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Incubation of boar spermatozoa in viscous media by addition of methylcellulose improves sperm quality and penetration rates during *in vitro* fertilization

David González-Abreu [a], Soledad García-Martínez [a], Vanesa Fernández-Espín [b], Raquel Romar [a, c], Joaquín Gadea [a, c, *]

[a] Department of Physiology, International Excellence Campus for Higher Education and Research (Campus Mare Nostrum), University of Murcia, Murcia, 30100, Spain
[b] Department of Physical Chemistry, International Excellence Campus for Higher Education and Research (Campus Mare Nostrum), University of Murcia, Murcia, 30100, Spain
[c] Institute for Biomedical Research of Murcia IMIB-Arrixaca, Murcia, Spain

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ABSTRACT

This work was designed to study whether viscous media can improve the *in vitro* sperm functionality in pigs by using methylcellulose as a thickener. Viscosity of porcine oviductal fluid (POF) was compared with culture medium (Tyrode's) supplemented with methylcellulose (MET 0, 0.5 and 1% w/v). Spermatozoa were incubated in the different media (0, 1 and 2 h) and sperm motion parameters, lipid membrane disorder, plasma membrane integrity and reactive oxygen species (ROS) formation were assessed. Fertilization results were assessed i) preincubating spermatozoa in the viscous media followed by gamete coculture in a non-viscous medium; and ii) gamete coculture in the viscous media. Viscosity of POF from early luteal phase was higher than late follicular phase. Medium without methylcellulose presented constant viscosity with increased shear rate, while viscosity of the POF and media with methylcellulose was reduced by increased shear rates. Methylcellulose improved sperm linearity, straightness and the proportion of fast-linear spermatozoa. Moreover, methylcellulose increased the rate of viable spermatozoa with intact acrosome and low lipid disorder, reducing the ROS generation. Preincubation in viscous media increased the penetration rate and the mean number of spermatozoa bound to the zona pellucida (both with 0.5 and 1% MET) and reduced monospermy with 1% MET. On the other hand fertilization in the viscous media reduced penetration rate and increased monospermy. The efficiency of the IVF system was not improved with the use of viscous media. The results show the relevance of increasing viscosity thus making the *in vitro* media more comparable to physiological conditions.

1. Introduction

In the last few years, many studies have been developed to investigate the way in which spermatozoa become orientated within the female genital tract and find their way towards the oocytes. At least three different potential sperm guidance mechanisms have been recognized: thermotaxis, rheotaxis and chemotaxis [1]. Specifically, the role of rheotaxis has become relevant due to the results shown in recent studies [2–4]. These studies reveal how rheotaxis leads to sperm orientation within a gradient of fluid flow, so that in the female reproductive tract, spermatozoa swim against the female genital fluid flow [4,5].

The rheotactic behaviour of sperm cells is influenced by several physical characteristics of the genital fluid. The viscosity of the female genital fluid changes during the oestrous cycle and is modulated by factors such as hormone concentration, most notably progesterone, dead cells and others factors like pH [5–9]. Mucins and glycosaminoglycans (GAGs) are important components of the oviductal fluid. Mucins, which are the main glycoproteins composing genital fluid, are long and flexible linear molecules and have an essential role in conferring viscosity, thus participating in establishing sperm orientation [6,10]. Besides, viscosity of the

* Corresponding author. Department of Physiology, University of Murcia, Murcia, 30100, Spain.
E-mail address: jgadea@um.es (J. Gadea).

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genital fluid is also due to the large size of GAGs, while elasticity results from the entanglement of the molecules [6].

Some authors have suggested that the fluid viscosity in the female reproductive tract could also help to select the best quality spermatozoa that reach oviduct in the proximity of the egg [2,11,12]. Spermatozoa swim into the flow, distribute in heterogeneous subpopulations, and remain within some regions of low shear to avoid being washed away by the flow resistance [2].

In the pig, there is no information related to the use of high viscosity media. Moreover, the viscosity of key biofluids, such as uterine or oviductal fluids that come into contact with the gametes, and are the natural media where relevant reproductive events take place, is unknown. However, to mimic *in vivo* conditions with the purpose of improving reproduction research and techniques, some *in vitro* studies have incorporated a viscous factor into incubation media, which are usually of low viscosity near to that of water [13]. In this sense acrylamide, powdered plant extracts, hyaluronic acid, carboxymethyl cellulose or methylcelluloses have been used to increase viscosity of the media for *in vitro* studies in different species [13–17]. Our group, using a powdered plant extract for increasing the viscosity of the media, concluded that increased viscosity is desirable to enhance the ability of spermatozoa to move, bind and penetrate the oocyte *in vitro* [13]. Among the mentioned macromolecules that can be used to increase viscosity, the properties of methylcellulose are unique, making this substance the appropriate additive to increase viscosity. Methylcellulose is long-chain substituted cellulose with ≈30% of its hydroxyl groups in the form of methyl ether. This substitution of cellulose by hydroxyl groups confers a higher resistance of the media to microbial attack. Besides, methylcellulose is essentially odorless and tasteless, has a uniform quality and consistency, is stable over long periods of time, easy to obtain and cheap. This is because this non-toxic substance is widely used in the pharmaceutical and food industries, and it has been already tested with sperm diluents in human *in vitro* studies [16,18]. It should be mentioned that studies carried out with human spermatozoa and oocytes have confirmed the security of using methylcellulose in ICSI procedures [19], showing there was no increase in sperm DNA damage resulting in sister chromatid exchanges.

