

Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris–glucose extender: In vitro and in vivo studies

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Abstract

The effects of medium exchange on motility parameters of chilled canine semen preserved in egg yolk Tris–glucose (EYTG) extender were analyzed over a 27-d period. Semen extender was exchanged at three time points (Days 11, 21 and 27) after collection, when motility parameters were demonstrated to significantly decrease from parameters observed at semen preparation (Day 0) or at day of previous extender exchange. In the absence of medium exchanges, motile spermatozoa were observed up to Day 16 (mean $\pm$ S.D. $1.5 \pm 0.3\%$ of motile spermatozoa). A stimulation of the different semen motility parameters was observed after extender exchange. Semen extender exchange at Day 11 allowed conservation of motility until Day 21, compared to 16 d in the absence of extender exchange. At Day 21, when spermatozoa appeared immobile or dead, a second extender exchange was performed, allowing the extension of motility conservation up to Day 27. The third extender exchange, performed at Day 27, was no longer associated with motility stimulation. Glucose content in the medium decreased slowly over time; a concomitant decrease in pH was also observed. No changes in osmolarity were observed over time. To verify the fertility of long-term conserved chilled semen, two groups of 10 bitches were inseminated either once (Group 1) or twice at 48-h intervals (Group 2) intra-vaginally with semen conserved chilled for a mean of 9 ± 1.8 d. Out of the 10 bitches inseminated once, 5 became pregnant, versus 7 in the group of animals inseminated twice. The present study reports the possibility to extend the conservation of chilled canine semen up to 3 wk

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with conservation of good fertility for at least 10 d. The role of energetic substrate and pH alteration is postulated and the classically accepted relation of semen motility/viability is raised.

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1. Introduction

Since the first successful artificial insemination in canine species using chilled semen in 1954 [1], several studies have been conducted in order to evaluate the effects of temperature over freezing point on semen conservation; the range of 4–5 °C was determined as the best temperature conditions for sustaining semen motility by reducing gamete metabolism [2–6]. This temperature was demonstrated to allow semen conservation for up to 4–5 d, as opposed to <12–24 h when semen was conserved either at room temperature or at 35 °C [4]. Different cooling techniques and semen extenders [2–7] were also analyzed. To further improve semen conservation at low temperature, even conservation with oviductal cells was proposed; this method allowing for an apparent extended motility up to 5 d without semen dilution [8]. However, this technique is far from being adapted to practical use and, except for basic research purposes, was rapidly abandoned.

Extenders are supposed to protect spermatozoa, conserving motility and fertility over time by stabilizing the plasmalemma, providing energy substrates and preventing deleterious effects of changes in pH and osmolarity [2–7]. Different commercial extenders are proposed for dog semen preservation. However, the results obtained using these extenders are often unsatisfactory as most of them were formulated for other species and are not adapted to canine semen. Semen conservation using these extenders is possible with motility preservation up to 4–6 d [9].

Among the laboratory-prepared buffers, egg yolk Tris–fructose was proposed as an extender [3]. However, results from our laboratory [9] have demonstrated an apparent superiority of the egg yolk Tris–glucose extender, in terms of motility conservation (assessed objectively using the Hamilton-Thorn computer-aided semen analyzer). The glucose extender was also evaluated by the ability to limit the acrosome reaction, as determined using chlortetracycline staining. Glucose has already been used as component of dog semen extenders [10,11]. However, no previous studies have reported any beneficial effects of glucose versus fructose. In this extender, good semen quality, characterized by >60% motile spermatozoa and <10% acrosome-reacted cells, was still observed after conservation for 10–13 d at 4 °C [9]. At the end of the storage period (17 d), no semen motility was observed, allowing the classification of semen as dead. Overall, quality of stored semen using glucose instead of fructose was improved, suggesting a major role of the energy substrate glucose in maintaining canine semen motility. This supports other observations and studies in other species in which several factors were proposed as being involved in aging of spermatozoa during the conservation period [3,7]. Factors that likely affect semen conservation include time-dependent metabolite accumulation with pH and osmolarity alteration and a depletion of energy substrate [3,7].

Taking into account the above observations, the aim of the present study was to analyze the effects of extender exchanges (after a few days conservation) on both motility and apparent fertility parameters of dog semen. The conserved medium was exchanged at different time intervals and pH, osmolarity, glucose content and motility characteristics were determined. In addition to these *in vitro* studies, the fertility of the long-term chilled semen was assessed *in vivo* after insemination of bitches with semen stored for 7–11 d.

