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Influence of glucose and fructose in the extender during long-term storage of chilled canine semen

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Abstract

The use of chilled, extended semen in dog breeding is becoming increasingly popular as preparation and transportation is less expensive and regulations are often less complicated than for frozen semen. Sugar is one of the main constituents in semen extenders, and glucose and fructose are metabolized in separate pathways by freshly ejaculated dog sperm. In this study, glucose, fructose or an equal mixture of both were used in an egg-yolk–tris (EYT) extender at two different concentrations (10 and 70 mM). EYT extender without sugar supplementation, providing only the glucose (3–4 mM) originating from the egg-yolk, served as a control. The longevity of the chilled semen at 5 °C was 23 days: the quality of physical and functional characteristics decreasing with time. Glucose and fructose had a strong influence on motility and movement patterns of chilled canine semen. The beneficial effect of 70 mM sugar concentrations compared to 10 mM and the control was pronounced, and maintained sperm motility $\geq$70% for 8 days of storage, compared to for 4 days in the control extender. Fructose maintained higher sperm motility than did glucose and the mixture. VAP values were higher in sugar-supplemented extenders ($P < 0.05$). Neither type nor concentration of the two sugars influenced sperm plasma membrane, acrosome integrity or the acrosome reaction following ionophore challenge (ARIC). Sugar consumption by dog sperm varied between the different periods of storage and with sugar concentrations provided in the extenders. Glucose consumption by dog sperm was greater than fructose consumption when both sugars were present in equal amounts, indicating that dog sperm used glucose in preference to fructose. In conclusion, the major influence of the two sugars on chilled semen was to support motility. EYT extender supplemented with fructose at a

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concentration of 70 mM was found to be the best of the tested extenders for long-term preservation of chilled canine semen.

Keywords: Glucose; Fructose; Sugar concentrations; Chilled semen; Dog; CASA

1. Introduction

Frozen canine semen can be stored practically indefinitely, and the techniques and extenders for dog sperm cryopreservation developed in recent years have resulted in post-thaw motility of up to 70% [1,2] and whelping rates of up to 85% [3–5]. However, pregnancy rates and whelping rates are generally lower with frozen semen than with chilled, extended semen, when the same optimal methods are used for AI [6,7]. It is technically easier to chill than to freeze semen, and the shipment of chilled semen is less expensive and regulations for import and export are often less complicated than for frozen semen. Consequently, the use and international shipment of chilled semen in dog breeding is becoming increasingly more common. One disadvantage with chilled semen is the relatively short survival time, which is thought to necessitate use within a few days after collection [8–11]. Therefore, the transportation of chilled semen to its destination requires careful planning so that it both remains fertile and is available on the most suitable insemination day for the bitch. To preserve viability and fertilizing capacity of sperm cells, semen to be stored or shipped needs to be extended and chilled. Many extenders for chilled dog semen have been evaluated, and egg-yolk–tris (YET) extenders appear superior to the other extenders tested in vitro and in vivo [11–19].

Mammalian spermatozoa require exogenous substrates for a variety of functions, e.g., to preserve intracellular energy reserves, cell components and most notably to support motility [20]. They can obtain energy through mitochondrial oxidative phosphorylation and glycolysis by the consumption of glycolysable sugars, such as glucose, fructose, mannose, and maltose [21]. Fructose is thought to be a major energy source for ejaculated spermatozoa [22], and together with glucose is found in seminal plasma in many mammalian species, but not in dogs; nevertheless, dog sperm can utilize these two sugars. In many species, glucose and fructose have been investigated for the different effects on gametes in terms of metabolizable energy and fertility potential, and the beneficial effects vary between species [23]. The effects of these two sugars on the metabolism of freshly ejaculated spermatozoa has been studied in dogs, and there is evidence that dog sperm metabolize glucose and fructose using separate pathways, resulting in separate systems of energy management as indicated by their different roles in glycogen metabolism [24], motility patterns [25], hexose metabolism [26], and glycogen deposition [27]. Glucose and fructose are two of the most commonly used sugars for canine semen extenders; however, the concentration of these sugars in the extenders for chilled canine semen varies markedly from 5 mM to about 120 mM [8,13,14,28]. In order to achieve the most efficacious use of chilled semen, it is important to study the influence of different sugars and their concentrations on chilled dog sperm.

In the present study, the aims were: firstly, to determine which of the two sugars, or their combination, and which sugar concentration (control: 10 or 70 mM) was most beneficial

for long-term preservation of chilled canine semen. Secondly, to investigate the effects of an EYT extender containing glucose and/or fructose on canine spermatozoa as evaluated by motility, plasma membrane and acrosome integrity, acrosome reaction following ionophore challenge, pH change and sugar consumption during long-term storage at 5 °C.

2. Materials and methods

2.1. Animals

Five privately owned, clinically healthy stud dogs between 2.5 and 8 years old were used in this study: one Appenzell Mountain Dog, one Bavarian Schweisshund, one Briard, and two Golden Retrievers. For the dogs to be included in the study, the percentage of motile and morphologically normal spermatozoa of the fresh semen had to be $\geq$70%.

2.2. Extenders

The extenders were used to dilute the samples of semen to be chilled. Two different concentrations (10 and 70 mM) of sugars in the extenders were tested. Seven EYT extenders were prepared in a single batch and stored frozen to avoid variation among batches (Table 1). An extender containing no sugar supplementation served as a control. Ten egg-yolks from hen's eggs were initially sampled and analyzed for differences in glucose and fructose content. Prior to sugar measurement, each egg-yolk was mixed in distilled water to the same final concentration as in the extenders, i.e., 20% (v:v). To make the extenders, fresh egg-yolks were pooled and mixed to reduce the biological variation found in the eggs. Particles of a size similar to that of sperm heads are a confounding factor with Computer Assisted Sperm Analysis (CASA) and results in over-estimated data of immotile sperm, therefore, all extenders were clarified from particles by centrifugation at 3,310 $\times$ *g* for 20 min at room-temperature. The osmolarity and pH of the extenders were determined (Table 1). A sample from each extender was also submitted for glucose and fructose analysis prior to use.

