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 *in vitro* fertilization (IVF). The aim of the present study was to investigate the effect of using a synthetic oviductal fluid (SOF) medium supplemented with either heparin-hypotaurine alone or in combination with progesterone (P4), 17β-estradiol (E_2) or bovine serum albumin (BSA), or just β-cyclodextrin, in replacement for estrous sheep serum (ESS) for ram sperm capacitation. Following incubation in the corresponding media for 15 (time 0) or 60 min, 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, following 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 are not suitable for replacing ESS in capacitation/fertilization media for ram spermatozoa.

Keywords: ram spermatozoa; capacitation; fertilization; estrus sheep serum; bovine serum albumin.

1. Introduction

Successful fertilization depends upon 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 (IVF) 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, several hormones present in uterine and oviductal fluid at the time of *in vivo* fertilization as well as those controlling the estrous cycle, such as progesterone or estrogens, may be good candidates for triggering *in vitro* sperm capacitation [7,8]. In this regard, addition of progesterone 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 bovine serum albumin (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 *in vitro* fertilization [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 progesterone, 17β-estradiol or BSA, or just β-cyclodextrin to replace estrus sheep serum (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). Rams were trained to semen collection by 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 phosphate-buffered saline (PBS). After incubation at 37ºC for 5 minutes, 5 µL of diluted sperm were placed between a pre-warmed 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 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-Alvarez 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 ºC for 30 s. 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 (20-22 ºC). Thawed semen was layered on top of the two Percoll® layers and centrifuged at 700 *x g* for 10 min [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 x 10^6 sperm/mL.

The following capacitating media treatments were tested: synthetic oviductal fluid [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) + 17β-estradiol (1µg/mL) (SOF_{E2}); SOF + heparin and hypotaurine (1 µL/mL of each) + progesterone (10 µg/mL) (SOF_{P4}); SOF + β-cyclodextrin (1 mM) + PVA (0.1%) ($SOF_{\beta cyclo}$). In some of the above media devoid of a protein source, PVA was added to

prevent sperm agglutination and sticking of gametes to various surfaces [27]). Stock solutions of heparin and hypotaurine were 10 mg/mL and 1 mg/mL, respectively.

Diluted sperm samples were incubated for 15 minutes (corresponding to time 0) or 60 minutes at 38.5ºC in a 5% CO_2 atmosphere. At each time point several sperm parameters were assessed, including motility parameters by computerized sperm motility analysis (CASA) and sperm function parameters assessed by flow cytometry (see below). The experiment was replicated three times.

Media in which sperm displayed capacitation-related changes such as high membrane fluidity, as well as high amplitude of lateral head displacement (ALH: µm) and low linearity index (LIN: %) were used to conduct an *in vitro* fertilization (IVF) test. Thus, SOF_{BSA} and SOF_{ESS} and two control media (SOF and SOF_{hh}) were chosen. The experiment was replicated five times per capacitating treatment.

2.4. Flow cytometry and sperm motility analyses

Flow cytometry analysis was carried out on sperm samples diluted and incubated in each of the capacitating media tested. Analyses of sperm viability, as well as membrane stability and fluidity were performed using YO-PRO-1, propidium iodide (PI) [40] and the hydrophobic dye Merocyanine 540 [41], respectively. For staining purposes, sperm samples were suspended to 1 x 10^6 sperm/mL in Bovine Gamete Medium (BGM-3) in flow cytometry polypropylene tubes. Then, 15 µM PI (stock: 7.5 mM in milli-Q water), 50 nM YO-PRO-1 (stock: 1 mM in DMSO) and 1 µM Merocyanine 540 (stock: 3.4 mM in DMSO) were added to each tube and samples were incubated for 30 min in the dark. Sperm samples were analyzed through a BD FACSFlow cytometer (Becton Dickinson, San Jose, CA, USA), using the following guidelines: the YO-PRO-1-/PI- sperm

subpopulation was considered viable with an intact membrane; and, the Merocyanine +/YO-PRO-1- subpopulation was considered viable with high membrane fluidity.

