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TECHNICAL NOTE

ARTIFICIAL INSEMINATION IN RABBITS: LABORATORY AND FIELD TRIAL WITH THREE DIFFERENT SEMEN EXTENDERS

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ABSTRACT: Today a great deal of research is focused on the development of new extenders that allow the refrigeration and maintenance of rabbit semen for a longer period of time. In this study, semen diluted with 3 different extenders (A, B, and C) and stored at two different temperatures (4°C and 38°C) were evaluated using both laboratory and in vivo tests. The best results were obtained with extender C (INRA 96) which, in the first 10 h of the test at 4°C, displayed about 80% higher motility compared to the other two extenders and preserved optimal seminal fluid motility for over 34 h after dilution. In the test at 38 °C, the motility of semen diluted with extender C was 52% in the fifth h *vs* 21% of the semens diluted with extenders A (Lepus) and B (Verdunnungsmischung). Another test involved artificial insemination (AI) of 1800 lactating does using refrigerated semen diluited with the extenders under study. The fertility rate and litter size obtained with semen diluted with extender C was higher (78.1% and 8.65, respectively) than those obtained with extenders A (71.2% and 8.15, respectively) and B (71.05% and 8.13, respectively) in both parallel and sequential tests. In conclusion, extender C offers greater vitality and motility to rabbit spermatozoa, thus higher fertility rates to rabbits does.

Key words: artificial insemination, rabbits, semen extenders.

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INTRODUCTION

Artificial insemination (AI) has become a routine practice in rabbit production (Alvariño, 2000). The technique offers significant benefits, including genetic selection, prolonged fertility even during unfavourable times of the year, cycle-based production, more efficient breeding programmes and last, but certainly not least, improved health monitoring (Bergonzoni *et al.,* 1994).

AI in rabbits is generally performed with 0.5 ml of extended semen. Theoretically, it is possible to obtain 30-40 doses per ejaculate, but in everyday practice it is preferable to have a dilution rate from 1:5 to 1:10, meaning approximately 10-15 doses/ejaculate, to ensure that there are at least 10 million viable, non damaged spermatozoa (Paufler, 1985; Facchin *et al.,* 1991; Facchin, 1995; Viudes De Castro and Vicente, 1997; Castellini and Lattaioli, 1999). After extension, insemination must take place within 24-48 hours, since the sperm survival strongly decrease after 36 hours and its fertilizing capacity tends to diminish after about 16 hours of storage (Paufler, 1985; Facchin *et al.,* 1991). Independently of semen dilution, however, it should be kept in mind that the type of extender used will have an impact on the reproduction rate (Kiprianidis and Facchin, 1994).

In fact, the survival of spermatozoa in a complex buffer solution is affected not only by the storage temperature but also by the interaction between temperature and its composition, in terms of cellular response to the culture medium. With the most widespread culture media currently employed, semen must be used within 36 to 48 hours after collection (Freychat *et al.,* 1989) when kept at refrigeration temperatures (Boussit, 1989).

Whereas frozen semen has been employed successfully for a long time in AI of cows, the storage and use of rabbit semen is still hampered by the need for refrigeration at 4ºC to 5°C and the restriction of storage for periods longer than 48 hours. Thus, it is not surprising that current research is pursuing two main objectives: formulation of extenders that permit longer storage periods of semen, and

optimisation of rabbit semen freezing (and/or cooling) processes.

Given that the storage of semen for periods longer than 24-36 hours after collection would be useful for AI programmes in intensive breeding farms, the purpose of our research was to verify the efficacy of three different semen extenders in both laboratory conditions and field trials, in order to offer new avenues of research aimed at improving rabbit reproductive performance through the study of new extenders.

MATERIALS AND METHODS

The trial was conducted on 180 bucks and 1800 does, all New Zealand Whites, aged between 4 and 18 months, housed on a rabbit farm at Bonefro (Campobasso, Italy). The farm consists of 15 sheds, 5 of which house the breeding females, one the breeding bucks and the remainder the growers. The does were fed on non-medicated commercial pellets (lactation feed) while the bucks were fed “ad libitum” (Purina®). All the rabbits were vaccinated against Myxomatosis and Rabbit Hemorrhagic Disease.

