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Estrous sheep serum enables *in vitro* capacitation of ram spermatozoa while preventing caspase activation

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ABSTRACT

Estrous sheep serum (ESS) is considered the most efficient agent for *in vitro* capacitation of ram spermatozoa. We have explored the relationship between caspase activation and capacitation in ram. Semen samples from 17 rams were cryopreserved. *In vivo* fertility was evaluated after intrauterine artificial insemination. Samples were submitted to four treatments: control, ESS (10%), caspase inhibitor (Z-VAD-FMK), and estrous ewe serum plus caspase inhibitor (I + E). Sperm samples were incubated for 30 minutes at 38.5 °C and 5% CO_2 and analyzed with flow cytometry for mitochondrial membrane potential (Mito-Tracker deep red), sperm viability and apoptosis-like changes (YO-PRO-1/propidium iodide), acrosomal status (peanut agglutinin–fluorescein isothiocyanate), membrane fluidity (merocyanine 540), and caspase activity (Vybrant FAM kits for polycaspases, caspase-8, and caspases 3–7). Estrous sheep serum induced changes compatible with capacitation, doubling the proportion of viable spermatozoa with increased merocyanine 540 and increasing YO-PRO-1^+ and acrosome-reacted spermatozoa ($P < 0.05$). Incubation increased the proportion of spermatozoa with activated caspases ($P < 0.05$), which was abolished by the treatments. We detected a simultaneous decrease in the proportion of the viable and caspase$^-$ spermatozoa after the incubation, which was prevented by the presence of estrous ewe serum ($P < 0.05$). The analysis of caspases 3/7 and 8 resulted in less marked differences. Fertility was positively related to viability and inactivated caspases and negatively to viable-capacitated spermatozoa and active caspases. *In vitro* induction of capacitation in thawed ram spermatozoa by using ESS suggests a downregulation in apoptotic pathways. However, males with the lowest fertility showed parameters similar to high-fertility males, suggesting that other factors were involved apart from capacitation and/or caspase activation.

1. Introduction

Mammalian spermatozoa are not able to fertilize immediately after ejaculation, but they must undergo a set of physiological changes, collectively called “capacitation,” to become fertilization competent [1]. These changes are

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the result of interactions with different factors of the seminal plasma, the fluids in the female genital tract, and the intimate association with epithelial cells in the oviduct. These result in profound changes in the composition and distribution of membrane components, loss of cholesterol, intracellular pH changes, activation of intracellular signaling pathways and, ultimately, phosphorylation of a wide range of proteins. These changes allow the spermatozoa to change their motility (hyperactivation) [2,3], release from the oviductal epithelium, interact with the ZP, develop the acrosomal reaction, and fuse with the oocyte membrane [4,5].

Capacitation can be induced *in vitro*, and in some species, it has been achieved in chemically defined media [6–9]. However, there are huge differences between species, and it is necessary to tune-up protocols to achieve acceptable induction of capacitation and, therefore, good IVF results [10]. In fact, attempts to capacitate ram spermatozoa in chemically defined media have failed until relatively recently [11,12], and in some species, nondefined additives must be used. In sheep, estrous sheep serum (ESS) is still considered the most efficient agent for *in vitro* capacitation and fertilization [13,14], although success with defined media has been reported [9]. Incubation of ram sperm samples in medium with ESS leads to changes that are consistent with an increase in the proportion of viable spermatozoa with high membrane fluidity and an increase in the sperm subpopulation characterized by a hyperactivated-like motility pattern [14].