Sperm motility was assessed by CASA as described by Tamayo-Canul et al. [42] on sperm samples diluted and incubated with each capacitating media. A pre-warmed (37ºC) Makler counting chamber (10-µm depth; Sefi-Medical Instruments, Haifa, Israel) was loaded with 5 µL of sample. The CASA system consisted of a triocular optical phase contrast microscope (Nikon Eclipse 80i; Nikon; Tokyo, Japan) and a Basler A302fs digital camera (Basler Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analyzed using the Sperm Class Analyzer® (SCA2002) software (Microptic S.L.; Barcelona, Spain). The sample was examined at ×10 (negative phase contrast) in a microscope with a warmed stage (37ºC). Software settings were adjusted to ram spermatozoa and at least five fields or 200 spermatozoa were saved and analyzed afterwards. For each sperm sample analyzed the following parameters were measured: motile spermatozoa (SM; %), curvilinear velocity (VCL; µm/s), linearity index (LIN; %), and amplitude of lateral head displacement (ALH; µm). The VCL, LIN and ALH measures were used to define hyperactivated motility [31,43].

2.5. In vitro fertilization test (IVF)

An IVF test was performed using those capacitating treatments (SOF_{BSA}, SOF_{ESS}) that yielded a significantly higher proportion of spermatozoa with high membrane fluidity, as well as higher ALH and lower LIN values (capacitation-related changes). Two other media were used as negative controls (SOF, SOF_{hh}). Sperm samples were incubated in each capacitating media for 15 minutes. The IVF test was replicated five times with a total of 380 oocytes used.

The IVF protocol was similar to that described by García-Álvarez et al. [44]. Briefly, sheep ovaries were collected at a slaughterhouse and transported to our laboratory in saline solution (25-30ºC) between 1-2 h after removal. Ovaries were sliced using a micro-blade and the follicle content was released in TCM 199 medium supplemented with HEPES (2.38 mg/mL), heparin (2 µL/mL) and gentamycin (40 µg/mL). Cumulus oocyte complexes (COC) were washed in TCM 199-gentamycin (40 µg/mL), and those with a dark homogeneous cytoplasm and surrounded by tightly packed cumulus cells were selected. COC were randomly placed in four-well plates containing 500 µL of TCM 199 supplemented with cysteamine (100 µM), FSH/LH (10 µg/mL), FCS (10%) and gentamycin (40 µg/mL), and matured at 38.5ºC in a 5% CO_2 atmosphere. After 24 h, cumulus cells were removed by gentle pipetting and denuded oocytes were randomly allocated among the 4 treatments tested (SOF, SOF_{hh}, SOF_{BSA}, SOF_{ESS}). For this purpose, oocytes were transferred into four-well plates containing 450 µL of each capacitating medium, under mineral oil. Sperm that had been preincubated in each of the capacitating media (SOF, SOF_{hh}, SOF_{BSA}, SOF_{ESS}) were then co-incubated with oocytes in the corresponding medium at a final concentration of 1 x 10^6 sperm/mL for 18 h at 38.5ºC in a 5% CO_2, 5% O_2 and 90% N_2 in a humidified air atmosphere. Presumptive zygotes were cultured in SOF enriched with amino acids and BSA [45] at 38.5ºC in the same atmospheric conditions used for IVF.

Fertilization was assessed by the cleavage rate at 48 hours post-insemination (h.p.i.) by phase-contrast microscopy (SMZ 1500 Nikon; Tokyo, Japan).

2.6. Statistical Analysis

Statistical analyses were performed using the R statistical package [46]. Prior to statistical analysis, data were evaluated for normal distribution by graphical methods

and the Kolmogorov-Smirnnov normality test. Differences were considered significant when $P < 0.05$, unless otherwise indicated.

A mixed effects model including the capacitating medium (SOF, SOF_{ESS}, SOF_{BSA}, SOF_{hh}, SOF_{E2}, SOF_{P4}, $SOF_{\beta cyclo}$) and incubation time (0 and 60 minutes) as fixed factors, and the replicate as random effect, was carried out to study the effect of sperm incubation in the different capacitating media on sperm parameters. When significant, comparison of means was carried out using the Bonferroni test.

Those capacitating media that had a significant effect on the proportion of spermatozoa with high membrane fluidity, low LIN and high ALH (capacitation-related changes) values were used to conduct an IVF test. Thus, SOF_{ESS} and SOF_{BSA} and two media as controls (SOF and SOF_{hh}) were chosen. A mixed effects model including the capacitation media (SOF_{ESS}, SOF_{BSA}, SOF and SOF_{hh}) as fixed factors and the replicate as random effect was carried out to study the effect of sperm incubation in the different capacitating media on cleavage rates at 48 h.p.i.