In the weeks previous to the experiment, semen samples were taken from all bucks to assess their characteristics and suitability for reproductive purposes according to standard seminal. Thirty bucks having poor semen quality were excluded from the experiment.

The semen was collected using an artificial vagina (mod ‘Amantea’, IMV Technologies, Piacenza, Italy), made of a plastic cylinder with a latex liner secured around the rim, so that warm water could be placed between the cylinder and the latex liner. The artificial vagina was connected to a plastic test tube and 5-6 ml of water at 50ºC to 55°C was introduced, ensuring a temperature of 40ºC to 42°C at the time of collection, despite heat dispersion due to the small size of the device.

Each ejaculation so collected was filtered through sterile pads to remove vesicle gland secretions which, being poorly soluble, could hamper the effectiveness of the dilution process (MACCARIO *et al*., 1992), then examined macroscopically to assess volume, density, colour and pH level. The ejaculates taken from the each rabbit bucks were mixed to obtain a pool of seminal fluid. The ejaculate collection was repeated 4 times at weekly intervals. A computerised evaluation of the pooled semen was then performed using a Hamilton Thorne Bioscences (IVOS, 100 Cummings Center, Suite, 465 E, Beverly, MA 01915, USA) sperm analyser. The parameters evaluated were: % total motility; % progressive motility; Straight Line Speed; Curvilinear Velocity (VCL); Amplitude of lateral head displacement (ALH) and Linearity (LIN); % of rapid spermatozoa. The concentration of the pooled semen was evaluated by Thoma camera.

The viability and morphology of the pooled semen was evaluated independently by two examiners.

The viability was assessed by staining with ethidium-bromide and acridine orange, at a 40x magnification (Figure 1). A coverslipped slide was prepared with 15 µl of pooled seminal fluid and 15 µl of dye; after an incubation period of 10 minutes in the dark at room temperature, 15 µl of china ink were added, then the

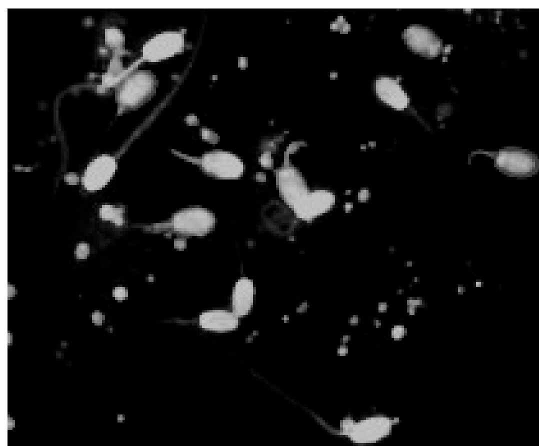

Figure 1: Sperm (40X) stained with ethidium bromide and acridine orange. Viable spermatozoa have a light gray head , while those of non viable ones were dark gray.

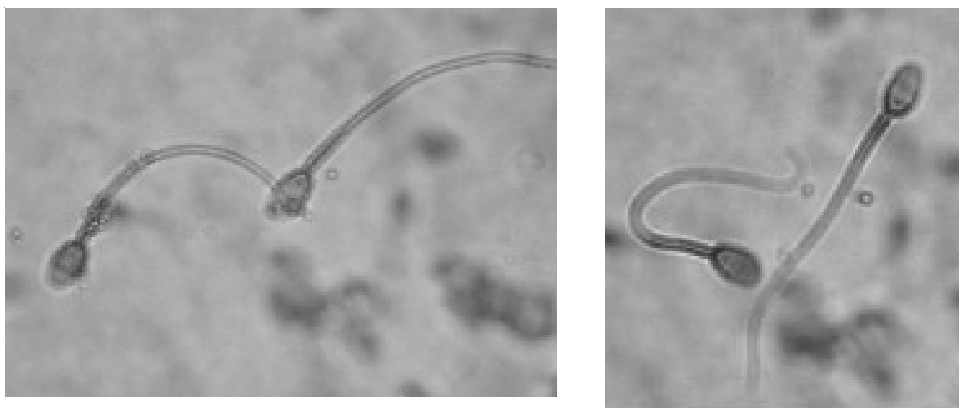

Figure 2: Morphological examination rabbit sperm (40X) using Rose Bengal and Victoria blue dyes. In the left panel two spermatozoa with abnormally thickened midpiece, whereas in the right two spermatozoa normal.

slide thus prepared was observed under fluorescence microscopy. The viable spermatozoa showed bright green heads, while those of non viable ones were orange-red.

