plant, *Arachis hypogaea* (FITC-PNA; 100 mg/mL), and 5 μL propidium iodide (PI) stock solution (500 mg/mL) at room temperature for 10 min [21]. Fluorescence was measured using a FL-1 sensor, a 525-nm band-pass filter to detect FITC-PNA, and a FL-2 sensor and a 575-nm band-pass filter to detect PI. Four sperm subsets were detected: live acrosome intact, live acrosome damaged, and dead spermatozoa with and without acrosome intact.

Assessment of viscosity

Oviductal fluid samples from animals at the preovulatory phase of the estrous cycle, stored at –80 °C in our laboratory and collected as described previously [22], were pooled to reach a final volume of 1.5 mL. Samples of 1.5 mL water, TALP and JHB solutions for sperm preincubation, and final IVF medium with either BSA or JHB were also prepared. Measurements were taken in a

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Bohlin Rheometer (Cone-Plate CP 4/40) at 38.5 °C setting a constant shear stress of 1 Pa.

Statistical methods

Data are presented as the mean ± SEM, and all percentages were modeled according to the binomial model of variables. The variables in all the experiments were analyzed by one-way ANOVA. A P value <0.05 was taken to denote statistical significance.

Results

In vivo study

Twelve immature animals were administered gonadotropin preparations and, at the predetermined time for surgical insemination 42 to 44 h after the hCG administration, 11 of these showed an ovulatory response. The number of ovulations ranged from 3 to 37 (mean, 9.3). However, only seven of the animals had ovulated on both ovaries and yielded oocytes from both oviducts, thereby permitting comparison of the two insemination treatments. A total of 102 oocytes was recovered and examined, 48 from the oviducts receiving a preparation of sperm in the colloidal suspension and 54 from the control tubes (Table 1).

Surgical insemination of the respective sperm suspensions into the caudal isthmus of the two oviducts revealed an incidence of penetration of 100% using both spermatozoa suspended in the colloidal preparation or in the physiologic medium. However, percentages of monospermy varied between the two groups, being 22.2% in 54 oocytes inseminated with the control suspension compared with 62.5% in 48 oocytes inseminated with the colloidal preparation (Table 1). Polyspermy was principally dispermic (94.1%) in the former group compared with 51.2% in the control, but trispermy, tetraspermy, and pentaspermy were also recorded in the control (35.2% of oocytes overall). The number of spermatozoa counted on or in the zona pellucida during the staining procedure varied from 28 to 100 in the controls but was conspicuously lower after insemination with the colloidal sperm preparation (3 to 50).

Table 1
Results of surgical insemination: Comparison of introducing the colloidal sperm preparation (JHB) into the isthmus of one fallopian tube and a control sperm preparation into the contralateral isthmus.

Group (N)	Proportion of eggs penetrated, %	Incidence of monospermy, %
Control (54)	100	22.2 ± 5.71^{a}
JHB* (48)	100	62.5 ± 7.06^{b}

* The full details of JHB (colloidal preparation) are given in Section 2.[a,b]Different superscripts in the same column indicate significant differences.

Table 2
Results of IVF: Comparison of incubating the sperm in the colloidal suspension (JHB) and in a control preparation.

Group (N)	Proportion of eggs penetrated, %	Incidence of monospermy, %
Control (178)	87.08 ± 2.52	32.69 ± 3.78^{a}
JHB* (177)	90.4 ± 2.22	10.63 ± 2.44^{b}

* The full details of JHB (colloidal preparation) are given in Section 2.[a,b]Different superscripts in the same column indicate significant differences.

A total of three primary oocytes was also recovered, each with a prominent germinal vesicle.

In vitro study

The results from four separate in vitro trials using a total of 355 oocytes indicated a different influence of the sperm treatment with the colloidal suspension for the penetration into the oocytes (Table 2). As in the in vivo situation, the penetration percentages were similar in both control and experimental groups. However, the incubation of the sperm with the colloidal suspensions led to a lower percentage of monospermy (10.6 ± 2.4 vs. 32.69 ± 3.8). In a more detailed analysis, a higher total number of sperm inside each penetrated oocyte was observed, resulting in a high number of pronuclei

Table 3
Details of the sperm binding and penetration after IVF: Comparison of incubating the sperm in the colloidal suspension (JHB) and in a control preparation.