3. Results

3.1. Flow cytometry analysis

There was an expected significant decrease of sperm viability (YOPRO-/PI-) after 60 minutes of incubation. Only those spermatozoa incubated with SOF and SOF_{ESS} maintained viability throughout incubation (Figure 1A).

The membrane phospholipid disorder as assessed by Merocyanine 540 was the sperm parameter most affected by the capacitating treatments (Figures 1B). Sperm samples incubated with SOF_{ESS} and SOF_{BSA} yielded a higher ($P < 0.05$) percent of the Merocyanine+/YOPRO-1- spermatozoa subpopulation at the beginning of incubation when compared to other media tested (Figures 1B). However, there was a significant

decrease in the percent of this sperm subpopulation after 60 minutes of incubation in both of these media (Figure 1B). Hence, differences among capacitating media in regards to the Merocyanine+/YOPRO-1- sperm population disappeared following 60 minutes of incubation.

3.2. Sperm motility analysis

For most capacitating media evaluated the percentage of motile spermatozoa (SM) remained similar throughout incubation (70.95%±13.87 *vs.* 57.13%±6.69, at 0 and 60 minutes, respectively). Only sperm samples incubated with SOF_{BSA} and $SOF_{\beta cyclo}$ showed a decrease ($P < 0.05$) in SM over time (73.19%±13.55 *vs*. 56.89%±8.01 for SOF_{BSA}; 88.76%±5.22 *vs*. 52.73%±13.28 for $SOF_{\beta cyclo}$, respectively). However, the pattern of sperm movement was significantly different depending on the medium (Figure 2). Thus, at the beginning of incubation VCL was lower ($P < 0.05$) for sperm in SOF_{ESS} than those in SOF, SOF_{hh}, SOF_{E2} or $SOF_{\beta cyclo}$. Conversely, there were no differences among VCL values for SOF_{ESS} and those obtained with SOF_{BSA} or SOF_{P4} (Figure 2A).

The effect of the different capacitating media on the sperm motility pattern was most evident after incubating the samples for 60 minutes (Figure 2B, 2C). Spermatozoa incubated with SOF_{ESS} and SOF_{BSA} displayed lower LIN and higher ALH ($P < 0.05$) than those in other media. This defines a motility pattern characteristic for sperm hyperactivation [43,31], which is consistent with the acquisition of fertilizing ability. Conversely, spermatozoa incubated 60 min in SOF_{P4} and $SOF_{\beta cyclo}$ showed the opposite response (higher LIN and lower ALH values), while there was no change for these motion parameters in sperm incubated in SOF, SOF_{hh} or SOF_{E2} (Figures 2B, 2C).

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276 *3.3. In vitro fertilization test (IVF)*

277 To carry out IVF, we selected SOF_{ESS} and SOF_{BSA}, media in which spermatozoa
278 showed changes related to sperm capacitation (high membrane fluidity, lower LIN and
279 higher ALH) throughout incubation. SOF and SOF_{hh} were used as negative control
280 media. Those spermatozoa treated with SOF_{ESS} rendered significant higher cleavage
281 rates at 48 h.p.i. compared to SOF_{BSA}, SOF and SOF_{hh} media, producing more than
282 twice the amount of embryos than SOF_{BSA} (Figure 3).

283

284 **4. Discussion**

285 This study showed that frozen-thawed ram spermatozoa responded differently to
286 various *in vitro* capacitating treatments, and this was subsequently reflected on the *in*
287 *vitro* fertilization rates. Notably, only those spermatozoa incubated in the presence of
288 bovine serum albumin (BSA) or estrous sheep serum (ESS) displayed changes
289 compatible with *in vitro* capacitation, such as modifications in the motility pattern and
290 membrane fluidity, as well as in the ability to achieve subsequent fertilization.
291 However, the cleavage rates achieved with spermatozoa treated with BSA were lower
292 than those with ESS.