The aims of this work were to measure the viscosities of experimental media and oviductal fluid around the time of fertilization, as the physiological medium where fertilization and early embryo development take place, and develop an artificial medium by adding methylcellulose which most resembles the natural fluid. With this approach we evaluate critically the use of methylcellulose as a potential viscosity-enhancing substance for use in media for porcine sperm fertilization. Due to the viscous nature of female genital fluids, we hypothesized that increasing the viscosity of the media by using methylcellulose before and during *in vitro* fertilization might enhance the fertilization quality of spermatozoa and their fertilizing ability. Experiments to test our hypothesis have been performed by adding the thickener to sperm suspension media and to *in vitro* fertilization media with *in vitro*-matured oocytes. Sperm motility, viability, membrane lipid disorder, acrosome status, reactive oxygen species (ROS) generation and fertilization results were assessed.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich Química, S.A. (Madrid, Spain) unless otherwise stated.

2.1. Sample collection and media for sperm processing

Semen samples were obtained from 6 fertility-tested boars (*Sus scrofa domestica*) from an artificial insemination centre (CEFUSA, Pliego, Murcia, Spain). Two ejaculates per boar, total 12 ejaculates, were used in this study.

Samples were collected by the gloved hand method and immediately transported to the laboratory [20]. The samples were diluted in Tyrode's sperm medium [21] supplemented with methylcellulose (MET, Sigma M0512, approx. 88 kDa, viscosity 4000 cP if dissolved 2% (wt/v) in H_2O) at 0 (control), 0.5% or 1% (w/v) to develop the experimental work.

The Tyrode's medium consisted of 116 mM NaCl, 3.1 mM KCl, 0.4 mM MgSO4, 0.3 mM NaHaP04·H2O, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 20 mM HEPES, 582.6 mM kanamycin and 334.38 mM phenol red. Before use Tyrode's medium was supplemented with 3 mg/mL bovine serum albumin (BSA) and 1 mM EGTA. pH of the medium was 7.4, and osmolality approximately 300 mOsmol/kg (freezing-point Osmometer Roebling 13DR, Messtechnik, Berlin. Germany).

Oviductal fluid samples were purchased from EmbryoCloud (Murcia, Spain. www.embryocloud.com). Specifically, porcine oviductal fluid from late follicular (NaturARTs POF-LF) and early luteal phase (NaturARTs POF-EL) were used in the experiments. Briefly, porcine oviductal fluid (POF) was obtained from late follicular phase (preovulatory) and early luteal phase (post-ovulatory) gilt oviducts recovered in the slaughterhouse, according to the method previously described [22]. Once aspirated, the POF was centrifuged at 7000 g for 10 min at 4 °C to remove cellular debris. Then the supernatant was immediately stored at −80 C until use for viscosity measurements.

2.2. Assessment of viscosity

The viscosity of porcine oviductal fluids and the sperm media supplemented with and without methylcellulose was measured using a MCR102 Rheometer (Anton Paar GmbH, Graz, Austria) with a cone-plate geometry (CP-25-1, diameter: 25 mm, angle: 1°). The viscosity (mPas) as a function of shear rate was measured from 0.1 to 500 s^{-1} at 38 °C.

2.3. Analysis of sperm functionality parameters

To examine the effect of sperm incubation in viscous media on sperm quality, boar spermatozoa were incubated in Tyrode's sperm medium i) without addition of methylcellulose that was the control group, and ii) with addition of 0.5% or 1% methylcellulose (w/v) to the Tyrode's sperm medium.

Final sperm concentration was 1×10^7/mL and samples were maintained for 30 min at 38 °C in a dry bath for sample equilibration before the evaluation starts (time 0). Sperm samples were incubated under the same conditions for 2 additional hours.

At the beginning of incubation (time 0) and after 1 and 2 h of incubation at 38 °C seminal samples were evaluated for i) motion parameters by CASA; ii) acrosome status by FITC-PNA and simultaneously plasma membrane integrity by propidium iodide; iii) lipid membrane disorder status by merocyanine 540 and plasma membrane integrity by Yo-Pro 1; and (iv) reactive oxygen species (ROS) formation by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) staining.

2.3.1. Assessment of sperm motion parameters by CASA

Analysis of post-incubation sperm motion parameters were determined using a Computer assisted sperm analysis (CASA) system (ISAS, Proiser, Valencia, Spain) according to protocol previously described [23]. The CASA-derived motility characteristics studied were total motility (%), progressive motility (%), curvilinear velocity (VCL, μm s^{-1}), straight-line velocity (VSL, μm s^{-1}), average path