Table 1
Compositions of chilled semen extenders used in the experiments

Ingredients	I	II	III	IV	V	VI	VII
Tris (g)	3.025	3.025	3.025	3.025	3.025	3.025	3.025
Citric acid (g)	1.7	1.7	1.7	1.7	1.7	1.7	1.7
Na-benzylpenicillin (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Streptomycin sulfate (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Egg-yolk (mL)	20	20	20	20	20	20	20
D-Glucose (g)	–	0.180	1.275	–	–	0.09	0.637
D-Fructose (g)	–	–	–	0.180	1.275	0.09	0.637
Distilled water (mL)	to 100	to 100	to 100	to 100	to 100	to 100	to 100
Osmolarity (mOsm)	261	273	330	275	332	272	337
pH	6.04	6.07	6.05	6.15	6.06	6.07	6.10

2.3. Experimental design

Three different series of experiments with the seven different extenders (Table 1) were performed to evaluate the most beneficial sugar type and concentration for chilled canine semen stored at 5 °C. Experiment Series A included extenders I–III. Experiment Series B included extenders I, IV, and V. Experiment Series C included extenders I, VI, and VII. All series of experiments (A–C) were repeated in triplicate. The semen samples were collected and pooled from three of the dogs on each of the nine occasions.

2.4. Semen collection and processing

The sperm-rich fraction of the ejaculates was collected by digital manipulation into a pre-warmed calibrated polypropylene vial [16]. The semen samples were pooled to allow for a sufficient number of sperm and to reduce an individual variation among the evaluated samples [1,3,29]. The sperm concentration was measured with a calibrated photocolorimeter (SpermaCue, Minitüb, Tiefenbach, Germany). The total number of spermatozoa in the pooled semen ranged between 2×10^9 and 2.8×10^9 cells. The percentage of motility in the pooled semen, assessed by Computer Assisted Sperm Analysis (Strömberg-Mika Cell Motion Analyzer SM-CMA, MTM Medical Technologies, Montreux, Switzerland), was 88.9 ± 4.2 (mean $\pm$ S.D. range: 80.8–95.1). The pooled semen was divided into three equal aliquots and placed in three screw cap closed sterile plastic tubes. The three aliquots were then centrifuged at $700 \times g$ for 8 min and the supernatants were removed, collected, frozen in Liquid N_2 and stored at -80 °C for glucose and fructose analysis. The resultant sperm pellets were re-suspended in the different extenders (Table 1) to give a final sperm concentration of 2×10^8 cells/mL. To avoid cooling too rapidly and to reduce the effects of cold shock to the spermatozoa, the extended semen samples were placed in a room-temperature cooler, which reached a temperature of 5 °C within 45 min. The cooling rate in the cooler was -0.5 °C/min. Subsequently, all samples were stored at 5 °C in a refrigerator throughout the whole period of study, i.e., until sperm motility was down to 0%. Chilled semen samples were evaluated daily during the first 4 days and every second day from Day 6 to Day 14, and then on Day 17, Day 20, and Day 23.

2.5. Semen evaluation

A 300-μl aliquot of each chilled semen sample was taken on each experimental day and was re-warmed to room-temperature for 15 min before evaluation.

2.6. Assessment of sperm motility and movement patterns

Spermatozoal motility was evaluated using CASA and at 37 °C. For each sample, at least 200 sperm cells from four randomly selected fields were evaluated. The following parameters were measured: the percentage of total motility (TM%); the percentage of linear motile spermatozoa (progressive motility; PM%); average path velocity (VAP, μm/s); straight line velocity (VSL, μm/s); and curvilinear velocity (VCL, μm/s). PM% was calculated from among the motile spermatozoa and the velocities were calculated from

spermatozoa showing linear motility. The settings for the motion analyzer were as follows: frames per second, 32; minimum number of frames, 16; acquisition time, 20 ms (50 Hz); minimum and maximum area of objects, 35 and 350 pixels; velocity class width, 5 μm/s; velocity limit for immotile objects, 10 μm/s; velocity limit for local motile objects, 25 μm/s; maximum radius for circles, 30 μm; minimum and maximum area for the immotile objects, 20 and 100 pixels; and depth of measuring chamber, 10 μm.

2.7. Assessment of plasma membrane integrity

Sperm plasma membrane was assessed with a dual fluorescent staining technique described by Garner et al. [30] and Harrison and Vickers [31], and modified by Rota et al. [17], using 6-carboxyfluorescein diacetate (C-FDA; Calbiochem, LabKemi, Stockholm, Sweden) and propidium iodide (PI; Sigma Chemical Company Ltd, St. Louis, MO, USA). A staining medium for the fluorescent probes was prepared within 1 h before use. For each sample stained with C-FDA/PI, a total of 200 spermatozoa were evaluated and classified into two categories (intact or damaged plasma membrane) [17]. In the results, data on damaged plasma membrane were presented as percentage.

2.8. Assessment of acrosomal integrity

After dual fluorescent staining with fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA, Sigma) and propidium iodide, acrosomal integrity was characterized with a protocol modified by Axnér et al. [32] from the procedure for FITC-PNA staining of cat spermatozoa [33], and the procedure described by Cheng et al. [34], for FITC-PNA staining of stallion spermatozoa. Two hundred spermatozoa were assessed in each smear and categorized according to their FITC-PNA patterns [34]. Spermatozoa with reacted acrosome or acrosome loss were presented as percentage.