The morphological examination of the pooled spermatozoa was performed using Rose Bengal and Victoria blue dyes (Figure 2). A drop of semen fixed in 10% formalin was placed on a slide and coverslipped, dried and stained with Rose Bengal dye (5 minutes) and Victoria blue (30-40 seconds), then rinsed with distilled water. The slide was examined under an optical microscope, at a 40X magnification.

The heterospermic pool was divided into three samples, each diluted 1:10 with three different semen extenders:

- extender A: Lepus (MEDI Chimica, Reggio Emilia-Italia);
- extender B: Verdunnungsmischung M III (MINITUB, Tiefenbach-Germany);
- extender C: INRA 96 (IMV Technologies).

Each of the three diluited samples was then further divided into two aliquots: one, was used for a storage test under refrigerated conditions (4°C), the other for a

storage test at 38°C.

In the first test, the three refrigerated semen samples were placed in an Equitainer™ (Hamilton Research, Inc., South Hamilton, MA 01982. USA) for 10 hours so that the temperature of the extended semen would drop from 37°C to 4°C according to a programmed cooling (Table 1) with an initial slow drop in temperature of –0.3°C/min (HUGHES and LOY, 1970; DOUGLAS-HAMILTON *et al*., 1984). After 10 h, the seminal material was placed in a refrigerator and kept steady at 4°C. The semen samples were then assessed hourly for the next 26 h using the HTB analyser. In the second test, the corresponding semen samples, placed in an incubator at 38°C, were evaluated using the HTB system at hourly intervals for 5 h. This time limit was purposely chosen because it is the time it takes the spermatozoa to swim up the female genital tract (BOUSSIT, 1989; GRILLI, 1994; DEL BOSCO and CASTELLINI, 2000).

The extended semen cooled with the three different extenders was also field tested using two protocols over a period of two months:

Table 1: Cooling curve in the Equitainer equipment

Time (hours)	Temperature °C	Time (hours)	Temperature °C
0	37	5.5	8
0.5	31	6	7
1	26	6.5	7
1.5	22	7	7
2	19	7.5	6
2.5	16	8	6
3	14	8.5	6
3.5	12	9	6
4	11	9.5	6
4.5	10	10	4
5	9		

1) AI of 3 lactating groups of does (600/ group), matched for age, weight, parity and litter size, with extended semen A, B, and C (Protocol 1).
2) the same group of 600 lactating does was inseminated at 1st cycle with extender A, at 2nd cycle with extender B, and at 3rd cycle with extender C (Protocol 2).

Prior to insemination, the does followed an oestrus synchronisation and ovulation induction protocol receiving 20 I.U. i.m. of PMSG (Ciclogonina®-Fort Dodge Animal Health S.p.A., Bologna - Italia) 72 h before AI and 200 μg i.m. of Gonadorelin (Cystoreline®- Ceva Vetem S.p.A., Agrate Brianza (MI) - Italia) at insemination. The individual dose of extended semen per doe was 0.5 ml, and contained at least 23-24 million motile spermatozoa.

Chi-square and Anova statistical tests were used.

RESULTS AND DISCUSSION

Prior to extension, the parameters of the pooled seminal fluid were considered and evaluated by the HTB sperm analyzer as shown in Table 2. The mean values found closely agree with those of Bergonzoni *et al.,* 1994 and Brun *et al.*, 2002 in healthy rabbit bucks.

The storage of the seminal material at refrigeration temperature affected the mass motility and the other parameters examined of the different semen extenders with a different time scale as detailed in Table 3 and Figure 3.