293 Capacitation is a key step through which sperm become fertilization-competent
294 and hence is essential for the success and development of assisted reproductive
295 techniques. We sought to investigate the effect of various components known to
296 facilitate sperm capacitation [9,10,12,16,27] for their effect on ram sperm, with the goal
297 of eliminating ESS from the capacitating medium. Specifically, we used a synthetic
298 oviductal fluid (SOF) medium to which we added heparin-hypotaurine alone or in
299 combination with progesterone (P4), 17β-estradiol (E_2), or bovine serum albumin

(BSA), or just β-cyclodextrin. To prevent sperm agglutination, SOF medium was supplemented with polyvinyl alcohol (PVA) in some of the media tested if these were devoid of a protein source, as previously reported [27]. While these authors found no effect of PVA on sperm protein tyrosine phosphorylation or *in vitro* fertilization rates in the mouse [27], others have reported that PVA may support bovine [47] or stallion [48] sperm capacitation. Our results confirmed the more accepted notion that PVA is unable to trigger capacitation, at least in ram spermatozoa, given that incubation of spermatozoa in the presence of this additive had no effect on capacitation or fertilization rates.

Previous studies have shown an improvement in IVF rates when frozen-thawed sperm from small ruminants were incubated in ESS-containing capacitating medium supplemented with heparin and/or hypotaurine [21,23,49]. Heparin is a potent glycosaminoglycan required for bovine sperm capacitation [14,17-19] that has been shown to improve the fertilizing ability of goat and ram spermatozoa when used in addition to ESS [50]. However, in our study heparin in combination with hypotaurine but in the absence of other additives did not have any effect on ram sperm capacitation. That is, spermatozoa incubated with heparin-hypotaurine (SOF_{hh}) alone did not display changes in membrane fluidity and or motility, nor they were able to carry out fertilization. In the bovine, glycosaminoglycans such as heparin are postulated to capacitate spermatozoa both in vivo and in vitro through their ability to sequester coating proteins termed bovine seminal plasma proteins (BSP); this, in turn, promotes cholesterol and phospholipid efflux from the sperm plasma membrane [19,51], leading to downstream molecular events that culminate with protein tyrosine phosphorylation [14]. The BSP protein family is expressed in all mammalian species studied and it is probably meant to play the same biological role in all of them [35,52,53]. However, the

homologous to BSPs present in ram are not closely related to those from the bovine [54]. This may be consistent with the notion that heparin-driven removal of proteins from the plasma membrane may not play a similar key role during capacitation in ram as it does for bull spermatozoa.

In our study there was no effect of P_4 or E_2 on *in vitro* sperm capacitation. Progesterone and estrogen-supplemented medium yielded low values of Merocyanine+/YO-PRO-1- spermatozoa, that displayed a sperm movement pattern different from hyperactivation, consisting of linear motility throughout incubation. Whereas, many authors reported that P_4 may act as a capacitating agent [9,12], others suggested that P_4 by itself had no effect on sperm capacitation in bovine [8] and human [11] unless heparin or BSA, respectively, were present in the medium. Therefore, while P_4 did play a role in inducing the acrosome reaction in these species, the capacitating effects of the media could be attributed to heparin and BSA both known to be capacitating agents for bovine and human sperm, respectively. In addition, we speculated that the lack of effect observed in our study for both media supplemented with P_4 and E_2, could relate to the composition of the sperm plasma membrane, which is species-specific [55,56]. These hormones act through receptors located on the surface of spermatozoa [57-59], which may be potentially lost or damaged during cryopreservation [60] to a different degree depending on the species [55,61].

Herein we also tested the effect of β-cyclodextrin, a non-physiological cholesterol acceptor, that was highly efficient in stimulating cholesterol efflux from sperm cell membranes [27,62-64], thereby inducing a downstream increase in protein tyrosine phosphorylation events [12,27,65]. Given that a previous report had shown no synergistic effect among heparin, hypotaurine and β-cyclodextrin [63] on buck sperm capacitation, we decided to evaluate the individual effect of β-cyclodextrin. In this

study, β-cyclodextrin addition to SOF medium yielded the poorest results in supporting ram sperm capacitation. These results are in agreement with those reported by Colas et al. [66] who showed the failure of β-cyclodextrin to support ram sperm capacitation as assessed by increases in protein tyrosine phosphorylation.

