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SHORT COMMUNICATION

Improve intra-uterine insemination in rabbits using ultra-high temperature skim milk as extender to keep semen at room temperature

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Summary

Two experiments were carried out to examine *in vitro* quality and *in vivo* fertility of rabbit semen diluted in ultra-high temperature (UHT) skim milk. In the first experiment, pooled ejaculates of 10 adult rabbits were divided in three aliquots. Each aliquot was diluted in saline solution, TrisC or UHTm extender and kept at room temperature for 24 h. Sperm quality assessment was performed during all the incubation periods. In the second experiment, 27 adult rabbit does were inseminated with semen incubated for 5 h. Embryo recovery was performed 96 h after insemination. Results showed that treatments diluted in UHTm registered the highest values of spermatozoon with total motility, intact and functional plasma membrane and greater number of embryos recovered in rabbit does. We conclude that UHT skim milk would be a good extender for improved intra-uterine insemination in rabbits and to keep sperm cells for several hours at room temperature.

Introduction

Artificial insemination (AI) is one of the most useful techniques of genetic improvement in animals of productive interest. In rabbits, AI is widely performed with fresh diluted semen, achieving fertility rates similar to natural mating (Alabiso *et al.*, 1996). Saline solution (SS) (0.9%) is the most usually used extender. However, good fertility rates are obtained only when insemination is performed immediately after semen collection.

Skimmed milk has been used for many years as a semen extender. Before dilution of semen, milk must be heated to inactivate lactenin (tindalisation), a nitrogenous molecule toxic to spermatozoon (Salamon & Maxwell, 2000). The ultra-high temperature (UHT) sterilisation process used in milk destroys lactenin and makes tindalisation unnecessary. The use of UHT skimmed milk as a fresh semen extender has been reported in several species (Hollinshead *et al.*, 2004; Salvador *et al.*, 2006). However, for our best knowledge, there are no reports of the use of UHT skimmed milk as a rabbit sperm extender.

The aims of the present study were to assess *in vitro* quality and *in vivo* fertility of rabbit semen diluted in UHT skim milk after incubation at room temperature.

Materials and methods

The study was carried out in the fields of Balcarce Experimental Research Station of the Instituto Nacional de Tecnología Agropecuaria, Argentina (37° 45′ south, 58° 18′ west).

To meet the objectives, two experiments were carried out. The first experiment was designed to assess *in vitro* quality of semen extended in UHTm, and the second experiment was performed to determine *in vivo* fertility of semen extended in UHTm. In both experiments, the same bucks were used as semen donors.

Semen collection and processing

Ejaculates were collected from 10 adult rabbit bucks (five Californian and five New Zealand White breeds) once a week and immediately evaluated for volume and mass motility. Ejaculates were pooled by male breed and divided into three equal aliquots. Then, aliquots were diluted (1 : 3) in SS, TrisC or UHTm extender and kept at room temperature for 24 h. Sperm quality assessments were performed throughout the incubation period (0, 2, 5 and 24 h). The extenders tested were as follows:

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TrisC: Tris-citric acid, Tris (hydroxymethyl) aminomethane (0.25 M), D (+) – glucose (47 mM), citric acid (88 mM).

UHTm: partially skimmed milk, ultra-high temperature processed.

Control group SS: saline solution (0.9% p/v).

All extenders contained streptomycin sulphate (80 mg l^{-1}).

Experiment 1: *In vitro* quality assessment

Total and progressive sperm motility was subjectively assessed from a sample placed under a coverslip in a prewarmed slide (37 °C) under light microscope at 400× (Nikon Diaphot, Tokyo, Japan). After staining with eosin–nigrosin (Mortimer, 1994), 200 spermatozoa in five different fields were counted using a light microscope to determine the percentage of spermatozoon with intact plasma membrane. Sperm plasma membrane functionality was assessed by hypo-osmotic swelling test (HOST), according to Jeyendran *et al.* (1984) revised by Bled *et al.* (2007). On a total of 200 cells, spermatozoa with swelling (functional plasma membrane) or straight tails (dysfunctional) were counted. Results were expressed as percentage of spermatozoon with a functional plasma membrane.

Experiment 2: *In vivo* fertility assessment

The 27 does were divided into three groups and inseminated with semen diluted in SS, TrisC or UHTm. All does were treated with eCG (60 UI, im, Novormon®; Syntex, Buenos Aires, Argentina) and licereline (0.3 ml, GnRH CIASA®; CIASA, Buenos Aires, Argentina) 48 and 4 h before insemination respectively.

After collection, semen was diluted and maintained at room temperature for 5 h until AI was performed. All rabbit does were inseminated once, 48 h before eCG treatment, and collection of embryos was performed 96 h after insemination. Anaesthesia was induced by xylazine 2% (5 mg kg^{-1}, im, Rompun; Bayer S.A., Argentina) and ketamine hydrochloride (50 mg kg^{-1} BW, im). For embryo recovery, uterine horns were flushed with phosphate buffer solution supplemented with BSA (2%). Collected structures were evaluated and classified in embryos or unfertilised oocytes.

