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Effect of different media additives on capacitation of frozen–thawed ram spermatozoa as a potential replacement for estrous sheep serum

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ABSTRACT

Capacitation is a key process through which spermatozoa acquire their fertilizing ability. This event is required for the successful application of assisted reproductive technologies such as IVF. The aim of the present study was to investigate the effect of using a synthetic oviductal fluid medium supplemented with either heparin–hypotaurine alone, in combination with progesterone (P4), 17β-estradiol (E_2), or BSA, or just β-cyclodextrin, in replacement for estrous sheep serum (ESS) for ram sperm capacitation. After incubation in the corresponding media for 15 (time 0) or 60 minutes, sperm function was evaluated by computerized sperm motility analysis and flow cytometry (plasma membrane status and fluidity). Treatments rendering the best results in regards to sperm function parameters related to capacitation were used for an IVF test. Herein, neither heparin–hypotaurine (alone), or in combination with P4, or E_2, nor β-cyclodextrin induced capacitation-related changes in frozen–thawed ram spermatozoa. Only the medium supplemented with heparin–hypotaurine–BSA was able to induce changes compatible with *in vitro* capacitation relating to sperm motility pattern and plasma membrane fluidity, comparable to those in ESS-containing medium. Both media yielded sperm parameter values that differed ($P < 0.05$) from those obtained in the rest of the media tested. However, after the IVF trial, BSA was unable to support cleavage rates (21.80%) comparable to those obtained with ESS (52.60%; $P < 0.05$). We conclude that heparin–hypotaurine, P4, E_2, β-cyclodextrin, or BSA is not suitable for replacing ESS in capacitation and fertilization media for ram spermatozoa.

1. Introduction

Successful fertilization depends on several interrelated physiological processes all of which must take place in a coordinated manner [1]. One such process is sperm capacitation [2], which involves modifications in membrane composition and fluidity, increases in intracellular cAMP, induction of tyrosine phosphorylation events, and the expression of hyperactivated motility [3–5]. Sperm undergo these changes within the female reproductive tract or, *in vitro*, when incubated in a medium that supports capacitation.

In vitro fertilization is a well-established technology with a variety of applications in basic and applied sciences [6]. To date, several media additives, both of synthetic and animal origin, have been successfully used to support *in vitro* capacitation of mammalian spermatozoa. For instance,

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several hormones present in uterine and oviductal fluid at the time of *in vivo* fertilization and those controlling the estrous cycle, such as progesterone (P4) or estrogens, may be good candidates for triggering *in vitro* sperm capacitation [7,8]. In this regard, addition of P4 to the capacitation medium exerted a positive effect on sperm membrane cholesterol efflux, hyperactivation, and the acrosome reaction [9–12]. However, the effect of estrogens on sperm capacitation has been controversial [7,10,13].

In the bovine species, heparin may promote both *in vitro* sperm capacitation and fertilization by binding to seminal plasma proteins that are incorporated to the sperm plasma membrane [14]. This induces changes in the properties of the plasma membrane that may stimulate increases in intracellular calcium, pH, and cAMP during capacitation [15–20]. Moreover, when added to bovine capacitation medium, heparin supported downstream time-dependent increases in protein tyrosine phosphorylation [15]. Similarly, hypotaurine, a precursor of taurine, added in combination with heparin further supported sperm motility and viability [10,21–23] and acted as an oxygen radical scavenger [24].

Another key component of media for mammalian sperm capacitation is BSA [1], which was shown to modulate capacitation through its ability to remove cholesterol from the sperm plasma membrane [25,26]. In fact, cholesterol efflux represents a key step of the intrinsic regulatory events leading to sperm capacitation [27]. Therefore, β-cyclodextrin, a compound that promotes cholesterol efflux from the sperm membrane [28], also supported capacitation and fertilization of human and murine spermatozoa [28–30].

Despite advances in knowledge regarding additives that may support mammalian sperm capacitation *in vitro*, in small ruminants, incubation of sperm in the presence of estrous sheep serum (ESS) is still required to achieve optimal rates of IVF [21,31–33]. However, a main drawback of using ESS is that it is not easily attainable given that it requires extraction and purification from blood samples and, therefore, its composition can be variable from batch to batch. Moreover, the presence of undefined compounds in IVF media is not desirable as it complicates any standardization of this technique. Therefore, there is a need to establish defined media conditions supporting *in vitro* ram sperm capacitation.