2. Materials and methods

The study was performed at the University of Liege, College of Veterinary Medicine, Small Animal Reproduction Clinic. Animal housing, care and experimentation were in accordance with Belgian regulations and the *NIH Guide for the Care and the Use of Laboratory Animals* [12].

2.1. Animals

2.1.1. *In vitro* study

Five fertile adult male beagle dogs aged from 3 to 6 yr were used. These dogs were obtained from the kennel of the Reproduction Section of the Department of Small Animal Clinical Sciences of the University of Liege, Belgium. The animals were housed in groups of two in indoor–outdoor runs with natural lighting, fed a commercial dry canine diet (Hill's Science Plan-Canine Maintenance, veterinary formulated) once daily, and given water *ad libitum*.

2.1.2. *In vivo* study

Twenty bitches with known fertility, aged from 3 to 8 yr, were used to test the fertility of long-term chilled semen.

2.2. Semen collection and analysis

Semen was collected by digital manipulation, as described by Linde–Forsberg [13] taking care to separate the three fractions. The sperm rich fraction of the five dogs collected were pooled to allow for the availability of large semen volume and to reduce variability due to individual ejaculate differences. Each pool was first analyzed using light microscopy, with subjective evaluation of motility parameters. The pooled volume was recorded and concentration was calculated using the Makler chamber (Sefi-Medical Instruments, Haifa, Israel) under a light microscope. Percentage of motility was subjectively determined. Percentage of live/dead spermatozoa and abnormal morphology was determined using eosin–negrosin stained smears, according to Christiansen's classification [14].

The Hamilton-Thorn computer-aided semen analyzer, version 10 Ivos (HTR analyzer, Hamilton-Thorn Research, Beverly, MA, USA), validated for dog semen analysis [15], was then used to objectively assess motility parameters and to analyze the effect of time and

treatments on semen characteristics. Software settings, allowing a clear separation and identification of canine spermatozoa motility, were as previously published [15]. For the analysis, a volume of 10 μL of semen was taken from the 4 °C conserved samples and warmed in the Makler chamber at 38 °C for 1 min, to analyze motility. The following parameters were measured: percentage of motile spermatozoa; velocity average pathway (VAP, the average velocity of the smoothed cell path in $\mu\text{m/s}$); velocity straight line (VSL, the average velocity measured in a straight line from the beginning to the end of track in $\mu\text{m/s}$); curvilinear velocity (VCL, the average velocity measured over the actual point-to-point track followed by the cell in $\mu\text{m/s}$); straightness (the average percentage value of the ratio VSL/VAP was calculated; the lower the value, the higher the deviation from a straight line).

According to low (LVV = 9.9 $\mu\text{m/s}$) and medium VAP cutoffs (MVV = 50 $\mu\text{m/s}$), the overall sperm population was subdivided into four categories: rapid spermatozoa with VAP > MVV; medium with LVV < VAP < MVV and slow with VAP < LVV and static gametes constituted by the fraction of all cells which were not moving during the analysis. The percentage of progressive spermatozoa includes cells moving with VAP > MVV and STR > So (So, straightness threshold cutoff to determine the progressive spermatozoa).

2.3. Semen extender

All chemicals used in this study, unless otherwise stated, were biological grade and purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). The egg yolk Tris–glucose (EYTG) based extender was prepared freshly before every trial. The components used were: tris(hydroxymethyl)-aminomethane (3.025 g), sodium citrate monohydrate (1.7 g), glucose (1.25 g), benzylpenicillin (100 mg), dihydrostreptomycin sulfate (100 mg), freshly collected egg yolk 20% (v/v) and distilled water (100 mL). Mean $\pm$ S.D. S.D. pH and osmolarity were 6.83 ± 0.031 and 381 ± 2.57 mOsm, respectively.

2.4. Semen processing and analysis

Pools of semen were processed and conserved identically for all manipulations. The major interest of using these pools, already used in similar studies in dogs and other species [16–18], is that all differences observed can only be attributed to the effects of time and/or extenders. This obviates the effects of individual dogs and processing variations. Finally, ejaculate pooling allows for the availability and use of a sufficient volume of identical materials. In the different studies, the pooled semen was diluted 1/5 (v/v) in the EYTG extender and was conserved in screw-cap closed sterile vials. Vials were placed into a glass tube filled with water and stored at 4 °C in a 3 m $\times$ 3 m cold room. The water in the external tube prevented both cold shock during the chilling process and temperature variations during the study period [19].