2.9. Induction of the acrosome reaction by calcium ionophore challenge

Calcium ionophore-induced acrosome reaction was observed as described by Hewitt and England [35] and Brewis et al. [36], slightly modified. The stock solution of calcium ionophore A23187 (C-7522, Sigma) was prepared in dimethyl sulfoxide (DMSO) (Sigma) at 5 mM concentration. This was divided into 10 μL aliquots and placed into 0.5 mL microcentrifuge tubes and further diluted in 90 μL phosphate-buffered saline (PBS) and stored frozen prior to use. A control solution was prepared with 10 μL of DMSO diluted with 90 μL of PBS, placed in a 0.5-mL microcentrifuge tube and stored frozen. The chilled semen sample was adjusted to a final sperm concentration of 20×10^6 cells/mL into canine capacitation medium (CCM) [37] and incubated for 2 h at 37 °C. Following incubation an aliquot (10 μL) of the sample was observed under a light microscope to confirm that the spermatozoa displayed hyperactivated motility and therefore, were capacitated. A 20-μL aliquot of capacitated spermatozoa was further incubated with a 20-μL ionophore challenge solution (10 μM A23187) or a 20-μL control solution for 1 h at 37 °C. After incubation, the cultures were evaluated for the presence of acrosome-reacted spermatozoa and stained with FITC-PNA/PI as described above. The percentage of spontaneous

reaction rates (control) and of A23187 induced reaction rates were then determined as described by Cummins et al. [38]. The difference between the two values was considered the percentage of sperm in the population that was capable of responding to ionophore challenge, which was termed the acrosome reaction following ionophore challenge (ARIC). In cases with a low response, the observed incidence in ionophore-stimulated suspensions was less than observed in the control. In these cases, ARIC was negative and, was therefore, considered essentially zero.

2.10. pH measurement

The pH values of the extenders before use (Table 1) and of the chilled semen samples on Day 23 were measured by a pH meter (PerpHect® METER, model 330, Orion Research, Inc., MA, USA).

2.11. Glucose and fructose measurement

A preliminary study indicated that the concentration of spermatozoa in the incubation media affected the amount of sugar utilization, therefore, a standardized sperm concentration of 200×10^6 cells/mL was used in this study. On semen assessment, the changes in glucose and fructose concentrations in the extenders during cool storage were measured fluorometrically (Fluorometer FL 600®, Bio-Tek Instruments, Inc., VT, USA), with a modified method by Lowry and Passonneau [39]. The same method was used in the preliminary studies on sugar content in egg-yolk. All enzyme components used in this study (glucose-6-phosphate dehydrogenase, hexokinase, and phosphoglucose-isomerase) were purchased from Roche Diagnostics Corporation (IN, USA). The sugar concentrations were expressed as mM. Both sugars were measured by sampling 50 μL of the supernatant from each sample, placing it into 1 mL microcentrifuge tubes that were lowered into liquid N_2 for 30 s and stored at $-80\ ^\circ C$ prior to sugar measurements. Sugar consumption by spermatozoa over time was calculated and compared.

2.12. Statistical analyses

Data were statistically analyzed using the SAS (Ver. 8e, SAS Institute Inc., Cary, NC, USA). Analysis of variance (PROC MIXED) was applied to the data. Except for pH, the statistical model for all parameters included: the fixed effects of sugar types (glucose, fructose, and a mixture of both); sugar concentrations (control: 10 and 70 mM); and time (Period I: Days $1 + 2 + 3$; Period II: Days $4 + 6 + 8$; Period III: Days $10 + 12 + 14$; and Period IV: Days $17 + 20 + 23$). Days were nested within periods and the interaction between sugar types, sugar concentrations and periods. The random effect of replications, nested within sugars, was also included in the model. For the analysis of pH values (on Day 23), the statistical model included the fixed effects of sugars, added concentrations and their interaction, as well as the random effect of replications, nested within sugars. In the results, mean ± standard deviations were presented, however, pairwise *t*-tests were performed to compare least-squares means. Changes in consecutive periods were compared to Period I. A value of $P < 0.05$ was considered statistically significant.

Table 2
Glucose and fructose concentrations (mM) in the semen extenders

Sugar concentrations	Semen extenders						
	I	II	III	IV	V	VI	VII
Glucose							
Prepared concentration	0	10	70	0	0	5	35
Measured concentration	4.7	14.0	74.0	3.9	4.9	8.2	38.2
Fructose							
Prepared concentration	0	0	0	10	70	5	35
Measured concentration	0.6	0.2	0.2	8.7	68.9	4.3	27.9

Extender I containing no additional sugars served as a control. Extenders II and III contained 10 and 70 mM glucose, respectively. Extenders IV and V contained 10 and 70 mM fructose, respectively. Extenders VI and VII contained equal combinations of glucose and fructose at final concentrations of 10 and 70 mM, respectively.

3. Results

3.1. Glucose and fructose contents in canine seminal plasma and in hen's egg-yolk

Glucose and fructose concentrations in pooled seminal plasma could not be detected. The mean glucose concentration (mM) in the 20% egg-yolk/distilled water mixture was 3.5 ± 0.5 (range: 2.8–4.0) and for fructose 0.4 ± 0.2 (range: 0–0.8).

3.2. Glucose and fructose contents in chilled semen extenders

Prior to use, all laboratory-prepared semen extenders were subjected to glucose and fructose analyses (Table 2).

3.3. Sperm motility and movement patterns

The mean TM% in freshly pooled semen was 88.9 ± 4.2 and PM% was 69.6 ± 12.6 ($n = 9$). TM% decreased with storage time in all treatments (Table 3, Fig. 1). There were no differences in TM% between sugars at the same concentration and in the same period. When pooled across time, however, type and concentration of sugar influenced TM% giving 55.9 ± 28.7 with fructose, 53.8 ± 28.9 with the mixture and 53.7 ± 29.1 with glucose. The addition of fructose to EYT extenders provided significantly better TM% than the other two extenders ($P < 0.05$). The overall percentages of TM were 58.9 ± 29.0 with 70 mM, 54.9 ± 28.9 with 10 mM and 49.1 ± 28.1 for the control ($P < 0.001$). Higher amounts of sugar showed a tendency of better TM% and this was more pronounced when the comparisons of TM% between the control and 70 mM sugars were made (Table 3). Storage time had a detrimental effect also on PM%. The decrease in PM% was found from Period II onwards ($P < 0.05$) (Table 4). The beneficial effect of higher sugar concentrations in maintaining higher PM% was consistent, and significant differences were found between 70 mM sugars and the control in Period I. The type of sugar tested had no effect on PM%.