Bovine serum albumin (BSA) is usually combined with calcium and bicarbonate in capacitation medium for mammalian spermatozoa [5]. BSA is supposed to act by removing cholesterol from the sperm membrane [30], and cholesterol depletion has been related to changes in membrane architecture and fluidity [67] that give rise to the capacitated state [30]. In our study, when SOF was supplemented with BSA-heparin-hypotaurine (SOF_{BSA}) the proportion of spermatozoa displaying high membrane fluidity was significantly higher at the beginning of incubation, when compared to all other media tested with the exception of SOF_{ESS} which yielded similar results. It was previously shown that ESS supported cholesterol efflux from the plasma membrane of ram spermatozoa [32]. Moreover, based on the LIN and ALH results at 60 minutes of incubation, spermatozoa treated with both BSA or ESS developed hyperactivated motility, one of the hallmarks of sperm capacitation [3]. In addition, spermatozoa capacitated with SOF_{BSA} were able to fertilize oocytes. However, the differences observed between BSA and ESS treatment in regards to cleavage rates suggest that their effects on spermatozoa are not identical, even after inducing similar changes in regards to sperm membrane fluidity and motility pattern. It is possible that the type or concentration of BSA used in our work might not have been optimal to capacitate ram spermatozoa. Furthermore, maybe only a small subpopulation of sperm were fully capacitated with the concentration used. Therefore, future studies should test the effects of a BSA concentration gradient for ram sperm capacitation, as it has been done in other species [64,68].

375

376 *4.1. Conclusions*

377 In summary, none of the substances tested in this study were able to overcome the
378 presence of ESS in the capacitation media for ram spermatozoa. While spermatozoa
379 incubated in the presence of BSA underwent changes compatible with capacitation, it
380 did not fully support the fertilizing ability of ram spermatozoa given that cleavage rates
381 following IVF were lower than those obtained in the presence of ESS. Future studies
382 must be directed at evaluating a range of BSA concentrations to assess their effect upon
383 ram sperm capacitation. Importantly, studies should be aimed at developing a
384 chemically defined medium to replace the ESS in capacitating media for ram
385 spermatozoa.

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599 Fig. 1. Flow cytometry parameters for sperm samples incubated with different
600 capacitating media. YOPRO-1-/PI-: intact spermatozoa (A); Merocyanine+/YOPRO-1-:
601 viable spermatozoa with high membrane phospholipid disorder (B). Bars represent
602 mean ± S.E.M. of different capacitating media. In each set of bars, lowercase letters
603 denote statistical differences ($P < 0.05$) among capacitating media. Capital letters
604 denote significant differences ($P < 0.05$) between times within each capacitating
605 medium.
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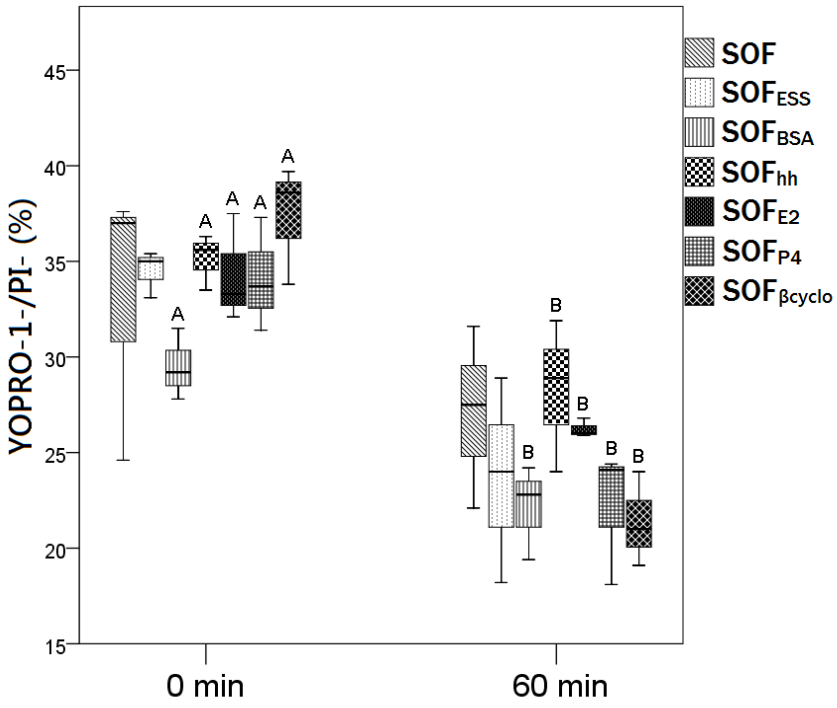
Fig. 2. Sperm motility parameters for sperm samples incubated in different capacitating media. VCL (µm/s): curvilinear velocity (A); LIN (%): linearity (B); ALH (µm): amplitude of lateral head displacement (C). Bars represent mean ± S.E.M. of different capacitating media. In each set of bars, lowercase letters denote significant differences ($P < 0.05$) among capacitating media. Capital letters denote significant differences ($P < 0.05$) between times within each capacitating medium.