Samples were analyzed daily for motility parameters using the computer-aided semen analysis. For each analysis, 100 μL of diluted semen was collected from the 4 °C stored samples after gentle re-suspension by agitation and slowly warmed to room temperature for 10–15 min before performing the different semen parameter evaluations.

At Day 11 of conservation, when motility parameters began to decrease significantly from initial values, stored samples were divided into two equal parts. The first half was kept unmodified at 4 °C in the cold room, whereas the second half was centrifuged at 4 °C at $300 \times g$ for 10 min. The supernatant was removed and the remaining pellet was gently re-suspended in the same volume of 4 °C freshly prepared EYTG extender. The new extender-exchanged samples were stored at 4 °C. Unmodified and modified samples were then analyzed every day simultaneously as described above. At Days 21 and 27 of storage, when all spermatozoa appeared immotile, the same procedure of centrifugation and extender exchanging was again applied on the samples treated on Day 11. For consistency of the results, the study was repeated five times at 1-mo intervals.

2.5. Glucose, pH and osmolarity measurements

In parallel to semen assessment, a glucose assay as described by Little et al. [20] was performed daily using the Vet-test 8008 analyzer (VETTEST S.A., Neufchatel, Switzerland) based on the Walter and Boguslaski [21] general principles of glucose analysis. The glucose measurements were performed by sampling 50 μ L of the supernatant from each sample. The 50 μ L aliquots were then diluted four times in double-distilled water for values to fit in the vet-test range of measurements. In parallel to the glucose measurement, the pH (pH meter, HI 9025, Hanna Instruments) and osmolarity (μ OSMETTE, Precision Systems) were recorded each day during the whole study.

2.6. Effects of long-term chilled semen preservation on fertility

To assess the fertility of long-term chilled semen, two groups of 10 bitches were inseminated either once (Group 1) when progesterone plasma concentrations were between 25.2 and 47.7 nmol/L (mean $\pm$ S.D. 31.2 ± 7) or twice (Group 2) at 48-h intervals, with the first insemination realized when progesterone plasma concentrations were between 15.9 and 27.6 ng/mL (mean $\pm$ S.D. 22.1 ± 3.1), using 7- to 11-d-old (mean $\pm$ S.D. 9 ± 1.8 d) semen. A volume of 2.5 mL of extended semen, including $\pm 200 \times 10^6$ spermatozoa/mL, was deposited intra-vaginally using the Osiris probe [22]. The bitches were examined every 3–4 d by ultrasonography (Hitachi Medical Corporation, model EUB-415, Tokyo, Japan) using a 7.5 MHz transducer, from Day 15 after the LH peak until pregnancy diagnosis. The number of puppies was counted at whelping.

2.7. Statistical analysis

Calculation of means, S.D., and statistical analysis of the results were performed using Statview 4.02 software (Abacus Concepts Inc., Berkeley, CA, USA). Analysis of repeated-measures of variance was done to compare semen motility parameters between groups and over time, followed by a Bonferroni significant difference test (to locate differences). Values were considered significant when $P < 0.05$ [23].

3. Results

The overall characteristics of the pooled fresh semen were: concentration (mean $\pm$ S.D.) $455 \pm 32 \times 10^6$ spermatozoa/mL, percentage of motility ranged from 85 to 95% (mean $\pm$ S.D. 93 ± 3) and proportion of morphologically normal spermatozoa $>85\%$. No significant differences were observed between pools done at different time intervals (data not shown).

3.1. Evolution of different semen motility parameters

Figs. 1 and 2 represent the percentages of motile and progressively motile cells over time, and the percentage of rapid and static spermatozoa conserved over time at 4°C in the EYTG extender. During the first 10 d, semen motility percentages (Fig. 1A) were not significantly different when compared to the initial values (Days 0–10; $P = 0.78$). From Day 11, motility percentage began to decrease significantly (Days 0–11; $P = 0.002$) and