Table 3
Mean percentages (±S.D.) over time of total motile spermatozoa in chilled canine semen in extenders containing glucose, fructose or a mixture of both at various concentrations

Periods	Sugar types and concentrations (mM)								
	Glucose			Fructose			The mixture		
	Control	10	70	Control	10	70	Control	10	70
I	78.5 ± 5.4^{a}	81.6 ± 3.6^{ab}	84.3 ± 2.6^{b}	78.4 ± 6.8^{a}	83.4 ± 1.8^{b}	85.0 ± 2.2^{bc}	77.1 ± 3.8^{a}	81.9 ± 3.4^{ab}	82.7 ± 3.6^{b}
II	64.6 ± 9.2^{a}	71.6 ± 4.6^{b}	78.3 ± 3.6^{c}	68.3 ± 9.4^{a}	77.0 ± 6.5^{b}	79.9 ± 4.5^{b}	67.9 ± 9.3^{a}	72.0 ± 8.1^{a}	77.1 ± 8.4^{b}
III	39.6 ± 13.0^{a}	49.8 ± 6.0^{b}	59.1 ± 7.2^{c}	44.0 ± 5.8^{a}	55.8 ± 8.2^{b}	60.9 ± 9.2^{c}	44.2 ± 13.7^{a}	53.3 ± 9.2^{b}	57.6 ± 11.2^{b}
IV	7.4 ± 7.8^{a}	9.0 ± 10.2^{a}	14.4 ± 14.2^{b}	10.5 ± 9.1^{a}	13.6 ± 11.0^{a}	13.9 ± 12.8^{a}	8.8 ± 10.0^{a}	9.6 ± 10.2^{a}	13.3 ± 14.8^{a}

Period I included Days 1, 2, and 3. Period II included Days 4, 6, and 8. Period III included Days 10, 12, and 14. Period IV included Days 17, 20, and 23. Significant differences were found between observation periods in all treatments ($P < 0.05$). Different superscripts (a–d) in the same row indicate differences between sugar concentrations of each sugar ($P < 0.05$).

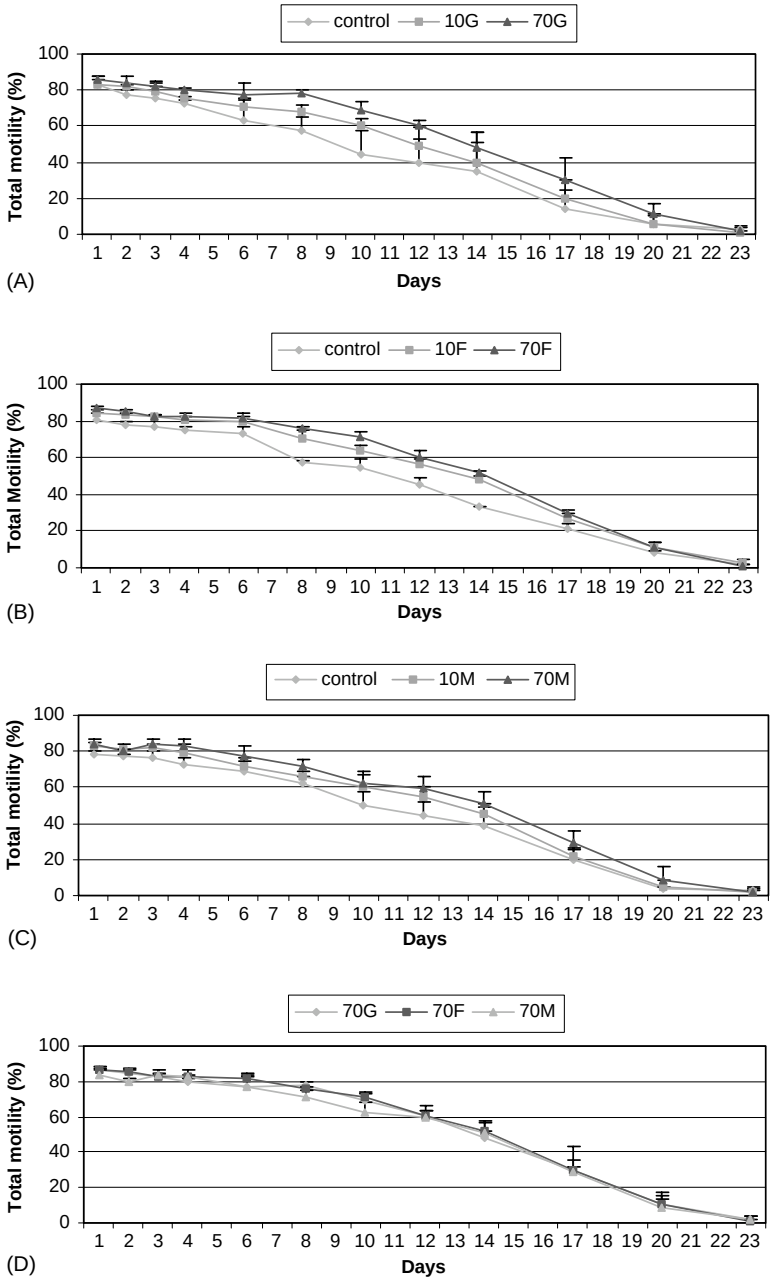

Fig. 1. Mean (±S.D.) values of total motility by observation day of chilled canine semen in extenders containing various concentrations (control: 10 and 70 mM) of glucose (A), fructose (B), and the mixture (C). Results of the best motility rate in each sugar were also compared (D).

