The effect of homologous prostatic fluid on motility and morphology of dog epididymal spermatozoa extended and frozen in Biladyl with Equex STM paste or Andromed

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Abstract

Although dog prostatic fluid decreases the longevity of ejaculated dog spermatozoa, it also increases their rate of motility and their fertility after vaginal insemination, as well as the fertility of epididymal spermatozoa after uterine insemination. These findings indicate a need to further characterize the effects of prostatic fluid on dog spermatozoa. This study was done to determine the effects ($P < 0.05$) of homologous prostatic fluid added prior to cooling, after thawing, or at both times to epididymal spermatozoa from 21 dogs. The effects of two extenders were also determined. The one extender was Biladyl* with Equex STM paste** (BilEq) and the other Andromed* (Minitüb, Tiefenbach, Germany (*); Nova Chemical Sales, Scituate, MA, USA (**)). The response variables were percentage progressively motile spermatozoa (Prog) and morphology after thawing. Prog was

measured at various times until 8 h after extension (unfrozen spermatozoa) or until 2 h after thawing.

Prog after thawing was higher with BilEq than Andromed, when no prostatic fluid was added prior to cooling, and when prostatic fluid was added after thawing. BilEq resulted in a higher mean percentage of spermatozoa with bent principle pieces than Andromed and the addition of prostatic fluid prior to cooling resulted in lower mean percentages of cytoplasmic droplets and bent principle pieces than when none was added. The optimal combination was BilEq with prostatic fluid added prior to cooling (in order to inhibit the development of bent principle pieces) and after thawing (to achieve higher motility until 1 h after thawing).

This study shows that BilEq is more suitable for the freezing of epididymal spermatozoa than Andromed and that prostatic fluid improves the freezability and post-thaw longevity of epididymal spermatozoa frozen in BilEq.

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1. Introduction

Since the work of Anderson in 1975 [1], it was accepted that frozen-thawed dog sperm should be inseminated into the uterus in order to obtain good fertility. Uterine insemination poses practical problems because it either requires one to pass a pipette through the cervix, which is sometimes impossible to achieve [2] and [3], or expensive fiber-optic equipment with which to catheterize the cervix under visual control [4], or surgical or laparoscopic exposure of the uterus [5] and [6]. Vaginal insemination, in contrast, is easy, requires simple, cheap, readily available equipment, and may be repeated over a number of days if necessary to ensure insemination at the time when the oocytes are fertilizable [7]. As early as 1993, Nöthling and Volkmann [7] showed that ejaculated dog sperm frozen in Triladyl® (Minitüb GmbH, Tiefenbach, Germany) with the surfactant Equex STM paste (Nova Chemical Sales, Scituate, MA, USA) and to which homologous prostatic fluid was added after thawing resulted in better fertility of vaginally-inseminated bitches than bitches inseminated with similar semen but to which no fluid was added after thawing. In support of the findings of Nöthling and Volkmann with respect to the group that received no prostatic fluid [7], Tsutsui et al. [8] reported very poor fertility in bitches that were inseminated intravaginally with high numbers ($(1.5–10) \times 10^8$) of progressively motile spermatozoa that were frozen in a similar extender (including a surfactant) to the one used by Nöthling and Volkmann [7], but

without prostatic fluid. The beneficial effect of prostatic fluid that Nöthling and Volkmann [7] observed could not be achieved by replacing prostatic fluid with albumin-free TALP, suggesting that prostatic fluid promotes the fertility of vaginally inseminated bitches via a mechanism other than merely changing the physical characteristics of the inseminate [9]. The mechanism through which prostatic fluid affects fertility is unknown but possibilities are that prostatic fluid interacts with spermatozoa or with the female reproductive tract or both. Several studies reported effects of prostatic fluid on spermatozoa. The addition of prostatic fluid to ejaculated dog spermatozoa results in a more rapid decrease in the percentage progressively motile spermatozoa compared to when no fluid [10], an egg yolk–Tris extender [10] and [11] or minimal essential medium [12] was added to the spermatozoa. In contrast to these findings suggesting a suppressive effect of prostatic fluid on motility, ejaculated spermatozoa to which prostatic fluid had been added before incubating them for 6 h at 37 °C moved more rapidly than spermatozoa to which minimal essential medium or an egg yolk–Tris extender was added [12]. Prostatic fluid prevents the exposure of a progesterone receptor on spermatozoa, which is suspected to prevent progesterone from inducing the acrosome reaction [13]. Prostatic fluid added to frozen-thawed ejaculated spermatozoa promotes their fertility [7] and [9], either because the prostatic fluid has an effect on the female reproductive tract or on the spermatozoa or both. There is, therefore, a need to further clarify the effects of prostatic fluid on the female reproductive tract as well as on spermatozoa. Seeing that epididymal spermatozoa have never been exposed to prostatic fluid, this study was done to determine the effects of adding prostatic fluid to epididymal spermatozoa before they are frozen or after they are thawed on some aspects of their in vitro quality.