Fig. 3. Cleavage rates at 48 hours post insemination (h.p.i.) for oocytes inseminated with frozen-thawed ram sperm samples incubated in different capacitating media (SOF_{ESS}, SOF_{BSA}, SOF and SOF_{hh}). Data are mean ± S.E.M. Different superscripts denote significant differences among treatments ($P < 0.05$).

672 Fig. 1. García-Álvarez et al.

673 **A)**



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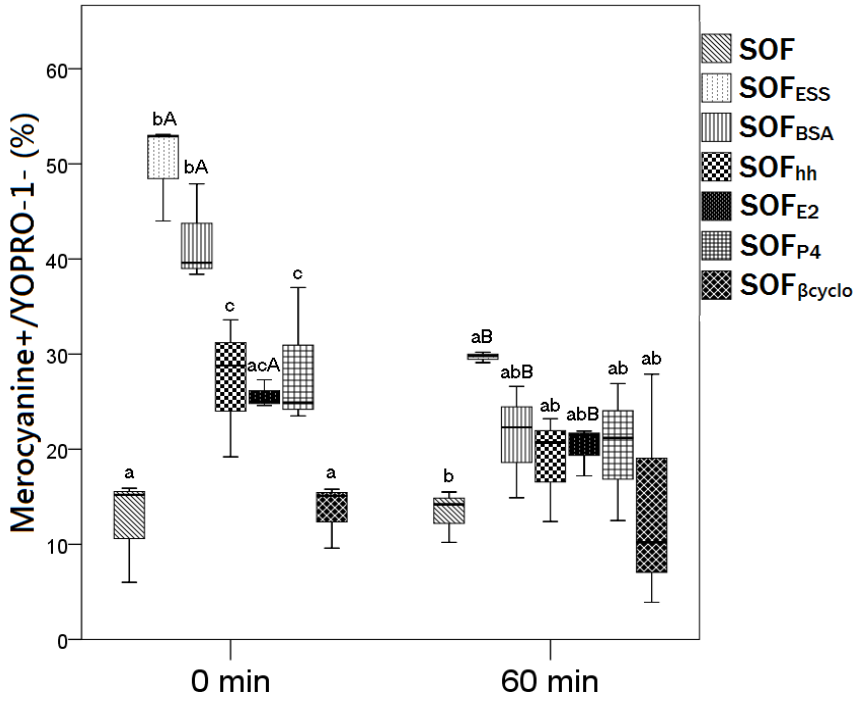
675 **B)**



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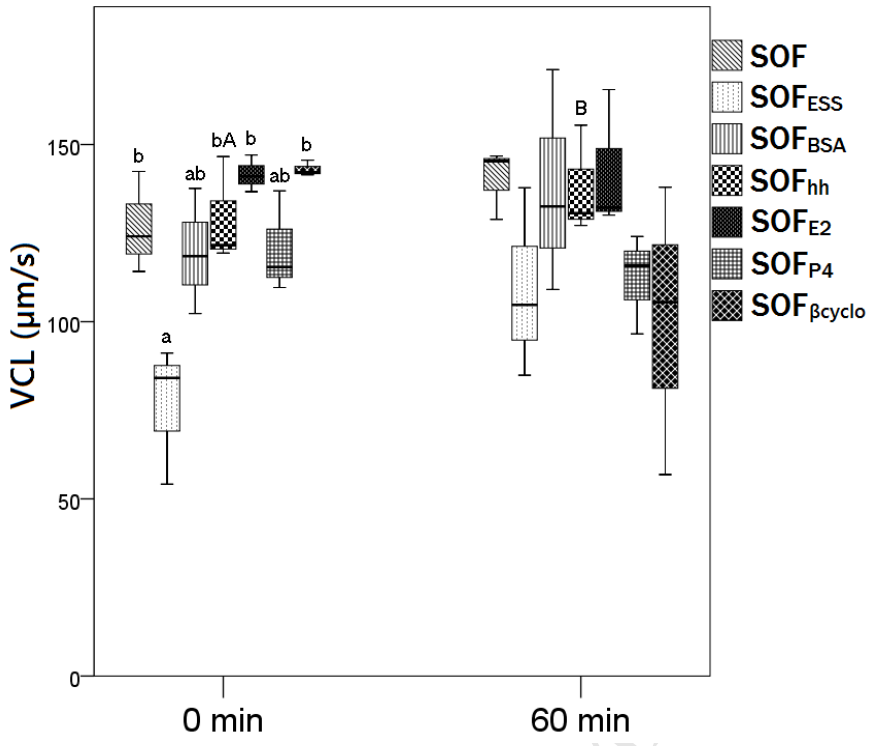
678 Fig. 2. García-Álvarez et al.