The aim of the present study was to investigate the effect of adding heparin–hypotaurine alone or in combination with P4, 17β-estradiol (E_2), or BSA, or just β-cyclodextrin to replace ESS in synthetic oviductal fluid (SOF) medium for capacitation of ram spermatozoa.

2. Materials and methods

2.1. Animals and reagents

Animal handling was performed in accordance to Spanish Animal Protection Regulation, RD 53/2013, which conforms to European Union Regulation 2010/63. Three rams of Manchega breed (age, >3 years) were used. Males were maintained and managed at the Regional Center of Animal Selection and Reproduction in Valdepeñas (Spain). The rams were trained to semen collection by an artificial vagina, which was performed at regular intervals of twice per week.

Flow cytometry equipment, software, and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). All other chemicals (reagent grade or higher) and the fluorescence probes, propidium iodide (PI) and Merocyanine 540 (Merocyanine), were acquired from Sigma (Madrid, Spain), unless otherwise stated. YO-PRO-1 was purchased from Invitrogen (Barcelona, Spain).

2.2. Semen collection and cryopreservation

Semen collection was performed using an artificial vagina, and the initial semen evaluation was performed shortly thereafter. Semen volume was assessed using a graduated conical tube, and concentration was determined using a spectrophotometer. Wave motion was subjectively scored from 0 to 5, where 0 was no movement and 5 was a strong swirling motion, on a wet mount of raw semen with no coverslip using bright-field microscopy at × 10 (BH-2 Olympus). Individual sperm motility and the quality of movement were also assessed in 5 μL of semen diluted in 200 μL of PBS. After incubation at 37 °C for 5 minutes, 5 μL of diluted sperm was placed between a prewarmed slide and a 22 × 22-mm coverslip and observed at × 400 under phase-contrast optics (Eclipse 50i, Nikon; Tokyo, Japan). The percentage of motile sperm was estimated subjectively with values ranging from 0%, when no motile spermatozoa (SM) were observed, to 100%, when all spermatozoa were moving.

Only those sperm samples with a minimum quality (percentage of motile sperm above 80% and quality of movement above 3.5) were cryopreserved. Cryopreservation was performed as described by García-Álvarez et al. [34] using the freezing extender, Biladyl (20% egg yolk and 6% glycerol; Minitüb, Tiefenbach, Germany).

2.3. Experimental design

Thawing was performed by placing straws in a water bath at 37 °C for 30 seconds. To eliminate individual differences, straws, one from each of three rams, were pooled and processed together as suggested by Ollero et al. [35]. Then, a sperm selection by discontinuous density gradient on Percoll (1 mL of 45% Percoll over 1 mL of 90% Percoll) was carried out at room temperature (20 °C–22 °C). Thawed semen was layered on top of the two Percoll layers and centrifuged at 700 × *g* for 10 minutes [36–38]. After centrifugation, the supernatant was carefully discarded. Sperm concentration was determined, and the sperm pellet was diluted with each capacitating media to a final concentration of 10×10^6 sperm/mL.

The following capacitating media treatments were tested: SOF [39] + polyvinyl alcohol (0.1%; PVA; SOF); SOF + heparin and hypotaurine (1 μL/mL of each) + PVA (0.1%; SOF_{hh}); SOF + heparin and hypotaurine (1 μL/mL of each) + ESS (2%; SOF_{ESS}); SOF + heparin and hypotaurine (1 μL/mL of each) + BSA (reference A9647; 7 mg/mL; SOF_{BSA}); SOF + heparin and hypotaurine (1 μL/mL of each) + E_2 (1 μg/mL; SOF_{E2}); SOF + heparin and hypotaurine (1 μL/mL of each) + P4 (10 μg/mL; SOF_{P4}); SOF + β-cyclodextrin (1 mM) + PVA (0.1%; $SOF_{\beta cyclo}$). In some of the