Table 4
Percentage change of progressive motility and movement patterns (VAP, VSL, and VCL) of chilled canine semen compared with the initial values of freshly pooled semen

Sugars (mM)	Periods	PM%	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)
Glucose					
Control	I	65.9[a]	102.2[a]	101.5[a]	131.0[a]
	II	36.3[b]	91.3[b]	85[ac]	140.9[a]
	III	23.7[c]	75.9[c]	70.4[bce]	132.7[a]
	IV	17.2[c]	61.3[d]	55.6[de]	107.6[b]
10	I	71.8[a]	106.0[a]	106.0[a]	127.3[a]
	II	38.9[b]	99.5[a]	95.2[ac]	145.3[bc]
	III	20.1[c]	85.3[b]	79.3[bce]	147.0[c]
	IV	13.5[c]	65.9[c]	58.1[de]	116.3[a]
70	I	89.9[a]	122.5[a]	124.8[a]	135.0[a]
	II	58.7[b]	113.6[b]	111.1[a]	146.0[a]
	III	23.4[c]	92.4[c]	85.3[b]	142.1[a]
	IV	13.9[c]	69.6[d]	62.9[c]	119.8[b]
Fructose					
Control	I	84.9[a]	110.2[a]	112.9[a]	130.3[a]
	II	52.1[b]	96.2[b]	94.7[ac]	136.9[a]
	III	33.9[c]	77.6[c]	72.8[bc]	125.8[a]
	IV	16.8[d]	54.0[d]	44.4[d]	98.6[b]
10	I	96.1[a]	124.8[a]	128.9[a]	139.0[a]
	II	56.1[b]	108.0[b]	106.8[ac]	148.1[ac]
	III	36.7[c]	86.4[c]	81.4[bc]	130.0[a]
	IV	16.5[d]	56.4[d]	47.9[d]	91.0[b]
70	I	101.4[a]	127.6[a]	132.0[a]	136.9[a]
	II	56.9[b]	113.6[b]	112.1[a]	148.6[b]
	III	34.3[c]	86.3[c]	80.3[b]	129.8[a]
	IV	26.1[c]	60.0[d]	51.8[c]	93.9[c]
The mixture					
Control	I	66.9[a]	108.4[a]	107.9[a]	133.1[a]
	II	60.3[a]	102.3[a]	102.0[a]	139.4[a]
	III	24.1[b]	80.1[b]	73.1[bd]	130.4[a]
	IV	18.5[b]	55.7[c]	54.7[cd]	94.8[b]
10	I	85.9[a]	124.7[a]	126.7[a]	143.0[a]
	II	61.6[b]	116.5[b]	117.3[a]	151.3[ac]
	III	20.2[c]	86.6[c]	79.8[b]	139.7[a]
	IV	13.8[c]	54.9[d]	47.8[c]	94.0[b]
70	I	87.7[a]	128.7[a]	130.6[a]	143.0[a]
	II	66.8[b]	121.9[a]	121.3[a]	148.9[a]
	III	21.9[c]	91.6[b]	84.5[b]	139.6[a]
	IV	13.2[c]	61.3[c]	54.3[c]	104.6[b]

Period I included Days 1, 2, and 3. Period II included Days 4, 6, and 8. Period III included Days 10, 12, and 14. Period IV included Days 17, 20, and 23. Data that do not have a common superscript in the same sugar and concentration indicate differences between observation periods ($P < 0.05$).

In the freshly pooled semen, the movement characteristics of VAP, VSL, and VCL were 107.3 ± 15.0 μm/s, 96.0 ± 14.6 μm/s, and 139.5 ± 16.7 μm/s, respectively. After extension and chilling, these values significantly increased in Period I, compared to initial values. Thereafter, in all treatments the values of VAP and VSL significantly decreased with storage time (Table 4). Supplementation with sugars (10 or 70 mM) produced better VAP results ($P < 0.05$), the biggest difference being seen between the control and 70 mM concentrations. Sugar concentrations had no effects on VSL and VCL values ($P > 0.05$), however, it was observed that increasing sugar concentrations induced higher VSL over time. The mean values of VCL increased in Period II (Table 4). At concentrations of 10 and 70 mM, the presence of glucose provided higher values of VCL than fructose in Period III and Period IV ($P < 0.05$) did.

3.4. Plasma membrane integrity

Extension, chilling and storage over time induced a reduction in plasma membrane integrity ($P < 0.05$). The mean percentage of spermatozoa with damaged plasma membrane in the freshly pooled semen was 19.2 ± 3.0, and after chilling the percentages increased over the periods ($P < 0.05$). When data was pooled across sugar and concentration, the respective mean percentages of spermatozoa with damaged plasma membrane were: 32.6 ± 5.7 (Period I), 40.7±4.1 (Period II), 54.2± 6.7 (Period III), and 76.1 ± 5.7 (Period IV). Neither type nor concentration of sugar had a protective effect on plasma membrane integrity.

3.5. Acrosomal integrity

Mean percentage of acrosomal loss in pooled ejaculates was 4.2 ± 1.0. When data was pooled across sugar and concentration, the respective mean percentages of acrosomal loss were 5.8 ± 2.6 (Period I), 9.6 ± 4.1 (Period II), 14.0 ± 5.5 (Period III), and 34.7 ± 15.0 (Period IV). There was no significant increase in percentages of acrosomal loss from that in freshly pooled semen compared to Period I. In most extenders, an increase in percentages of spermatozoa with acrosomal loss was found in Period III ($P < 0.05$). No significant differences in the percentages of acrosomal loss were found between sugar types or concentrations throughout the study.

