

Motility and Functional State of the Membrane of Caprine Capacitated Spermatozoa under Different Chemical Agents

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ABSTRACT

The objective of this study was to test the use of a commercial extender (Triladyl) as a diluent in caprine semen refrigerated at 15°C, using caffeine (CF), heparin (HP), synthetic oviductal fluid (SOF) and triladyl (TRY) as capacitating chemical agents at different times. Twenty ejaculates of caprine semen were collected using an artificial vagina. The ejaculates were diluted and refrigerated by three days. Evaluated the progressive motility (PM) and the functional state of the sperm plasma membrane through fluorescein CTC staining, counting 200 spermatic cells (non-capacitated spermatozoa NCS, capacitated CS and reacting spermatozoa REA) of caprine in two capacitating agents: CAF and HEP; one culture medium: SOF and a commercial extender: TRY at 60, 120, 180 and 240 min of incubation, during 24, 48 and 72 hs. PM was high under TRY, and CS was high under the HEP treatment. TRY could be an alternative to capacitate caprine spermatozoa, keeping PM for a longer time than HEP or CAF.

Keywords: Spermatozoa; Capacitation; Caprine; Chemical Agents

1. Introduction

Artificial insemination (AI) is probably the reproductive technology most widely used, because it is simple and the highest benefit: Cost ratio when tested bucks are used for reproduction [1]. Semen from tested bucks could be used fresh, freeze or frozen. However, fresh semen must be used immediately after its collection, because motility and viability of the spermatozoa is reduced in a short time. However, frozen semen could be maintained longer than 48 hs [2], providing a greater flexibility of their use in AI programs. frozen semen could be carry from the animal breeding center or a given ranch to the desire one [3]. Preservation of ovine semen kept at 15°C [4] caprine semen at 5°C - 21°C [5] and canine semen [6] has been notified. However, it has been found that the production of reactive oxygen species (ROS) occurs mainly during the cooling period at 5°C [7].

In this context, semen has been diluted in Tris base extenders using as cryoprotector agent non-penetrant lipoproteins of yolk egg and milk proteins because both act against the osmotic effects of the cooling process, pro-

ducing that the hypertonic medium cause the exit of water of the cells, increasing dehydration and reducing the possibility of intracellular crystal ice formation. However, even though this diluent length the lifetime of the spermatozoa and allow the sperm capacitation [8], this does not happen during the cryopreservation process of the caprine spermatozoa, because the interaction of the egg yolk [9] and descreamed milk [10] with the seminal plasma produce a set of nocive reactions for the spermatozoa [11], and irreversible damages in the structure and function of the membranes during the froze and defroze processes [12], which limits the use of AI in caprines.

The *in vivo* sperm capacitation occurs during migration in the reproductive tract of the female, whereas *in vitro* capacitation requires the exposition of fresh or freeze semen to specific capacitating agents [13]. The beginning of this process implies the remotion of cholesterol and probably other sterols from the plasmatic membrane of the spermatozoa, changes in the membrane, flux and redistribution of the proteins modulation in the intracellular ion concentration (Ca^{2+} , HCO_3^- , K^+ and Na^+), hyperpolarization of the plasmatic membrane associated with K^+ flux, pH increase and increase of phosphoryla-

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tion of the protein tyrosine [14].

Many methods have been applied to measure the changes that occur in the membrane, during the *in vitro* capacitation process. The most common method used is chlortetracycline staining (CTC) [15]. This fluorescent antibiotic binds to the membrane linking cations, specially Ca^{+2} and then shows an increase of fluorescence on the segments of the membrane where these cations accumulate. CTC staining has demonstrated to interact with spermatozoa of different species of mammals, that present different fluorescent patterns on the spermatozoa head, which are thought to express different stages of the capacitation process [16].