The motility of the spermatozoa diluted with extender A was about 70% and it remained at this level for the first 10 hours, after which there was a rapid decline (Figure 3). The semen diluted with extender B featured steady mass motility for a similar time to extender A; initially the motility tapered off slowly, until around the

Table 2: Means and standard deviation (SD) of the pooled seminal fluid before dilution collect in four replicates.

PARAMETERS	Mean ± SD
Total pool volume (ml)	114 ± 15
Color	Milky
Odor	Odorless
pH	7.12 ± 0.13
Total motility(%)	83.52 ± 2.72
Progressive motility (%)	78.8 ± 1.7
Concentration 108/ml	6.00 ± 0.51
VSL (μm/sec)	43.16 ± 0.43
VCL (μm/sec)	140.82 ± 14.23
LIN (%)	54.8 ± 1.8
ALH (μm)	4.1 ± 2.3
% rapid sperm	63.2 ± 2.9
Viability (%)	92.2 ± 1.8
Abnormalities (%)	2.4 ± 0.6

16th hour, then not only did the motility decline, but all the other parameter values also dropped suddenly, settling at around 0 at the 34th hour (Figure 3). The seminal fluid diluted with extender C displayed consistently greater motility in the first 10 hours, compared to the other two extenders (about 15% higher), after which slight decreases were observed up to the 28th hour, when the mass motility reached 58% and settling at around 0 after the 38th hour (Figure 3). Table 3 shows that at 4°C, extender C preserved optimal seminal fluid motility for over 34 hours after dilution. Concerning the viability of the spermatozoa at 4°C, there was no statistically significant effect of the extenders (Table 3). However, at 34 h the viability of the spermatozoa diluted with the three different extenders was close to 60% (Table 3).

Table 3: Mean and standard deviation of rabbit pooled semen collected in four replicates and diluted with three different extenders (A, B, and C) after 0, 15, 25, and 34 hours of storage at 4°C.

	extender A				extender B				extender C			
	T0	T15	T25	T34	T0	T15	T25	T34	T0	T15	T25	T34
Total motility (%)	72.3±2.5^{a}	48.4±3.2^{a}	7.8±2.4^{a}	0^{a}	74.2±4.1^{a}	55.2±1.9^{a}	32.1±1.5^{a}	0^{a}	81.1±3.1^{b}	71.5±2.2^{b}	61.2±2.6^{b}	41.1±3.1^{b}
Progressive motility (%)	78.5±1.5^{a}	35.2±1.8^{a}	4.8±1.1^{a}	0^{a}	81.4±1.7^{a}	45.2±1.1^{a}	29.5±2.3^{b}	0^{a}	94.6±1.2^{b}	87.5±2.3^{b}	87.1±2.5^{c}	54.2±4.2^{b}
VSL (μm /sec)	46.5±0.5^{a}	44.2±1.2^{a}	45.7±2.3^{a}	0^{a}	55.8±0.4^{a}	54.5±1.1^{a}	43.2±1.5^{a}	0^{a}	56.5±0.7^{a}	61.5±2.3^{b}	43.3±1.8^{a}	41.3±1.4^{b}
VCL (μm/sec)	159.5±13.3^{a}	132.5±12.5^{a}	129.6±12.9^{a}	0^{a}	168.2±14.5^{a}	140.6±15.1^{a}	152.6±13.8^{b}	0^{a}	185.5±12.7^{a}	158.1±15.3^{b}	159.0±14.7^{b}	142.2±13.2^{b}
LIN (%)	34.2±1.8^{a}	19.3±1.5^{a}	44.2±0.9^{a}	0^{a}	41.5±2.1^{a}	29.2±1.5^{b}	31.6±1.2^{b}	0^{a}	48.8±2.3^{a}	36.2±1.9^{b}	30.5±1.4^{b}	34.3±1.2^{b}
ALH (μm)	6.5±1.5^{a}	4.2±1.1^{a}	8.4±2.3^{a}	0^{a}	6.1±1.7^{a}	6.9±1.3^{b}	6.9±1.8^{a}	0^{a}	7.6±1.6^{a}	7.8±1.9^{b}	5.4±1.5^{a}	6.5±1.9^{b}
% rapid sperm	55.9±2.8^{a}	31.4±1.9^{a}	7.4±1.7^{a}	0^{a}	58.5±2.6^{a}	38.2±2.2^{a}	39.2±1.8^{b}	0^{a}	69.8±2.9^{b}	63.1±1.9^{b}	61.6±1.6^{c}	32.6±2.3^{b}
Viability (%)	90.2±2.1^{a}	82.3±3.5^{a}	70.3±3.9^{a}	60.5±4.1^{a}	90.5±3.2^{a}	82.6±3.1^{a}	70.5±1.8^{a}	59.3±3.6^{a}	89.4±3.5^{a}	82.6±4.1^{a}	75.2±3.1^{a}	62.7±1.9^{a}
Abnormalities (%)	2.3±0.3^{a}	2.5±0.5^{a}	2.7±0.5^{a}	2.8±0.6^{a}	3.2±0.4^{a}	3.5±0.6^{a}	3.5±0.6^{a}	3.6±0.7^{a}	3.4±0.3^{a}	3.6±0.5^{a}	3.6±0.5^{a}	3.6±0.5^{a}