3.6. Induction of acrosomal reaction by calcium ionophore challenge

The acrosome reaction following ionophore challenge (ARIC) in fresh pooled semen was 27.8 ± 15.0. After extension and chilling, there was a significant decrease in mean ARIC values in Period I. Neither type nor concentration of sugars significantly affected the ARIC value (Fig. 2). However, the beneficial effect of glucose on this value was most pronounced at 70 mM sugar concentration.

3.7. Change in pH

Pooled seminal plasma had a mean pH value of 6.4 ± 0.2 ($n = 9$): the pH values of the seven different extenders are shown in Table 1. Mean pH values recorded on Day 23 were

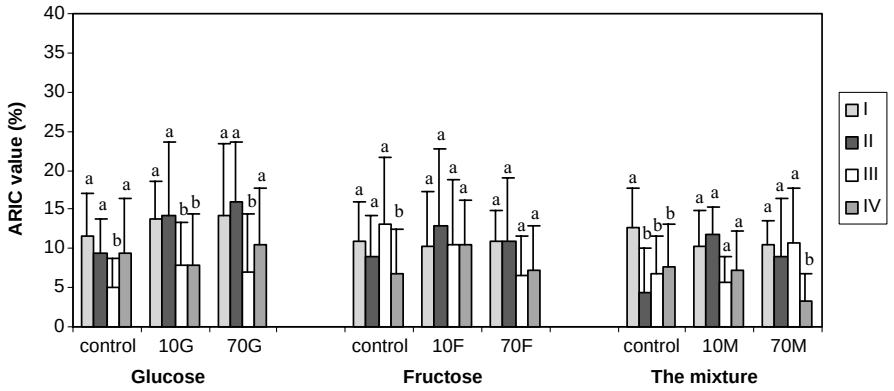

Fig. 2. The effect over time on the mean percentages (±S.D.) of the acrosome reaction following ionophore challenge (ARIC) of glucose (G), fructose (F), or the mixture (M) extenders at concentrations of 10 and 70 mM, compared to control extenders; (I–IV) represent the periods. Period I included Days 1, 2, and 3. Period II included Days 4, 6, and 8. Period III included Days 10, 12, and 14. Period IV included Days 17, 20, and 23. Different letters (a, b) indicate significant differences compared to Period I ($P < 0.05$).

altered from the mean pH value in the extenders prior to use (Fig. 3). The mean pH in 70 mM sugar extenders was significantly lower than at 10 mM sugar. The mean pH value was lower in the presence of glucose than in the presence of fructose ($P < 0.05$).

3.8. Consumption of glucose and fructose by canine spermatozoa

During the 23 days, mean sugar consumption (mM) in glucose containing extenders was 1.5 ± 2.0 and in fructose containing extenders 1.3 ± 2.9 ($P > 0.05$) (Fig. 4). In the extenders containing equal amounts of both sugars, mean consumption of glucose was 1.0 ± 1.3 and of fructose 0.4 ± 0.7 ($P = 0.005$) (Fig. 5). Sugar consumption was greater in Period I than in the following periods. Glucose and fructose consumption by chilled dog sperm increased with the increasing sugar concentrations provided in the extenders,

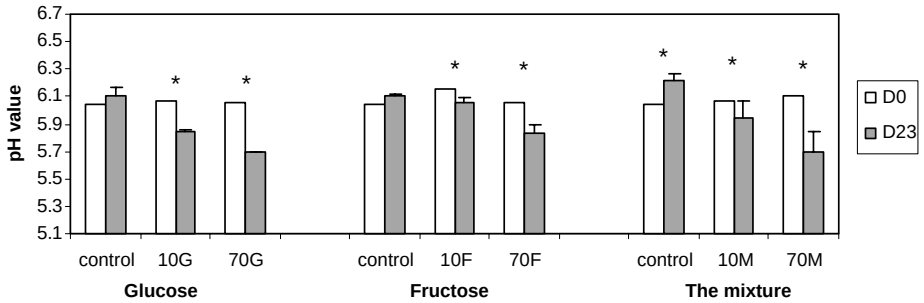

Fig. 3. Changes in pH value (mean ± S.D.) of extenders containing three different sugars and two different concentrations (10 and 70 mM) before use (D0) and of chilled canine semen stored at 5 °C in corresponding extenders on Day 23 (D23). The asterisks indicate significant differences ($P < 0.05$).

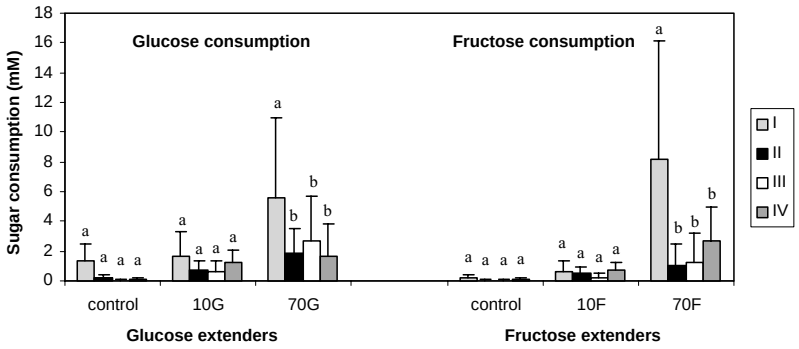

Fig. 4. Glucose or fructose consumption (mean ± S.D.) over time in the extenders containing glucose (G) or fructose (F) at concentrations of 10 and 70 mM; (I–IV) represent the periods. Period I included Days 1, 2, and 3. Period II included Days 4, 6, and 8. Period III included Days 10, 12, and 14. Period IV included Days 17, 20, and 23. Different letters (a, b) indicate a significant difference compared to Period I ($P < 0.05$).