679 **A)**



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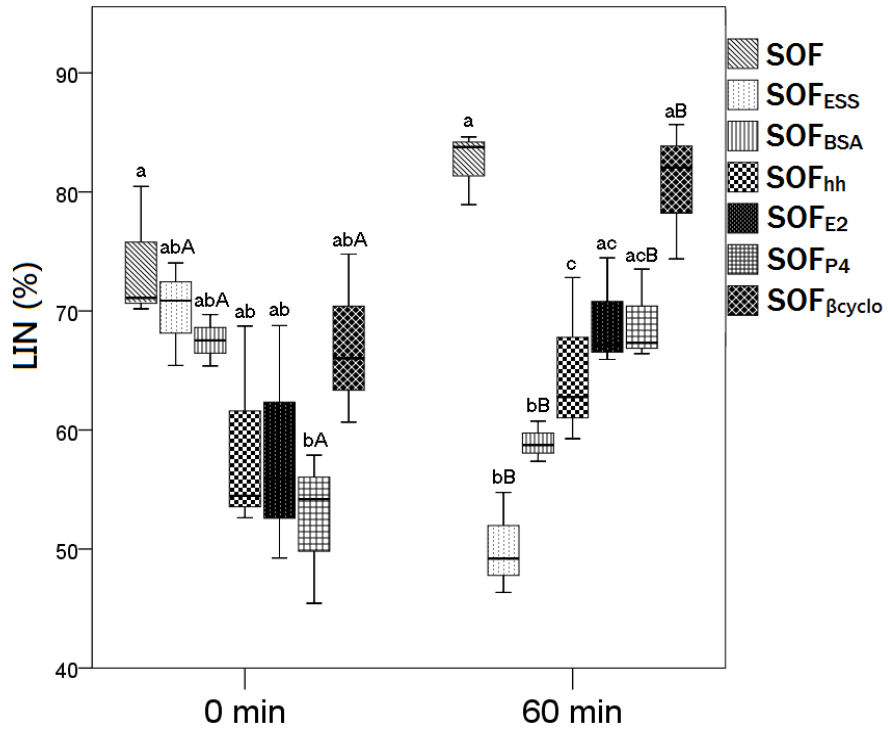
681 **B)**