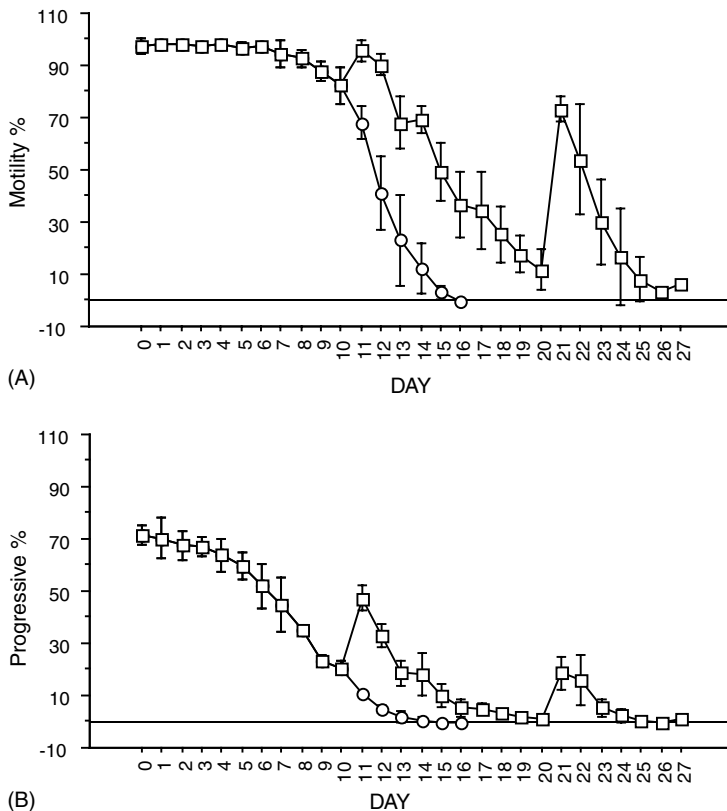


Fig. 1. Percentage of motile (A) and progressive (B) spermatozoa in dog semen conserved at 4°C in supplemented ($\square$) and non-supplemented EYTG extender ($\circ$) samples. Extender was changed at Days 11, 21 and 27.

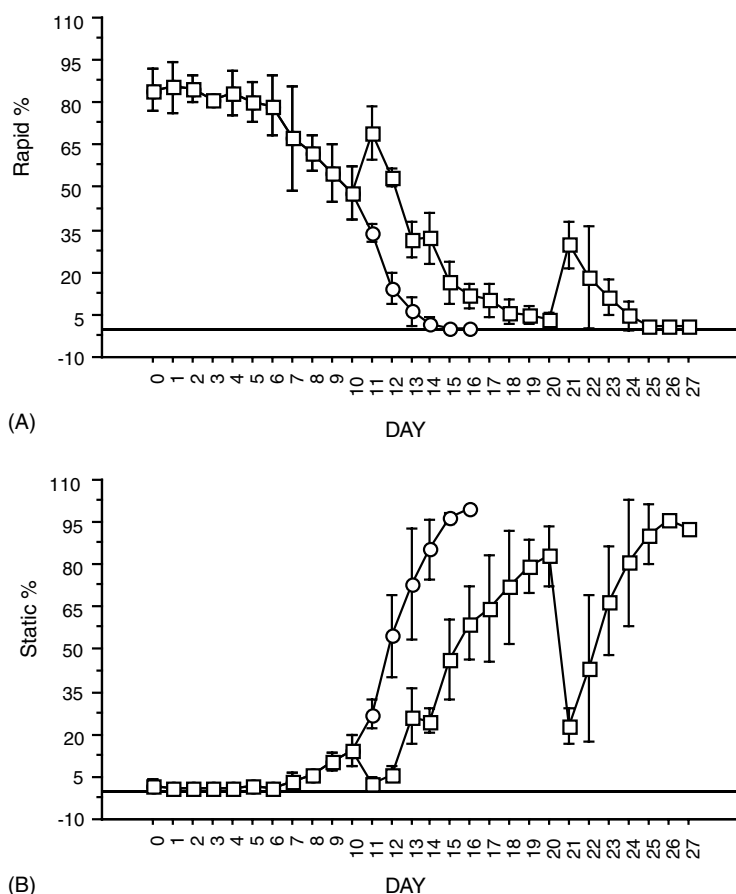


Fig. 2. Percentage of rapid (A) and static (B) spermatozoa of dog semen conserved at 4 °C in supplemented (□) and non-supplemented EYTG extender (○) samples. Extender was changed at Days 11, 21 and 27.

was reduced to zero around Days 16–17 (mean $\pm$ S.D. 16.4 ± 0.9) in the non-supplemented samples.

In extender-exchanged samples, at Day 11 (immediately after medium exchange) the motility percentage significantly increased to peak value not significantly different from initial values measured on Day 0 (Day 0 = 97.3 ± 3.05 , Day 11 = 95.6 ± 4.1 ; $P = 0.6$), but they were significantly different from the values before medium exchange (68 ± 7 versus 95.6 ± 4.1 ; $P < 0.001$). Thereafter, a progressive decrease in motility was observed, characterized by a slope similar to the one previously observed. Values significantly different from the ones observed at Days 0 and 11 were obtained after Day 13 (13.7 ± 1.2 ; $P < 0.001$). In these samples, motility decreased and was, in the absence of further extender exchanges, totally absent around Day 20 (19.9 ± 1.4).