Statistical analysis

Sperm quality parameters were analysed using a statistical model that included the main effects of rabbit breed, extender, incubation time and their interactions. When fertility was analysed, only the effect of the extender was considered. Data were scrutinized using proc GLIMMIX

Table 1 Semen quality parameters of ejaculates obtained from Californian and New Zealand rabbit bucks, diluted in Tris C, UHTm and SS extenders at 0, 2, 5 year 24 h of incubation

	Breed		Incubation time (h)				Extender		
Parameters (%)	Californian	New Zealand	0	2	5	24	TrisC	UHTm	SS
Total sperm motility	54.7 ± 5.2	43.4 ± 4.6	70.7 ± 4.9	60.6 ± 4.5	49.6 ± 4.1	26.4 ± 3.1	49.4 ± 6.1	58.8 ± 6.6	39.7 ± 5.7
Forward Progressive motility	35.7 ± 5.4^{a}	21.4 ± 4.2^{b}	56.1 ± 5.6^{a}	42.1 ± 4.9^{b}	31.3 ± 4.3^{c}	7.9 ± 2.4^{d}	30.1 ± 5.7^{ab}	38.3 ± 6.7^{a}	18.3 ± 5.3^{b}
Plasma membrane integrity	81.9 ± 1.2	80.7 ± 1.2	83.2 ± 1.3^{a}	83.5 ± 1.3^{a}	81.4 ± 1.3^{a}	77.2 ± 1.2^{b}	80.0 ± 1.4^{b}	84.7 ± 1.5^{a}	79.3 ± 1.4^{b}
Plasma membrane functionality	31.8 ± 2.3	41.2 ± 2.7	50.2 ± 2.9	37.5 ± 2.5	33.4 ± 2.3	28.5 ± 2.3	26.6 ± 2.7	49.7 ± 3.5	36.9 ± 3.0

UHT, ultra-high temperature; SS, saline solution.

Data are means ± SEM. Values in the same row with different superscripts (a, b, c, d) are statistically different ($P < 0.05$).

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Table 2 Embryos and oocytes recovery from rabbit does inseminated with semen diluted with three different extenders: TrisC, UHTm and SS

	TrisC	UHTm	SS (control)
Rabbit doe flushed for embryo recovery	9.0	9.0	7.0*
Total structures	53.0	57.0	39.0
Number of structures/ rabbit does	5.9 ± 3.7	6.3 ± 4.2	5.6 ± 4.7
Total embryos	28.0	43.0	17.0
Number of embryos/ rabbit does	3.1 ± 3.2[ab]	4.8 ± 4.6[b]	2.4 ± 3.1[b]
Total unfertilized oocytes	25.0	14.0	20.0
Number of oocytes/ rabbit does	2.8 ± 2.2[a]	1.6 ± 1.4[b]	3.1 ± 3.4[a]

UHT, ultra-high temperature; SS, saline solution.

Data are means ± SEM. Values in the same row with different superscripts (a, b) are statistically different ($P < 0.05$).

*Data from two females of SS group were eliminated, due to problems in the uterine flushing.

with a Poisson distribution. The least square means were compared using Tukey–Kramer test. Statistical significance was established at $P < 0.05$.

Results

Sperm quality parameters were affected by the combination of incubation time and extender. Semen diluted in TrisC and UHTm registered higher values than semen diluted in SS at all evaluation periods. According to incubation time increases, motility was declined. Spermatozoa with progressive motility from Californian bucks were higher than those of New Zealand bucks, inverse of what happened with values of spermatozoon with intact plasma membrane. The percentage of spermatozoon with functional plasma membranes diluted in TrisC decreased as storage time increased; meanwhile, those extended in SS decreased significantly after 2 h of incubation (Table 1). The number of embryos recovered was higher in rabbit does inseminated with semen diluted in UHTm than those inseminated with semen diluted in SS. No differences were detected between UHTm and TrisC. The percentage of unfertilised oocyte was lower in UHTm than SS extender (Table 2).

Discussion

In the present study, semen diluted in UHTm and TrisC showed a similar decline in total sperm motility as incubation time increased. These were smaller than values reported by Rosato *et al.* (2006). Differences could be due to the highest incubation temperatures used in our study that allows highest sperm metabolic activity. As we expected, semen diluted in complex extenders showed higher progressive motility than diluted in SS, which does not contain energetic substrates. Milk proteins would have a protective effect over sperm plasma membrane (Alfonso Sánchez *et al.*, 2006). Taking into account the correlation between progressive motility values and fertility rate found by Lavara *et al.* (2005), we would expect good fertility rate using UHTm or TrisC. Sperm fertilisation ability depends on the functional integrity of plasma membrane. All extenders of the present study were able to preserve integrity for 5 h of storage and decreased after 24 h. We consider that semen with those values could be use in AI.

The HOS test is an estimator of sperm plasma membrane functionality. The percentages registered at time zero were similar to those reported for Bled *et al.* (2007) and Amorim *et al.* (2008).

Good fertility rates were reported in mare (Saporiti, 2005) and ewes (Bidinost & Abad, 2007) when UHT milk was used. To our knowledge, this is the first report about the use as rabbit sperm extender.

In conclusion, our data of quality and embryo production with semen diluted in UHT skim milk suggest that it would be a good extender for improved intra-uterine insemination in rabbits and to keep sperm cells for several hours at room temperature. Moreover, this product has the following advantages: does not require prior preparation, can be stored at room temperature for several months and can be easily purchased.

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