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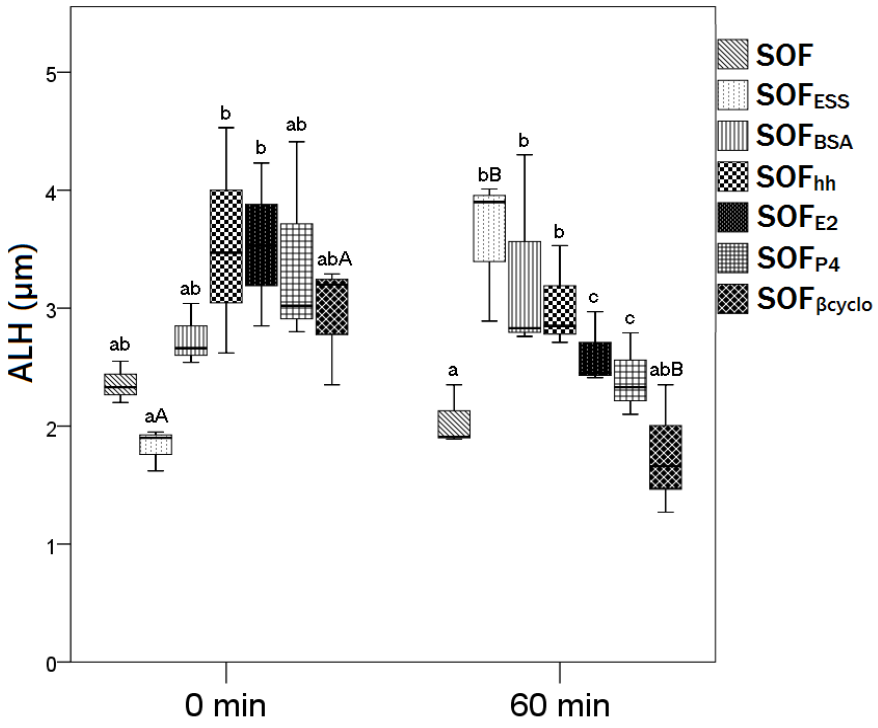
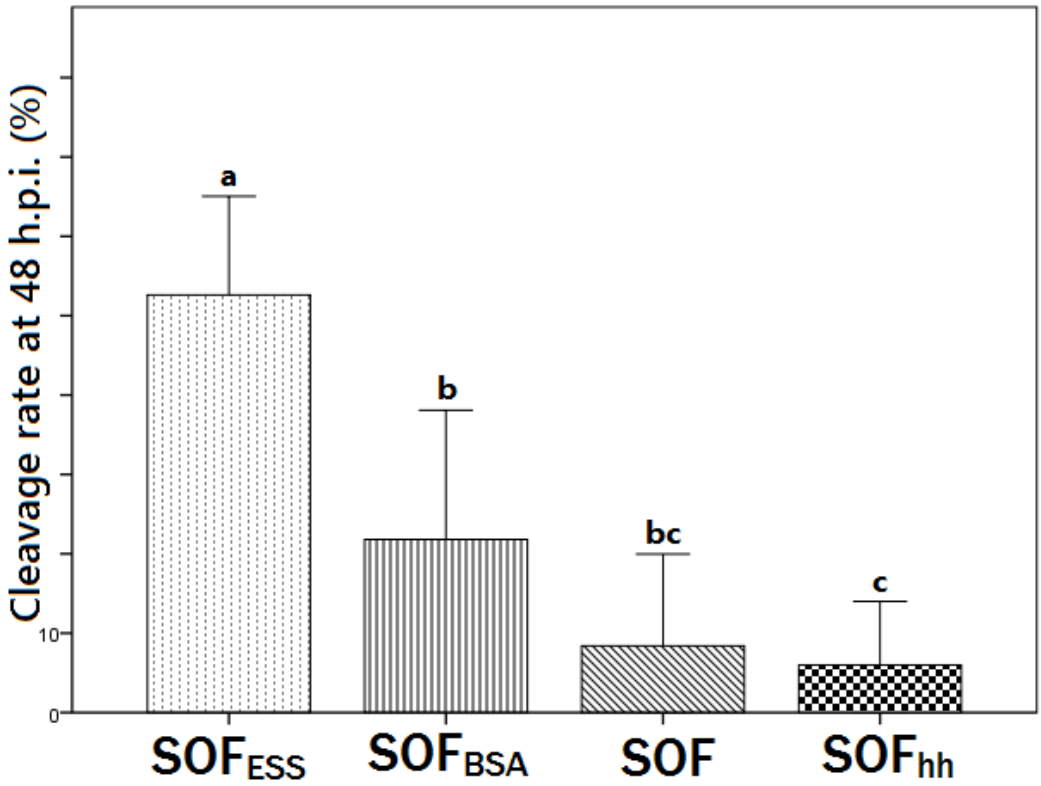
C)



699 Fig 3. García-Álvarez et al.



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Highlights:

- The effect of different substances to capacitated frozen-thawed ram spermatozoa was assessed.
- The sperm motility pattern and membrane fluidity were chosen as parameters related to capacitation process.
- The in vitro fertilization was also used as benchmark of sperm capacitation.
- The media of capacitation, SOF supplemented with heparine and hypotaurine, progesterone, 17β-estradiol, or β-ciclodextrin were unable to trigger capacitation
- The medium of capacitation with BSA was able to trigger capacitation in a similar way than estrous sheep serum (ESS) based on sperm motility pattern and membrane fluidity.
- However, the spermatozoa capacitated with ESS rendered significant higher fertilization rates compared to those spermatozoa treated with BSA.