At Day 21, when apparent and computer-aided controlled motilities were not significantly different from zero, the samples were again supplemented with the EYTG

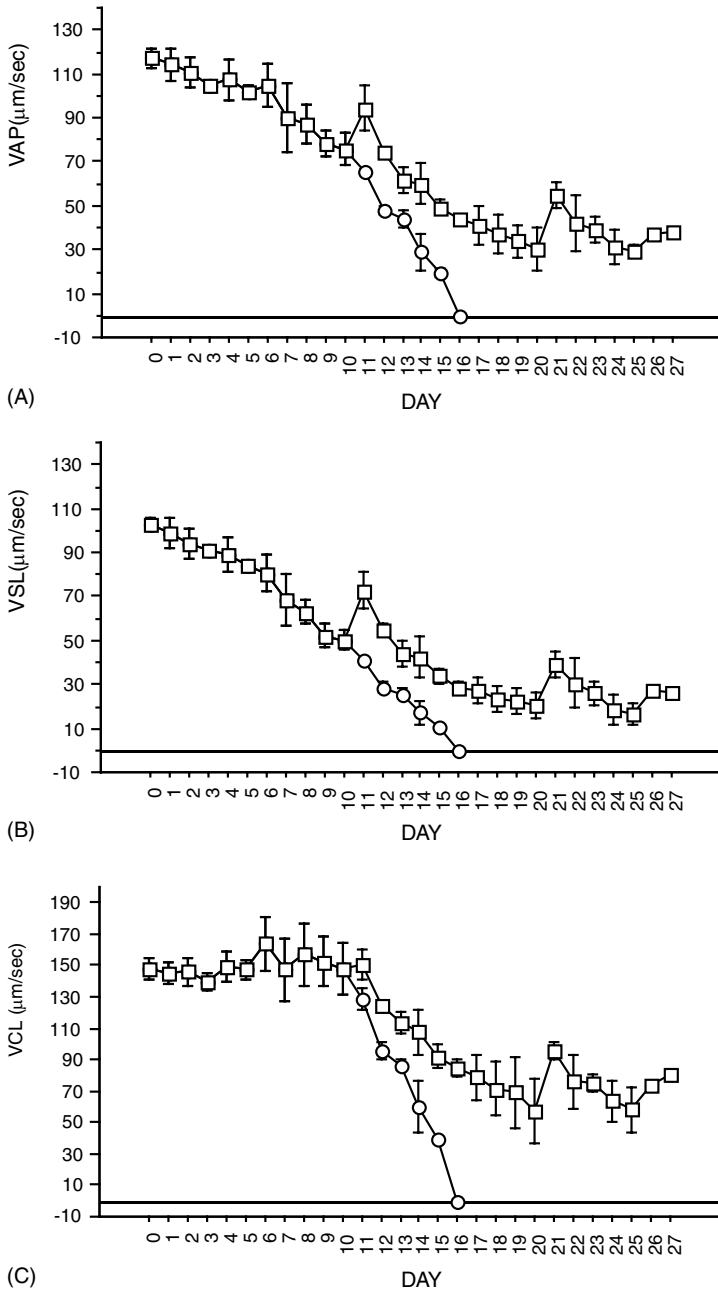


Fig. 3. Plots of VAP (A), VSL (B) and VCL (C) for dog spermatozoa conserved at 4 °C in supplemented ($\square$) and non-supplemented EYTG extender ($\circ$) samples. Extender was changed at Days 11, 21 and 27.

extender. Apparently immotile spermatozoa were re-activated and the motility percentage reached value of $73 \pm 4\%$ (mean $\pm$ S.D.), significantly lower than that observed at Days 0 and 11 ($P < 0.05$), but higher than the one observed the day before ($P < 0.001$). Thereafter, motility decreased, becoming absent around Day 27 of storage. No motility reactivation was observed when a third extender exchange was performed at that time.

A similar evolution was observed for the percentage of spermatozoa with motility that was progressive (Fig. 1B) and rapid (Fig. 2A). The values of these two parameters in the non-supplemented samples became different ($P < 0.01$) when compared to the initial values (Day 0) at Days 5 (mean $\pm$ S.D. 57.6 ± 8 , 76.6 ± 11.5) and 7 (mean $\pm$ S.D. 48 ± 6.9 , 69 ± 15.9), respectively, and equivalent to 0 after 14 d of storage. At Days 11 and 21 of extender exchanges, these two parameters peaked (progressive = 47.3 ± 5.03 and 18.66 ± 6.4 ; rapid = 69.33 ± 9.6 and 29.66 ± 8.38 at Days 11 and 21, respectively) and slowly decreased thereafter. No changes were observed after the third extender exchange.