In vivo the oviductal fluid of the reproductive tract of the female is used as cholesterol-acceptor because is rich in albumins and high density lipoproteins (HDL), capable of withdrawing cholesterol from the membrane of the spermatozoa [17]. Similarly, the pass through the reproductive tract cause that the spermatozoa get rid of the seminal plasma keeping only those proteins or descapacitating factors (muco-polysaccharides and proteins) that cover the spermatid surface [18], which are removed in the oviductal crypts [19] and by progesterone receptors ones initiated the capacitation process [20]. *In vitro* studies, glycosaminoglycans (GAGs) have been identified as efficient inductors of the spermatozoa capacitation. This group of carbohydrates (polysaccharides) formed by repetitive units of disaccharides include the heparin, heparin sulphate, chondroitin sulphate, keratan sulphate and hyaluronic acid [21]. GAGs promote the capacitation binding and removing the proteins of the seminal plasma fixed by adsorption to the plasma membrane of the spermatozoa and inhibit the capacitation [22]. Heparin has been the most potent inductor of capacitation, because it attaches to the spermatozoa through union proteins situated in the cell membrane, driving to the affluence of Ca^{+2} , which increases the synthesis of AMPc and consequently produce the protein phosphorylation [23]. Caffeine is an alkaloid composed of the methylxanthin group used *in vitro* as capacitating agent because it inhibits the phosphodiesterase nucleotide which is responsible of the degradation of AMPc; inducing an increase in its concentration [24]. This has conducted to find new ways to improve the spermatozoa capacitation results. Bergqvist *et al.*, [16] have pointed out the use of oviductal fluid collected *in vivo*, as spermatozoa capacitation medium for bovines. The use of this medium based on synthetic oviductal fluid (SOF) of ovine has not been used in caprines. The use of only the extender triladyl on caprine frozen semen and spermatozoa capacitation with caffeine, heparin, SOF and extender triladyl have also not been evaluated.

The objective of this study was to test the use of a commercial extender (Triladyl) as a diluent in caprine

semen refrigerated at 15°C, using caffeine (CF), heparin (HP), synthetic oviductal fluid (SOF) and triladyl (TRY) as capacitating chemical agents at different times.

2. Materials and Methods

Twenty ejaculates from a buck (Saanen) were collected using an artificial vagina. The semen was evaluated in terms of its micro and macroscopic characteristics and thereafter diluted 9:1 in a commercial base TRIS (Triladyl, Minitüb, Tiefenbach, Germany) extender and refrigerated at 15°C. In order to evaluate the spermatozoa capacitation of the cool semen under different conditions, the experimental protocol was carried out during three consecutive days.

Semen samples (3 ml) diluted in TRIS were divided in four aliquots, which were incubated at 37°C by 15 min. Samples were swim-up three times consecutively. Semen was centrifuged at 750 rpm by 5 min at room temperature. The semen fluid was decanted and reposed twice with equal volume of mDM medium and homogenized gently. In the third wash the pill was incubated with mDM for 45 min to allow to up and select the spermatozoa with greater movility [25].

The supernatant was separated and placed in independent vials to get the different treatments of the study: Treat 1) upper fraction + mDM (Vol/vol) + Caffeine (concentration 5 mM; CA); Treat 2) upper fraction + mDM (Vol/vol) + Heparin (50 µg/ml; HE); Treat 3) upper fraction + synthetic oviductal fluid (SOF; Vol/vol); and Treat 4) upper fraction + Triladyl (TR; Vol/vol).

Evaluation of the functional state of the spermatid membrane: It was done through fluorescent CTC staining, counting 200 spermatid cells. The evaluation of the functional state of the membrane and PM of the spermatozoa was carried out in 150 µl of each treatment sample at 60, 120, 180 and 240 min. At all times the treatments stayed in incubation at 37°C. The protocol was repeated 24 hs (day 2) and 48 hs (day 3) with diluted semen and refrigerated in Triladyl.

The functional state of the membrane was classified according to staining portion in the following categories: a) Non-capacitated spermatozoa, with uniform fluorescence in the head and intact acrosome; b) Capacitated spermatozoa, with fluorescence concentrated in the acrosomal region, a band without fluorescence in the post-acrosomal region and the presence of intact acrosomes; c) Spermatozoa with acrosomal reaction, without fluorescence in the head, except by a thin band in the equatorial region [26].

Statistical analysis: The effect of chemical agent caffeine (CF), heparin (HP), synthetic oviductal fluid (SOF) and triladyl (TRY) extender, culture time (60, 120, 180 and 240 min), day of evaluation (1, 2 and 3) and simple

interactions among them on progressive motility (PM), non-capacitated spermatozoa (NCS), capacitated spermatozoa capacitated (CS) and reacting spermatozoa (REA) was tested. Data were analyzed using the general linear model procedure of SAS [27].

3. Results

The statistical analysis showed effects of treatment ($P > 0.0001$), day ($P > 0.0001$) and culture time ($P > 0.0001$). However, there was no effect of the treatment* day ($P > 0.476$) and treatment* time interaction ($P > 0.995$).

Treatment effect: PM, CS, NCS and REA were affected by treatment. A greater PM and NCS spermatozoa was observed for TRY and SOF treatments ($P < 0.01$), respectively (Table 1). CS was better for HEP and CAF; whereas better REA was found for CAF.