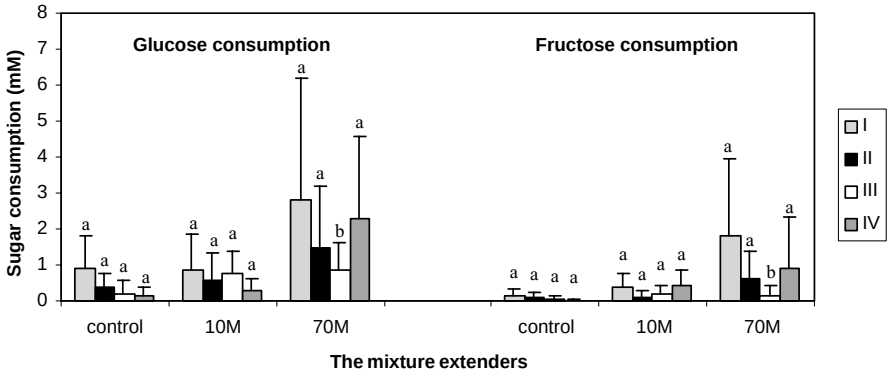

Fig. 5. Glucose or fructose consumption (mean ± S.D.) over time in the mixture extenders (M) containing equal amounts of glucose and fructose to give final concentrations of 10 and 70 mM; (I–IV) represent the periods. Period I included Days 1, 2, and 3. Period II included Days 4, 6, and 8. Period III included Days 10, 12, and 14. Period IV included Days 17, 20, and 23. Different letters (a, b) indicate a significant difference compared to Period I ($P < 0.05$).

although the difference between the control and the 10 mM concentration was not significant ($P > 0.05$).

4. Discussion

Two different concentrations (10 and 70 mM) of sugars were tested and compared to the control, because it has been claimed that low concentrations (1–10 mM) have more intense and stronger positive effects on motility patterns and hexose metabolism than higher concentrations (50 mM) do [25,26]. The hexose metabolism is intimately correlated with intracellular energy reserves in freshly ejaculated dog sperm. In contrast, for semen

preservation, the relatively high concentrations (for example, 70 mM) of sugars commonly used in EYT extenders have given satisfactory results when evaluated by sperm motility, plasma membrane and acrosome integrity, survival time [13,17,40] and whelping rate after AI [16].

The results from the present study clearly demonstrated that the major effect of glucose and fructose in semen extenders on chilled canine semen was to support sperm motility and movement patterns. Motility is an important indicator of sugar utilization by spermatozoa as the sugars provide the external energy source essential for maintaining motility. In this study, TM% (81.4%) and PM% (58.0%) remained high during the first 3 days and subsequently, as could be expected, decreased with storage time. The decrease in the percentage of total motile spermatozoa in Period I (81.4%) compared to in freshly pooled semen (88.9%) may be due to that there are subpopulations of dog spermatozoa with different sensitivities to changes in the environment, particularly reduced temperature. Decreased sperm motility after chilling and re-warming has been postulated to be due to temperature sensitivity of the ATPase-linked sodium–potassium pump and subsequently a leakage of ions [41].

Preservation with increasing amounts of sugars resulted in better maintenance of sperm motility and movement patterns. The addition of 70 mM sugars to EYT extenders produced notable beneficial effects on chilled semen. Sperm motility, considered an important criterion in canine semen quality evaluation, should exceed 70% in a normal sample [10]. Overall TM% was significantly higher when 70 mM was added to the extenders and TM of >70% was maintained until Day 8 of storage. This is in agreement with a previous study [40] using a similar sugar concentration. Although our results still showed a high motility rate after 8 days of storage, fluctuations in container temperature during semen transportation might affect semen quality [42]; thus, the results may differ in vitro and in vivo. A 70-mM extenders provided significantly higher PM% over time, and significantly higher VAP. The mean values of VAP, VSL, and VCL increased significantly in Period I after extension and preservation, compared to the freshly pooled semen, thus suggesting that sugars in the extenders activate sperm velocity.

Fructose appeared preferable to glucose or the mixture by inducing significantly higher percentages of TM% compared to glucose and the mixture, and, from Day 1 onwards, addition of 10 mM fructose to the extender produced a better effect on TM% than the addition of 10 mM glucose. Fructose provided higher sperm motility over time compared with glucose and the mixture at all concentrations. It has been stated [25] that neither glucose nor fructose modify dog sperm motility in raw ejaculates observed for 60 min at 37 °C, and that the most positive effect is observed at the relatively low concentration of 10 mM. The difference in results compared to our study could be due to differences in observation time (60 min versus 23 days) and in sugar metabolism of fresh spermatozoa compared to spermatozoa preserved at 5 °C. Lowering of the temperature is a widely accepted means of slowing down both cell metabolism and chemical reactions, thus prolonging cell life span [21].

As motility is an indicator of the viability of spermatozoa, the results in this study demonstrated that dog sperm stored in a suitable semen extender, and in cool conditions, can survive for approximately 3 weeks. Dog sperm preserved in autologuous seminal plasma at 4 °C survive for only 2 days [17]. In most studies on chilled canine semen

[11,14,15,17,19], motility is observed for up to 4 days, and the motility rate on Day 4 of storage varies between 4 and 80%. There are few observations over a longer period. Chilled canine semen stored in an EYT extender at 4–5 °C displayed zero motility on Day 10 [43], Day 17 [40] and, in the present study, Day 23. The variation in motility rate and storage life of chilled canine semen could be due to several factors. These include: differences in motility in the fresh ejaculates prior to processing (ranging between 75 and 90% in the studies cited above); differences in composition of semen extenders; differences in preservation procedures, such as the cooling rate, prostatic fluid removal and sperm concentration; and differences in methods of estimating the percentage of motility (subjective or objective).