The evolution of static percentage (Fig. 2B) was inversely proportional to the other semen motility parameters. The proportion of static spermatozoa increased over time and was maximum at the end of the storage period in non-supplemented samples (Day 16) or at Days 21 and 27 in the supplemented ones.

The different semen velocities (Fig. 3) decreased slowly during the storage process, as did the other semen motility parameters, to be completely reduced to zero at the end of each conservation period. When compared to initial data (Day 0), VAP, VSL and VCL started becoming significantly different at Days 5, 3 and 11, respectively, in the non-supplemented samples, respectively. The values of the supplemented samples remained statistically lower than the initial data (Day 0), even after medium exchange. Table 1 summarizes the values of the different semen motility parameters at Day 0, before and after each medium exchange.

3.2. Evolution of glucose concentration, pH and osmolarity

Glucose concentration (Fig. 4) decreased abruptly from 9.66 ± 0.08 g/L (mean $\pm$ S.D.) the first day of the study to 7.47 ± 0.03 g/L the second day ($P < 0.001$). Thereafter, this

Table 1

Mean ($\pm$ S.D.) semen motility parameters at Day 0 and at days before (B) and after (A) extender (EYTG) was exchanged

Parameter	Day 0	Day 11 B	Day 11 A	Day 21 B	Day 21 A	Day 27 B	Day 27 A
Motility (%)	97.3 ± 3	68 ± 7^b	95.6 ± 4.1^a	11.6 ± 7.6^b	$73.3 \pm 4.9^{a,b}$	1.1 ± 0.2^b	1 ± 0.5^b
Progressive (%)	71.6 ± 3.7	10.6 ± 0.5^b	$47.3 \pm 5^{a,b}$	1 ± 1^b	$18.6 \pm 6.4^{a,b}$	0.7 ± 1^b	0.5 ± 1^b
Rapid (%)	84.3 ± 7.5	34 ± 3.6^b	$69.3 \pm 9.6^{a,b}$	3 ± 2.6^b	$29.6 \pm 8.3^{a,b}$	1.5 ± 1^b	1 ± 1^b
Static (%)	1.6 ± 2	27 ± 5.5^b	$2.6 \pm 2^{a,b}$	83 ± 10.5^b	$23 \pm 6.5^{a,b}$	95 ± 3.1^b	97 ± 2^b
VAP ($\mu\text{m/s}$)	117 ± 4.3	65.6 ± 0.7^b	$94 \pm 10.4^{a,b}$	30.4 ± 9.9^b	$45.7 \pm 5.4^{a,b}$	19.3 ± 2.5^b	17.5 ± 3.2^b
VSL ($\mu\text{m/s}$)	102.8 ± 2	41.3 ± 0.7^b	$72.8 \pm 7.9^{a,b}$	20.7 ± 5.8^b	$39.2 \pm 6^{a,b}$	17.2 ± 3.2^b	15.7 ± 2.8^b
VCL ($\mu\text{m/s}$)	147.9 ± 7.2	128.9 ± 7.4^b	150 ± 9.6^a	57 ± 20.8^b	$95.6 \pm 5.1^{a,b}$	21.3 ± 4.1^b	18.3 ± 2.9^b

A, after extender exchange and B, before extender exchange.

^a Different ($P < 0.05$) relative to before EYTG exchange.

^b Different ($P < 0.05$) relative to Day 0.

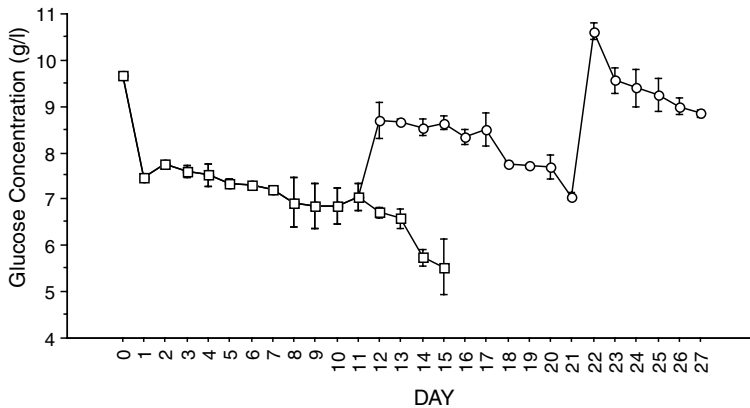


Fig. 4. Glucose concentration of chilled dog semen in the supplemented (○) and non-supplemented (□) EYTG samples. Extender was changed at Days 11, 21 and 27.