Group	Total number of spermatozoa per oocyte	Mean number of male pronuclei per oocyte	Mean number of decondensed sperm heads per oocyte	Mean number of spermatozoa on the zona pellucida
Control	2.7 ± 0.2^{a}	2.7 ± 0.1^{a}	0.7 ± 0.1^{a}	6.2 ± 0.4^{a}
JHB*	5.8 ± 0.3^{b}	3.2 ± 0.9^{b}	3.3 ± 0.3^{b}	12.2 ± 0.7^{b}

* The full details of JHB (colloidal preparation) are given in Section 2.[a,b]Different superscripts in the same column indicate significant differences.

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Table 4
Motility parameters measured by CASA: Comparison of incubating the sperm in the colloidal suspension (JHB) and in a control preparation.

Group	Progressive motility, %	Percentage motility, %	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Control	11.43 ± 3.26	32.96 ± 4.29	50.11 ± 2.28	14.61 ± 1.92	22.64 ± 1.29	24.36 ± 1.33	52.89 ± 1.70	43.93 ± 1.90	1.71 ± 0.25	2.82 ± 0.47
JHB*	44.67 ± 4.50	73.54 ± 2.33	57.36 ± 2.07	20.93 ± 1.17	35.50 ± 1.90	35.89 ± 1.37	58.32 ± 1.32	61.25 ± 1.93	2.04 ± 0.06	5.96 ± 0.22
P value	<0.01	<0.01	0.02	0.01	<0.01	<0.01	0.01	<0.01	0.21	<0.01

VCL, curvilinear velocity (μm/sec); VSL, straight-line velocity (μm/sec); VAP, average path velocity (μm/sec); LIN, linearity of the curvilinear trajectory (ratio of VSL/VCL, %); STR, straightness (ratio of VSL/VAP, %); ALH, amplitude of lateral head displacement (μm); WOB, wobble (ratio of VAP/VCL, %); BCF, beat cross-frequency (Hz).
* The full details of JHB (colloidal preparation) are given in Section 2.

and swollen sperm heads (Table 3). Dispermic and trispermic penetration represented the predominant anomaly in the control group, but tetraspermic, pentaspermic, sexaspermic, and highly polyspermic penetration were frequent for the JHB treatment. Finally, the total number of sperm bound to the zona pellucida was also higher for the group fertilized with the sperm incubated in the colloidal suspension, ranging from 1 to 48 spermatozoa (Table 3).

Significant differences were found for all the motility parameters analyzed except the amplitude of lateral head displacement (ALH), with always higher values for the sperm incubated in the colloidal suspension after the in vitro capacitation treatment than for the sperm on the control suspension (Table 4).

Sperm membranes were more stable in terms of both capacitation and spontaneous acrosome reaction after sperm incubation for 20 min in TALP medium containing JHB than in the medium containing BSA (Tables 5 and 6). Sperm viability was also higher and ROS generation was lower in the JHB preparation (Tables 5, 6 and 7).

Viscosity data

Viscosity of the oviductal fluid samples was 29.7 mPa sec. TALP and JHB solutions for sperm pre-incubation gave viscosity values of 2.74 mPa sec and 38.7 mPa sec, respectively. The viscosity in the final IVF medium (after adding 100 μL sperm suspension to 150 μL IVF TALP, as explained in Section 2, and then to 250 μL IVF TALP containing the oocytes) was 2.70 mPa sec and 5.8 mPa sec for the control and JHB treatments, respectively. Water viscosity at the set-up conditions was 2.69 mPa sec.