Once capacitated, spermatozoa express an altered motility pattern called hyperactivation [44]. Capacitation-like changes are found in chilled canine semen as determined by the chlortetracycline assay together with alterations of motility characteristics (mainly VCL and lateral head displacement; LHD) [28]. In dogs, VCL and LHD reflect hyperactivated movement, usually occurring during capacitation, and the chilling procedure initiates and accelerates these changes [28]. Similarly, the VCL values in this study increased with storage time from Day 1 onwards, compared to the initial value. From Day 10 onward, addition of glucose maintained higher VCL values than fructose did. Although the interaction among capacitation, storage time and sugars was not evaluated, a relation between the higher mean values of VCL, a longer storage time and the presence of glucose was found. If these results indicate that dog sperm undergo capacitation when they are stored and exposed to cool conditions, thus reducing longevity, inseminations using chilled semen should be undertaken in the most fertile period of the bitch, i.e., 2–5 days after ovulation, and the semen should be deposited in the uterus to facilitate sperm migration. This theory is supported by a recent finding that intrauterine, as compared to intravaginal, insemination with chilled canine semen increases whelping rate and litter size [45].

Egg-yolk has been reported to contain some glucose [46], and in 20% egg-yolk solution in distilled water, the glucose concentration was found to be 3–4 mM. The addition of egg-yolk to the extenders was considered necessary, even though this increased the glucose content, because egg-yolk is an essential ingredient in semen extenders. The mechanisms by which egg-yolk provides a protective action to spermatozoa have been extensively studied [47–51]. Interestingly, dog sperm, which were preserved in a control extender providing only the glucose originating from egg-yolk, were able to maintain $\geq$70% motility during the first 4 days of observation and to survive for up to more than 21 days of storage, thus demonstrating that the small amount of glucose contained in the egg-yolk component of the EYT extender may be sufficient to preserve chilled dog sperm over a considerable period of time. Supplementation with glucose or fructose, however, resulted in better motility rates over time, thus, if semen is to be preserved for a longer period, sugars should be added to the EYT extenders.

Dog sperm are sensitive to osmotic stress [52], however, spermatozoa can tolerate a moderate range of osmolarities without a reduction in fertility [53]. In this study, the osmolarities of semen extenders were within the range of physiological values for canine seminal plasma (315.9 ± 21.8 mOsm) [17,54] and below that proven to suppress dog sperm motility [52,55]. Therefore, the difference in osmolarities among the extenders is not expected to have any detrimental effect on the chilled semen.

Damages to the plasma membrane occurred earlier and were more pronounced than damages to the acrosome. This indicated that the sperm membrane was more sensitive to the cool storage conditions than the acrosome was. Our findings were similar to those of previous studies [56,57] that used scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Although stimulation of the AR by the ionophore A23187 is a non-physiological process, it has been widely used in many species to diagnose potential fertility [33,38,58]. In dogs, this test has been established as a simple method of predicting cryopreservability of semen from individual donors [59]. In the present study, this value appeared to decrease with time. This suggested that the ability of dog sperm to elicit AR following a longer storage time was reduced.

On Day 23 of the experiment, the mean pH in the sugar-supplemented extenders had decreased, compared to the initial values. The pH values in 70 mM extenders were also lower than in 10 mM extenders. This indicated that addition of sugars in extenders prolonged sperm life and metabolism. Incubation of freshly ejaculated dog sperm at 37 °C with fructose, which is intimately associated with sperm motility, induces greater amounts of CO_2, an acidic end-product, than glucose does [26]. In the present study, sperm preservation with glucose at 5 °C resulted in a more pronounced decline of pH than with fructose. This implied that when the motility was depressed by cold storage, dog sperm utilized more glucose for other cellular activities than fructose.

Clearly, sugar consumption by dog sperm depended on type and concentration of sugars available in their environment. The consumption in Period I was greater than that in the following periods ($P < 0.05$), indicating that the slowing down of the sperm metabolic rate in the early stage of preservation was not as efficient as during the later periods, and could also reflect a decrease in the percentage of living cells. Sugar concentration affected the rate of consumption. This could depend on the saturation kinetic of monosacharides, which is a characteristic feature of a carrier-mediated transport system [60]. The preference of dog sperm for the consumption of glucose rather than fructose was demonstrated when both sugars were present in equal amounts and the utilization of glucose was greater than that of fructose. This is in agreement with previous studies on other species [22,61,62–64]. The higher capacity of the glucose transport system possibly explains the preference for glucose utilization. Independent glucose and fructose transport systems, and locations in the dog spermatozoon, have been reported [26]. It is still unclear why extenders containing relatively large amounts of sugar preserved dog sperm better. In contrast, we demonstrated that relatively small amounts of sugars were utilized by dog sperm throughout the study period. The greater amounts of sugars in extenders for chilled semen may play a particular and different role in cold stored dog sperm, compared to in freshly ejaculated spermatozoa. The mechanisms of dog sperm for managing intracellular energy when preserved in cool conditions warrant further investigation.

Based on the results in this study, it is possible to use chilled dog sperm, stored at 5 °C for at least up to 8 days, when spermatozoa are extended in an EYT extender containing 70 mM glucose or fructose. However, further studies are needed to test the fertility of long-term chilled dog sperm in vivo. One study on chilled canine semen, preserved in an EYT extender and stored at 4 °C for 4 days, demonstrated that the mean number of spermatozoa that bound to homologous zona pellucida in vitro was reduced from 4.8 on Day 1 to 0.9 on

Day 4 [19]. Although this difference was non-significant, the data indicates that with increasing storage time the binding capacity to zona pellucida of chilled, stored dog sperm is reduced.

Clinically, it would be useful if chilled semen could be preserved while maintaining its fertilizing capacity for longer than a few days so that it could be stored awaiting the optimal insemination time of the bitch, which is important considering the extended fertile period (2–4 days) in this species, and to allow for sufficient time for semen transportation. Furthermore, repeated inseminations in dogs have been reported to result in higher pregnancy rates than a single insemination [4,65,66]. Therefore, it would be of advantage if the number of spermatozoa in chilled semen from one ejaculation could be divided into, at least, two insemination doses, to be kept stored at 5 °C for repeated inseminations in the same cycle of the bitch.

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