In contrast, apoptosis is a basic biological principle of programmed cell death that occurs in almost every cell type [15]. In spermatozoa, apoptosis is not strictly a physiological event but rather a spontaneous consequence of the changes underwent in the female genital tract and the engagement in the process of fertilization [16]. In humans, presence of apoptotic markers has been associated with low fertilization and implantation rates in assisted reproductive techniques [17]. Caspases (cysteinyl-aspartate-specific proteases) are the main transducers and effectors of the apoptosis signal leading to programmed cell death [18]. They comprise a family of cysteine proteases that cleave proteins after aspartic acid residues. In mammals, caspases are divided into two groups: initiator caspases, such as caspases 2, 8, 9, and 10, and effector or executioner caspases, such as caspases 3, 6 and 7. Reproductive cells can undergo apoptosis during their development and even in their mature state [16].

Spermatozoa are highly specialized cells and are considered transcriptionally inactive, which conditions the kind of apoptotic events taking place and their consequences [16]. Apoptosis has been proposed as a default position for spermatozoa because their fate is to undergo apoptotic death if they fail to fertilize the oocyte [16]. Thus, the key question that several authors invite to answer is not "what induces apoptosis in spermatozoa?" but rather "what prevents this process from occurring?" Interestingly, it seems that a balance between the capacitation and the apoptosis signaling systems could be taking place in spermatozoa because the activation of capacitation in human spermatozoa seems to prevent apoptosis [18].

Therefore, our objective in this study is to test if this balance is taking place in ram spermatozoa. We hypothesized that the effect of ESS *in vitro* would be not only to induce capacitation but also to block apoptosis. We aim to provide new insights into the effects of ESS on physiological changes induced in ram spermatozoa during *in vitro* capacitation.

2. Materials and methods

2.1. Reagents and media

Flow cytometry equipment, software, and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The rest of the chemicals (reagent grade or higher) and the fluorescence probe propidium iodide (PI) were acquired from Sigma (Madrid, Spain). Other fluorescent probes and caspase analysis kits were purchased from Life Technologies (Barcelona, Spain). Stock solutions of the fluorescent probes were PI, 1.5 mM in Milli-Q water; YO-PRO-1, 50 μM in DMSO; merocyanine 540 (M540), 3.4 mM in DMSO; MitoTracker deep red, 1 mM in DMSO; peanut agglutinin–fluorescein isothiocyanate (PNA-FITC) 1 mg/mL. All fluorescent stocks were kept at −20 °C in the dark until needed. The freezing extender was prepared in our laboratory as described by Fiser et al. [19] using reagent-grade chemicals purchased from Panreac Química SA (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, MO, USA).

Synthetic oviduct flui0d (SOF) was composed of 107-mM NaCl, 7.17-mM KCl, 1.19-mM KH_2PO_4, 1.71-mM Ca_2Cl, 0.49-mM $MgCl_2$, 25.07-mM $NaHCO_3$, 3.3-mM Na lactate, 0.3-mM Na pyruvate, and 200-mM glutamine. Osmolality was 270 to 280 mOsm/kg and pH 7.2 to 7.3 [20].

2.2. Animals and sperm collection

All animal procedures were performed in accordance with Spanish Animal Protection Regulation RD 53/2013 which conforms to European Union Regulation 2010/63/UE. Adult males belonged to the Regional Center of Animal Selection and Reproduction in Valdepeñas (CERSYRA, Spain). In total, 17 rams (Manchega breed, age: >3 years) were used. Semen collection was performed using an artificial vagina. Volume, concentration, wave motion (0: no movement to 5: strong wave movement), and sperm motility were assessed shortly after collection. Only ejaculates with wave-motion values of 4 or 5 and sperm motility higher than 80% were frozen.

2.3. Cryopreservation of semen

After initial semen evaluation, each ejaculate was diluted with the freezing extender. First, sperm samples were diluted in fraction 1 of the diluent down to a concentration of 400×10^6 spermatozoa/mL and slowly cooled from 30 °C to 5 °C for 2 hours. Then, sperm samples were further diluted with the same volume of fraction 2 at 5 °C and held for equilibration for 2 hours more. At the end of the cooling and equilibration period, the extended semen was loaded into 0.25-mL plastic straws and frozen with a