Time effect: PM was greatest at 120 min and CS at 240 min as compared with other culture times (Table 2). No significant difference was found with respect to REA.

Effect of day (Storage time): Evaluation day had a significant effect on PM and CS; as day of evaluation increased PM decreased but CS increased (Table 3).

Table 1. Effect of treatment on progressive motility and functional state of the plasmatic membrane of caprine spermatozoa.

Variable (%)	Caffeine	Heparin	SOF	Triladyl	SE	P-value
PM	29.2 ^a	34.8 ^a	59.9 ^b	65.9 ^b	1.67	0.0001
CS	59.5 ^{ab}	63.2 ^a	55.7 ^{bc}	54.2 ^c	0.95	0.0011
NCS	31.2 ^a	29.2 ^a	37.1 ^b	37.8 ^b	1.09	0.0002
REA	9.3 ^a	7.7 ^{ab}	7.2 ^{ab}	7.8 ^b	0.32	0.04

^{abc}Different literals in the same row mean statistical significant effect. PM = progressive motility; CS = capacitated spermatozoa; NCS = non-capacitated spermatozoa; REA = reacting spermatozoa.

Table 2. Effect of incubation time with different chemical agents on progressive motility and functional state of the caprine spermatozoa membrane.

Variable (%)	Incubation time (min)				SE	P-value
	60	120	180	240		
PM	57.4 ^a	49.6 ^b	42.7 ^c	40.1 ^c	1.73	0.0001
CS	52.9 ^a	56.3 ^{ab}	60.1 ^{bc}	63.3 ^c	1.71	0.002
NCS	40.1 ^a	35.9 ^a	31.4 ^{bc}	27.9 ^c	1.65	0.0001
REA	6.9 ^a	7.8 ^a	8.4 ^a	8.7 ^a	0.53	0.08

^{abc}Different literals in the same row mean statistical significant effect. PM = progressive motility; CS = capacitated spermatozoa; NCS = non-capacitated spermatozoa; REA = reacting spermatozoa.

Table 3. Effect of incubation day with different chemical agents on progressive motility and functional state of the caprine spermatozoa membrane.

Variable (%)	Evaluation day			SE	P-value
	1 (0 h)	2 (24 h)	3 (48 h)		
PM	55.6 ^a	49.2 ^b	37.7 ^c	1.45	0.0001
CS	56.1 ^a	55.3 ^a	62.3 ^b	0.82	0.002
NCS	37.2 ^a	35.8 ^a	28.4 ^b	0.95	0.0001
REA	5.8 ^a	8.1 ^b	9.3 ^b	0.28	0.0001

^{abc}Different letters in the same row mean statistical significant effect. PM = progressive motility; CS = capacitated spermatozoa; NCS = non-capacitated spermatozoa; REA = reacting spermatozoa.

4. Discussion

Capacitation process is a prerequisite step for sperm to bind to the zona pellucida, it experiment acrosomic reaction in response to natural agonistics and express hypermotility, a special movement that allow spermatozoa to move in the viscose fluid of the oviduct and get into the zona pellucida [28].

The results of this study indicate that caprine semen diluted in Triladyl could be preserve at 15°C until 48 hs maintaining an acceptable motility ($P > 0.0001$). Probably the presence of lipoproteins of egg yolk and milk may not be required because the extender *per se*, compose of TRIS, sugar, glycerol and citric acid, is capable of providing the nutrients that the spermatozoa need for its metabolism. It also gives protection against pH variation [26], an adequate osmotic pressure for the spermatozoa, and avoids-dehydration damage through the stabilization of the lipid bilayer. *In vivo* the pH of luminal fluid in the reproductive tract has effects on the whole process of reproduction, including spermatogenesis, sperm capacitation, fertilization, and early stage embryo development. The epithelia lining along the reproductive tract wall of both males and females actively perform considerable transepithelial transport of acid-base equivalents to tightly control the pH of luminal fluid milieu along the entire reproductive tract [29]. Because sugars (sucrose, raffinose, trehalose and lactose) are non-penetrating cryoprotectors of high molecular weight they are useful when applied at high cooling speeds, because the cryoprotector action is associated with its dehydrate activity and its specific interaction with the phospholipid membrane [30]. In this context, the type and sugar concentration used can modify not only the sperm motility but also the viability and acrosomal integrity [31]. In general the monosaccharides in addition to act as cryoprotector play other functions. They provide a source of energy for the spermatid cell and maintain the osmotic pressure [32].