Discussion

The results of these studies performed both in vivo and in vitro indicate that altering the physical condition of the medium in which ejaculated boar spermatozoa have been resuspended prior to insemination can act to modify the penetration ability when compared with a control sample from the same ejaculate. Although it may appear that the results from the in vivo and in vitro experiments are contradictory, a detailed analysis of these results leads us to propose one hypothesis: increased viscosity in the medium apparently enhances sperm membrane stability, viability, and ability to move and penetrate the oocyte, whereas a hyperviscosity (above the physiologic levels) could reduce the mentioned ability.

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Table 5
Membrane lipid packing disorder. Subpopulations of spermatozoa after staining with merocyanine 540 and Yo-Pro 1 and examined by flow cytometry; comparison of incubating the sperm in the colloidal suspension (JHB) and in a control preparation.

Group	Viable low lipid disorder, uncapacitated, %	Viable high lipid disorder, capacitated, %	Dead, %
Control	16.00 ± 2.140	32.04 ± 2.58	51.96 ± 3.48
JHB*	25.52 ± 3.43	38.89 ± 3.54	35.59 ± 3.07
P value	0.02	0.12	<0.01

* The full details of JHB (colloidal preparation) are given in Section 2.

Table 6
Acrosome reaction. Subpopulations of spermatozoa after staining with FITC-PNA and PI and examined by flow cytometry; comparison of incubating the sperm in the colloidal suspension (JHB) and in a control preparation.

Group	Intact acrosome and viable, %	Acrosome reacted and viable, %	Acrosome reacted and dead, %	Intact acrosome and dead
Control	51.87 ± 4.43	1.75 ± 0.28	9.60 ± 1.08	36.78 ± 3.64
JHB*	66.03 ± 3.01	1.30 ± 0.16	7.52 ± 0.73	25.16 ± 2.37
P value	0.02	0.15	0.11	0.01

* The full details of JHB (colloidal preparation) are given in Section 2.

Under the conditions of the current experiments, the numbers of spermatozoa penetrating the oocytes after surgical insemination into the caudal isthmus of the oviducts was reduced when the colloidal suspension was used, as is indicated by the levels of polyspermy (38% with spermatozoa suspended in JHB and 78% with the control sperm suspension). It was previously demonstrated that surgical insemination of boar spermatozoa directly into the isthmus at varying intervals before ovulation produced polyspermy (reviewed in [23]). The degree of polyspermy increased dramatically when the sperm were inseminated by 17 to 18 h before ovulation compared with 26 to 40 h, and it was as low as 2% with insemination 1 to 2 h before ovulation. In the current work, insemination was done around the ovulation time, but polyspermy still ensued, probably because the number of sperm introduced was very high (0.5×10^6 in the 0.01 mL of suspension introduced). However, when the colloidal suspensions were employed, sperm penetration of the oocyte was less effective. A possible explanation for this result can be found in the much-increased viscosity of the fluid in which sperm and oocytes met, due to simultaneous influences of (i) the viscous glycoprotein secretions in the oviduct around the ovulation time, with values that would be around the 29.7 mPa sec measured in our samples, and (ii) the extra viscosity provided by the sperm JHB suspension, which reached values of 38.7 mPa sec. As indicated in Section 1, hyper-viscoelasticity in human seminal fluid is associated with a lower percentage of motile spermatozoa and infertility [15], and this could have been the case in the current experiments. Whereas the 38.7 mPa sec viscosity in the JHB suspension could have resulted in beneficial effects for the spermatozoa, the addition of the viscosity in the JHB suspension (38.7 mPa sec) to the viscosity in the oviductal fluid (29.7 mPa sec) could have exceeded the physiologic threshold for the ''comfortable'' swimming of the spermatozoa in viscous solutions having a detrimental influence on the chances to reach the oocytes. In fact, hyper-viscoelasticity has been associated with a lower percentage of motile spermatozoa as well as with lower VCL, VAP, and ALH [15]. At the same time, and although viscosity in the oviductal fluid under physiologic conditions could not be measured, data from other physiologic fluids such as seminal plasma indicate that values of 1.59 mPa sec correspond with fertile men, whereas infertile men showed viscosity values around 1.99 mPa sec in their samples of seminal fluid [24]. Yet assuming that the physiologic oviductal fluid close to the time of ovulation is more viscous than seminal plasma, and that infertility in the men from the study of Aydemir et al. was not directly caused by the higher viscosity in their seminal fluids,

Table 7
Reactive oxygen species generation (mean channel of fluorescence) by boar spermatozoa: Comparison of incubating the sperm in the colloidal suspension (JHB) and in a control preparation.