Comparison between extenders within times. Means in the same row with differenlettersts differ significantly ($P<0.05$).

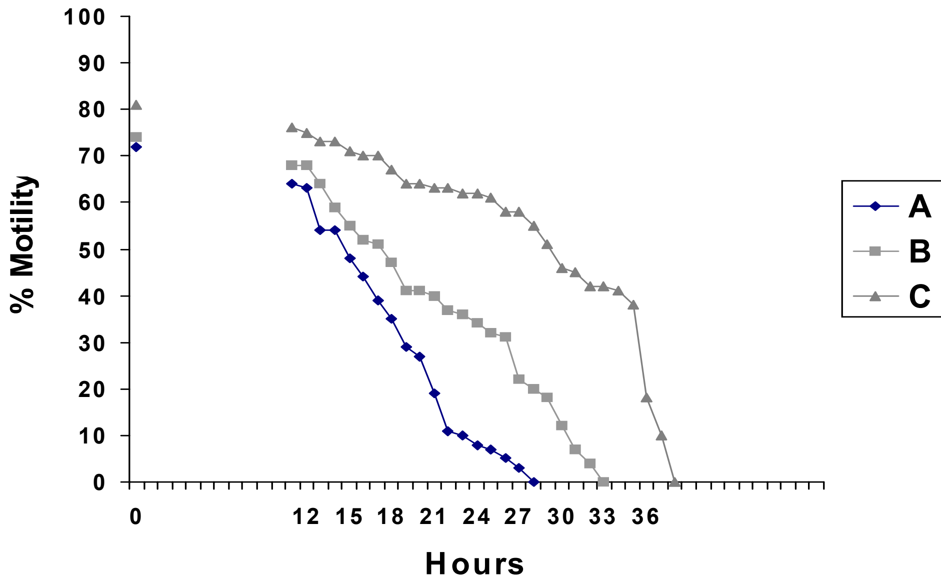

Figure 3: Motility values in the refrigeration test of the semen diluted with extenders A, B, and C.

The most popular and best known extenders employed in rabbit AI are: Ringer Locke, Salisbury, Tris Buffer, Spermasol, Lepus® (MEDI Chimica, Reggio Emilia-Italia) and Dilap 2000® (IMV Technologies-Italia) (DURANTI *et al.*, 1993). The first two preparations are used for 1:8, 1:10 dilutions. The Ringer Locke extender has a very unusual effect on the spermatozoa, based on the stimulation of the electrolytes contained in the saline solution, which enhance the kinesis and consequently the fertilizing capacity of the sperm (PEREZ and PEREZ, 1993). The Tris Buffer is used for AI using fresh semen; it is associated with reproductive performance rates that are equal to or better than those obtained by natural mating. The results achieved with this extender are comparable to those obtained with the use of 0.9% saline solution, when the AI is done immediately after collection-dilution, as regards the reproductive performance of the doe and the number of pups in each litter. The number of live births was higher even in respect to the different seasons (CASTELLINI, 1990). A new extender created by the Lavipal Center, with a slightly acid pH (6.6-6.8) buffered with lactose and peptone, has achieved better results in terms of the number of live births and weaned pups in an artificial insemination programme, compared to the Tris buffer.