concentration decreased slowly to reach a value of 5.5 ± 0.6 g/L ($P < 0.001$) at Day 16 when motility was absent in the non-supplemented samples. In the supplemented samples, this concentration increased immediately after EYTG addition, in parallel to the different semen motility parameters and then slowly decreased to reach a value of 7.06 ± 0.08 g/L at Day 21. The second supplementation at Day 21 induced a similar increase in glucose concentration; it reached 10.6 ± 0.17 g/L, and then decreased to 8.85 ± 0.07 ($P < 0.001$) at Day 27 of storage.

The pH in the EYTG extender during the storage period is shown in Fig. 5. A decrease over time in pH values occurred, parallel to the motility parameters and glucose concentration evolution. When mediums were exchanged, pH returned to values not significantly different from Day 0 values. The pH decrease curves after medium exchanges were similar to that observed for the other parameters studied. Osmolarity remained stable and did not have any significant changes over time (data not shown).

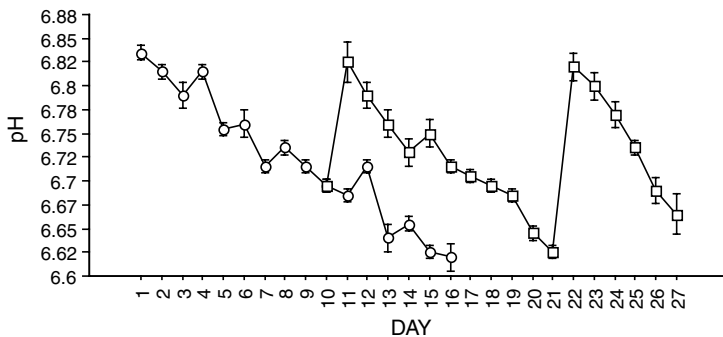


Fig. 5. Plot of pH of chilled stored dog semen in supplemented (□) and non-supplemented (○) EYTG samples. Extender was changed at Days 11, 21 and 27.

3.3. *Effects of long-term chilled semen on fertility preservation*

Among the 20 inseminated bitches, 12 females were pregnant (5 and 7 in Groups 1 and 2, respectively). Litter size in Group 1 was significantly lower than in Group 2 (2.3 ± 1.4 versus 4.6 ± 2.8).

4. Discussion

Results presented in this paper provide interesting information concerning conservation of chilled semen in canine species, including: (1) the possibility of conserving motile spermatozoa for extended periods up to 20 d, (2) the importance of energy substrate adjunction and medium exchanges and (3) the apparent conservation of fertility potential of long-term chilled semen, at least during the first 9 ± 1.8 d of storage. In addition, it was clear that motility was a poor means of assessing sperm viability.

As objective evaluation of semen characteristics is important in selecting extenders and sperm processing techniques, computer-aided semen analysis (CASA) was used in both the previous [9,15] and the present study as an accurate and reliable technique for objective evaluation of the different semen motility parameters.

During the last decade, the use of chilled semen appeared as an interesting alternative to freezing for short- to medium-term semen conservation, particularly where the high costs of materials (e.g., nitrogen containers) and complex custom/shipment procedures can be avoided. However, the main disadvantage of this technique was, up to now, the short-term conservation of motility and apparent fertility parameters [2–7]. Studies to improve chilled semen conservation were therefore warranted. Recent results [9] have demonstrated that semen motility can be extended up to 10–13 d in cooling conditions using EYTG extender.

During this study, evolution of the different semen motility parameters in the non-supplemented samples were similar to that described in our previous reports [9]. In the extender-exchanged samples, the rapid and progressive percentages did not totally recover initial values after medium exchanges, demonstrating that if reactivation is possible, there are probably some spermatozoa that have lost some physical properties and have changed categories. This clearly illustrated the percentage of static spermatozoa; although it decreased after reactivation, it did not regain initial basal values. A 10–20% shift from highly motile spermatozoa to static and probably dead spermatozoa was observed. However, it was particularly noteworthy to observe that the first extender supplementation prolonged the survival of the spermatozoa up to Day 21 (compared to Day 16 in the non-supplemented samples) and that apparently non-motile spermatozoa could be reactivated and motility parameters could be re-stimulated. At Day 21, when motility appeared completely absent, a second supplementation was performed and also re-stimulated motility parameters, even though it was not as pronounced and was of shorter duration. At that time, some spermatozoa were unable to respond.