Group	ROS
Control	28.52 ± 1.08
JHB*	24.03 ± 1.15
P value	0.01

* The full details of JHB (colloidal preparation) are given in Section 2.

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these results and other from Suarez and Dai [25], showing that mouse sperm movement was depressed when viscosity increased, could support our hypothesis.

However, alternative explanations should not be discarded. The colloidal material could have been acting at the surface of the zona pellucida to alter the kinetics and/or extent of so-called sperm-zona binding, even though the approximate number of sperm binding sites on the zona pellucida of an ovulated oocyte has not yet been characterized. One further consideration is that the colloidal material might have had a stabilizing influence on the sperm plasma membrane and/or endosalpingeal microvilli, to some degree functioning as a macromolecule. This, in turn, might have reduced the chances of simultaneous completion of capacitation and initiation of the acrosome reaction, thus reducing the number of spermatozoa competent to penetrate the zona pellucida more or less simultaneously.

Some answers for the above questions were found in the results of the in vitro experiments. Use of the JHB preparation in the insemination solutions prepared after the Percoll selection of the spermatozoa indicate that the increased viscosity of the JHB medium in which the sperm were incubated for 20 min produced an increase in their penetration ability as well as in their motility. It has been shown that hyperactivated sperm are better able to penetrate viscoelastic substances [26] and, although not mimicking with the hyperactivated sperm patterns, spermatozoa in JHB suspensions in the current experiments showed a significant increase in the motility parameters, which could have assisted in acquiring the hypermotility in the culture dish and reaching the oocyte. Thus, the effect of JHB on motility in vitro would not explain the differences found in the in vivo experiments (i.e., reduced polyspermy) due to the high differences in the final viscosity under in vivo and in vitro conditions. On the contrary, the increased motility for the JHB samples observed in the in vitro experiments does explain the increased polyspermy after IVF. From our visual impressions, the pattern of motility in the JHB samples could be subjectively described as ''three-dimensional,'' whereas in the control samples it seemed ''bidimensional.'' This pattern was revealed as advantageous to penetrate the oocyte in vitro. Indeed, after only 15 min of contact among sperm and oocytes, a higher number of sperm could be observed bound to the zona pellucida in the JHB group than that in the control.

The data about capacitation, production of ROS, and spontaneous acrosome reaction corroborate the results obtained from the motility and IVF assays. The JHB samples showed an increased viability and a higher membrane stability with lower generation of ROS than that of the control samples, guaranteeing the presence of a higher number of viable spermatozoa in the culture medium ready to confront the zona pellucida at the insemination time. Similarly, higher numbers of viable spermatozoa ready to undergo the acrosome reaction once bound to the zona pellucida were expected in the JHB group at any time in the light of the current results.

Nevertheless, returning to the beginning of this discussion, it seems that viscosity of the medium is an important parameter to bear in mind when in vivo or in vitro fertilization experiments are performed, while the precise evaluation of this physical characteristic in the fluids remains a challenge for future work. Additional experiments testing the effect of increased levels of viscosity in the sperm preincubation and IVF media will be performed in our laboratory in the near future. Almost certainly, when considered at the level of individual sperm cells, the viscosity of a medium will be in a dynamic state in the microenvironment.

Acknowledgments

We gratefully acknowledge the skilled assistance of Mrs. Antje Frenzel, Mrs. Petra Weidemann, Mr. Rolf Poppenga, Mr. Juan Antonio Carvajal, and Dr. Primoz Klinc. Mrs. Frances Anderson kindly prepared the manuscript in Scotland. These experiments were generously supported by the Foundation of the Friends of FAL and by the Spanish Ministry of Science and Innovation and FEDER(AGL2006-03495).

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