Table 4: Mean and standard deviation of rabbit pooled semen collected in four replicates and diluted with three different extenders (A, B, and C) after 0, 1, 3, and 5 hours of storage at 38°C.

	extender A				extender B				extender C			
	T0	T1	T3	T5	T0	T1	T3	T5	T0	T1	T3	T5
Total motility (%)	72.4±2.6^{a}	41.4±2.9^{a}	12.6±1.6^{a}	2.1±1.1^{a}	74.5±3.2^{a}	55.7±2.1^{a}	35.4±1.7^{b}	21.3±2.2^{b}	81.1±3.2^{b}	74.6±1.9^{b}	58.3±2.2^{b}	52.6±4.1^{b}
Progressive motility (%)	78.5±1.9^{a}	43.7±2.2^{a}	15.6±3.2^{a}	4.5±1.3^{a}	81.4±2.2^{a}	60.6±2.8^{b}	40.4±1.9^{b}	38.4±3.2^{b}	94.6±1,7^{b}	80.9±3.5^{c}	65.1±3.2^{c}	45.6±1.9^{b}
VSL (µm /sec)	46.5±1.1^{a}	46.5±0.9^{a}	46.1±2.1^{a}	45.7±1.7^{a}	55.8±2.1^{a}	54.8±1.9^{a}	49.4±1.6^{a}	43.6±3.2^{a}	56.5±0.9^{a}	53.2±1.8^{a}	51.3±2.7^{a}	48.8±3.1^{a}
VCL (µm/sec)	159.1±15.1^{a}	156.5± 14.7^{a}	139.4±13.8^{a}	129.5±11.9^{a}	168.2±14.8^{a}	162.7±13.9^{a}	156.4±12.7^{b}	152.6±16.1^{b}	185.5±16.1^{b}	185.5±14.5^{b}	184.9±13.7^{c}	184.5±13.9^{c}
LIN (%)	34.2±2.3^{a}	34.1±3.1^{a}	34.5±2.1^{a}	33.7±1.9^{a}	41.5±2.2^{b}	38.9±1.9^{a}	35.3±3.1^{a}	31.7±3.2^{a}	46.8±1.9^{b}	42.7±2.2^{a}	35.3±3.5^{a}	29.6±2.9^{a}
ALH (µm)	6.5±2.3^{a}	6.1±2.2^{a}	5.3±1.9^{a}	4.9±2.7^{a}	6.1±2.1^{a}	6.4±2.3^{a}	5.9±3.1^{a}	5.8±2.9^{b}	7.6±3.1^{a}	7.1±2.7^{b}	6.5±1.8^{a}	6.1±1.7^{b}
% rapid sperm	55.9±2.9^{a}	45.3±3.1^{a}	18.9±1.7^{a}	3.9±1.5^{a}	58.5±2.3^{a}	48.9±2.6^{a}	31.1±1.9^{b}	19.8±1.7^{b}	69.8±2.5^{b}	62.3±2.6^{b}	55.3±3.1^{c}	47.7±3.5^{c}
Viability (%)	90.7±2.5^{a}	81.8±2.8^{a}	72.8±2.9^{a}	65.7±1.9^{a}	90.6±2.3^{a}	81.6±2.9^{a}	71.5±3.2^{a}	67.1±1.9^{a}	89.5±2.5^{a}	81.7±3.2^{a}	70.5±1.9^{a}	69.4±2.2^{a}
Abnormalities (%)	2.3±0.5^{a}	2.4±0.5^{a}	2.7±0.7^{a}	3.2±0.8^{a}	3.2±0.4^{a}	3.4±0.6^{a}	3.4±0.6^{a}	3.6±0.7^{a}	3.1±0.4^{a}	3.3±0.6^{a}	3.4±0.6^{a}	3.5±0.7^{a}

Comparison between extenders within times. Means in the same row with different letters differ significantly ($P<0.05$).