The glycerol has been used as a penetrating cryoprotector agent since its discovery by Polge *et al.* [33]. It has

been shown that their presence in the extenders reduces damage in the spermatozoa, because it avoids the build of intracellular crystal ice, as well as the excessive dehydration caused by the slow cooling [34], and improve fertility [8]. The differences between studies respect to the use of glycerol, are due to alterations in the organization and viscosity of the cytoplasm of the spermatozoa, to the permeability and stability of the membrane, and alteration of the protein and phospholipids organization [35]. However, the previous could be due to many factors such as glycerol concentration, the use of different diluents, different cryopreservation protocols, and the use of other cryoprotectors; as well as the different criteria and methods used to evaluate sperm quality [36].

Sugar and glycerol act in a way that their hydrogen bond to the polar group of the head of the membrane lipids, which have the ability to replace the water molecules normally found in the polar groups, which help to stabilize the membrane during the temperature transition [8].

On the other hand, the results of CS (56.1%) and PM (55.6%) here obtained indicated that the spermatozoa can be conserved during one day only, this is due to that citric acid and sugar compounds provide a source of energy enough for the sperm metabolism and to increase motility [37] but can decrease viability. Previous studies indicate that the use of Triladyl can provide substrates such as citric acid, which through a series of chemical reactions favor sperm motility due to an increase of ATP production [38]. Spermatozoa require a permanent production of ATPs in order to maintain the cell structure, the composition of intracellular ions and motility. It has been hypothesized that composition of ions participate in the capacitation process [39].

The decreasing effect of HEP on PM and the increasing effect of CAF on CS agree with the results of Zhou *et al.* [40], who evaluating the effect of heparin in fresh semen during caprine spermatic capacitation by 120 min observed a significant reduction of motility and membrane integrity. El Gaafary *et al.* [41] using CAF obtained acceptable results of sperm motility of fresh bovine semen during 1 h; however, motility decreased as incubation time increased (up to 6 hs).

Previous research suggested that the reduction of the motility of *in vitro* capacitated spermatozoa treated with heparin or caffeine may be due to reduction of the glycosylable substrates like glucose and/or fructose, and also to a reduction in the availability of pyruvate and lactate, substrates that during the cell metabolism are primordial to produce ATPs [42]; which make difficult its conservation and use for longer times.

Respect to the medium known as synthetic oviduct fluid (SOF) which composition is based in the biochemical analysis of ovine oviduct fluid plus HEPES and

polyvinyl alcohol (PVA). The incubation results with the SOF medium showed a greater PM than for CAF and HEP but the capacitation percentages was similar. The mechanism by which the motility vary has not been established. However, it is known that motility is particularly dependent of the mitochondrial function [43]. Mitochondria are strategically distributed around the mid-piece of the spermatozoa to provide energy to dynein that propel the microtubules. The mitochondria provides the main source of oxidative energy throughout the production of ATPs via the electrons transport chain. In the other hand, the response to the capacitation could be due to the fact that the medium is a source of intracellular ions (NaCl, KCl, KH₂PO₄, MgCl₂, NaHCO₃, CaCl₂) pyruvate and lactate, that cause changes in the properties of the plasmatic membrane, particularly of the ionic conductance which has been shown to be powerful regulator of the metabolism and consequently of capacitation and sperm motility [44]. Also, it is known that bicarbonate plays an important role in the capacitation and fertilization processes both *in vivo* and *in vitro*, because it increases the distabilization of the lipid membrane [45], and regulates and stimulates the adenylcyclase activity that increases the concentration of AMPc and speed up the A1 cyanase protein (PKA1), which phosphorylate some proteins [46]. In the other hand, Huo *et al.* [47] made reference to the fact that the elimination of the seminal plasma by centrifugation and sperm dilution could cause destabilization of the spermatic membrane. Therefore the hydrophobic an adjustment among membranes of the proteins and the "lipidic rafts" cause small changes in the membrane thickness [48] and protein interactions with lipidic rafts. This may allow the exteriorization of receptors of the ionic channels that participate in the activation of the mechanism of calcium transduction flux, AMPc synthesis, and protein phosphorylation diphosphorylation [49].

5. Conclusion

In summary, TRY without egg yolk and milk could be an option for refrigerating caprine sperm and flexible option to use in caprine AI programs. The use of SOF medium and TRY extender as chemical agents maintained an acceptable motility and sperm capacitation for longer time compared with HEP or CAF. However, further FIV test are required to determine the fecundate capacity of the spermatozoa.

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