The mechanisms by which the reactivation and prolongation of motility characteristics are obtained are probably related to glucose supplementation, to fresh egg yolk addition and the removal of degradation products present in the medium. Similar observations have

been made for other different types of cultured cells for which medium exchanges allowed for extension and conservation of viability parameters [24].

The changes in glucose concentration appeared to occur approximately concurrent with different semen motility parameters. The initial rapid drop in glucose concentration was probably due to higher consumption during the first 24 h of storage (due to high gamete activity during this period) before a reduction of both motility and metabolism by low temperature (4 °C). However, glucose was probably not the only parameter involved in sustaining motility. Indeed, at Days 16, 21 and 27, when motility was no longer significantly detectable, glucose concentrations were still 5.5 ± 0.6 , 7.06 ± 0.08 and 8.85 ± 0.07 g/L, respectively. Increase of glucose content in the medium appeared to be associated with the percentage of static spermatozoa. In fact, glucose consumption certainly diminished with the reduction of the percentage of motile spermatozoa and thus with the increase of static ones. Furthermore, glucose content was far from being reduced to zero when apparent spermatozoa motility had already disappeared. Improvement of motility at each supplementation thus cannot only be attributed to glucose addition. The exchanges of extenders certainly allowed for a reduction of the deleterious effects of a decrease in pH during storage (Fig. 5), which had already been demonstrated as involved in the toxic effects of medium on the semen [3,25]. The pH toxic effects were certainly related to metabolite accumulation; these metabolites were discarded by the medium exchanges. The role of egg yolk supplementation in semen reactivation was not determined, but because of the energy substrate that it provides as well as the addition of phospholipids, egg yolk is probably involved in these changes.

The results reported in the present study describe, for the first time, storage of chilled dog semen for extended intervals, with acceptable motility parameters for at least 2 wk (and probably longer). This long duration was related to the use of the EYTG and extender supplementation at Days 11 and 21. These findings are important; they clearly showed that apparent motility or immotility is not a reliable criterion to estimate fertility potential of dog semen. Non-motile gametes could still recover motility and remain fertile if adequate medium is added. This may to some extent explain the apparent contradictions found in the literature concerning the correlation between motility and fertility [26,27]. Low-motility spermatozoa may be reactivated by medium supplementation *in vitro* or *in vivo* when interaction with the vagino-utero-oviductal secretion occurs. Studies in canines have yet to demonstrate whether or not such interaction or effects can exist. However, some interesting observations have already been made in clinical situations where poor quality semen (and sometimes apparently dead semen) was used for insemination and pregnancy ensued. In view of the present data, the conventional and classical association between apparent motility and fertility is certainly questionable and warrants further studies.

During the present work, 20 bitches were inseminated to verify the conservation of the fertility potential of the long-term chilled semen. Pregnancy was obtained with semen that had been stored for 7–11 d. Similar results have not been previously reported; it appears that chilled canine semen remains fertile for at least 11 d. Fertility in Group 1 seemed lower than in Group 2, apparently due to the timing of insemination and numbers of living spermatozoa inseminated. Fertility rate in Group 1 (50%) was acceptable for a single insemination with a low number of spermatozoa (200×10^6) and a reduced volume of semen (2.5 mL) deposited in the vagina. The 70% success rate after two inseminations with

chilled semen seemed slightly inferior than that usually achieved with fresh semen; it was probably related to some reduction of fertility after storage for >7 d, but was still highly acceptable. Presumably, fertility would have been better with intra-uterine insemination and with a larger number of spermatozoa. However, the objective of the study was not to achieve the highest pregnancy rate, but rather to demonstrate that fertility was conserved after semen was stored >7 d.

In conclusion, the present study is the first report of long-term storage of chilled dog semen, as well as the beneficial effects of extender supplementation. *In vivo* studies demonstrated that the long-term chilled semen remained fertile at least 11 d of storage, which confirms present and previous *in vitro* studies [9]. To improve the fertility rate of long-term stored samples, the interest of medium exchange, both before assessing semen quality as well as before AI, warrants further studies.

It would have been interesting to test the viability and fertility of the semen after >11 d of storage. However, this is clinically difficult to achieve in a species characterized by a proestrus of 7–10 d and a fertile period occurring on Days 10–13. Indeed semen was collected and treated during the days of the first signs of proestrus. Finally, other *in vitro* studies are needed, particularly to characterize (microscopically and biochemically) the changes associated with this long-term conservation.

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