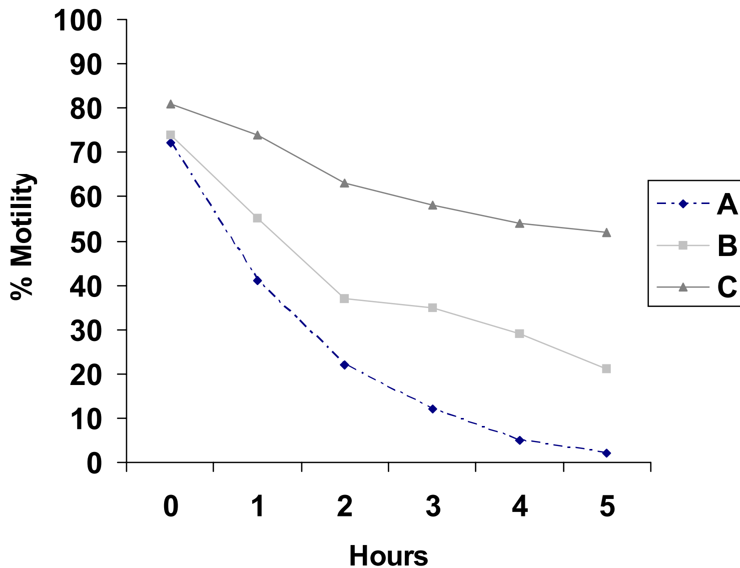

Figure 4: Motility values in the refrigeration test at 38°C of the semen diluted with extenders A, B, and C.

Table 4 summarises the most significant values for the test in which the extended semens were stored for 5 h at 38°C. The semen diluted with extender A featured a mass motility of 72%; and then rapidly declined to about 2% 5 h later (Figure 4). The semen diluted with extender B showed a slower decrease in motility than extender A as shown in Figure 4. In the first 2 h, the semen diluted with extender C dropped from 81% to 63%, but then slowly declined to 52% 5 h later (Figure 4).

There were no statistically significant differences in the viability of the semen diluted with the three extenders in the test at 38°C which gradually declined during the first 5 hours from 90% to 65% (Table 4).

The results of the field test with the two different protocols are shown in Table 5. The fertility rate (Protocol 1) for the does inseminated with semen diluted with extender A was 71.5%, with an average litter of 8.1. Those inseminated with semen diluted with extender B displayed a fertility rate of 72.2% and the average litter was 8.2 (Table 5). Conversely, insemination with semen to which extender C was added gave a fertility rate of 77.8% and a litter size of 8.5 (Table 5).

Table 5: Fertility and litter size in relation with the extender used to dilute the refrigerated semen and the field protocol.

		Extender A	Extender B	Extender C
Protocol 1	Fertility	71.5[b]	72.2[b]	77.8[a]
	Litter size	8.1[a]	8.15[a]	8.5[a]
Protocol 2	Fertility	70.9[b]	71.6[b]	78.3[a]
	Litter size	8.2[a]	8.1[a]	8.65[a]

Means in the same row with different superscripts differ significantly (P<0.05).

The protocol with a single group of does (Protocol 2) evidenced that: insemination with semen diluted with extender A produced a fertility rate of 70.9% and litter size of 8.2; with extender B, the fertility rate was 71.6% and the litter size 8.10; with extender C. the fertility rate was 78.3% and the litter size 8.7 (Table 5). There are no statistically significant differences in the fertility and litter size obtained with the two protocols.

CONCLUSION

This study shows that under the operating conditions illustrated above, semen diluted with extender C preserved a higher concentration of viable and motile spermatozoa both in the refrigeration test and in the test at 38°C. The aforesaid results were also confirmed by the field test, in which higher fertility rates were observed.

In the light of the results obtained on the farm, semen extender C would appear to offer benefits in terms of management efficiency and profitability.

From the management standpoint, the use of this extender could make it possible

to perform two artificial insemination procedures per 24 hours, using the same pool of seminal material. The rabbit seminal material cooled with the Equitainer system could be transported over large distances. Concerning the financial benefits, dilution of the seminal fluid with semen extender C involves operating only on the males, thus the fertility rate is higher than the farm standard rate.

In conclusion, it can be stated that this encouraging preliminary trial needs to be backed up by further studies in the light of the biochemical composition of the three extenders, in order to optimise new ones based on the formula of extender C.

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