Triladyl, as used to demonstrate the beneficial effect of prostatic fluid on fertility of frozen-thawed ejaculated spermatozoa [7] and [9], contains egg yolk, Tris, citric acid, fructose and glycerol, which are all familiar substances in canine freezing extenders [14], and a combination of tylosin, gentamycin, spectinomycin and lincomycin. Equex STM, which Nöthling et al. [7] and [9] added to the Triladyl, prolongs the maintenance of motility [15], [16], [17] and [18], membrane integrity [15] and [19] and acrosomal integrity [17], [20] and [19], calcium uptake [18] and fertility after intrauterine insemination [8] of frozen-thawed dog spermatozoa. Biladyl® (Minitüb, GmbH,

Tiefenbach, Germany) contains the same substances but, unlike Triladyl that is a proprietary extender, the concentrations of the substances in Biladyl are known (see Section 2). Biladyl with the same amount of Equex STM paste added as the amount used by Nöthling and co-workers [7] and [9], abbreviated as BilEq throughout the remainder of this article, has been shown to be an effective extender for the freezing of ejaculated dog sperm [21].
Andromed (Minitüb, GmbH, Tiefenbach, Germany) is a patented extender containing phospholipids, Tris, citric acid, fructose, antioxidants, buffers, glycerol and the same combination of antibiotics as Triladyl and Biladyl. Unlike the latter two extenders, Andromed contains no egg yolk or animal substances, which reduces the risk of introducing infectious agents into semen via the extender. The suitability of Andromed for dog spermatozoa has not yet been determined.
The primary aim of this study was to determine whether homologous prostatic fluid has any effect on the percentage progressively motile spermatozoa after thawing, longevity (maintenance of motility at 37 °C), and morphology after thawing of epididymal spermatozoa of dogs. The secondary aim of this study was to compare the effects of BilEq and Andromed (Minitüb, Germany) as freezing extenders for epididymal spermatozoa of dogs.

2. Materials and methods

2.1. Extenders and prostatic fluid

The extenders used in this study were BilEq (all fractions of Biladyl combined plus Equex STM paste) and Andromed. Biladyl was made up in deionised water and consisted of 20% (v/v) of egg yolk, 933.7 mM glycerol, 199.8 mM tris(hydroxymethyl)aminomethane, 65.7 mM citric acid monohydrate, 55.5 mM fructose, 0.0625 mg/mL tylosin, 0.3125 mg/mL gentamycin, 0.1875 mg/mL lincomycin, 0.375 mg/mL spectinomycin. In addition, 0.5 mL Equex STM Paste was added to 100 mL of Biladyl [21] to provide 100.5 mL of BilEq. The osmolality of BilEq was 1.4805 osmol/kg. Andromed contains no egg yolk but, because it is a proprietary extender, no more is known about its constituents than what is listed in Section 1.

The post-sperm fractions of the ejaculates of seven healthy dogs (six German shepherd dogs and one Dobermann), aged 2–5 years, that belonged to the South African Police Service were collected, pooled, centrifuged at 1000 × *g* for 10 min and the supernatant frozen in a domestic freezer until use.

The testes with their associated epididymides were obtained from 21 privately owned, sexually mature dogs immediately after routine castration and allowed to cool to room temperature (about 25 °C). The castrations were performed under general anesthesia that was induced with sodium thiopentone (Intraval, Merial, Randburg, South Africa) and maintained with halothane (Fluothane, Zeneca, Woodmead, South Africa) in oxygen. The dogs were five German shepherds, eight German shepherd crosses, two Chow crosses, one each of Boerboel cross, Rottweiler cross, Collie cross, Great Dane and Staffordshire bullterrier and a mongrel of unidentifiable origin.

Throughout the experiment, all tubes or straws with spermatozoa, as well as all slides prepared for assessment of motility and all sperm smears were identified by four-digit random codes that made it impossible for the person evaluating the spermatozoa to know the treatment the spermatozoa had been exposed to.

The tail of one epididymis together with a few centimeters of its vas deferens was dissected free. The epididymal duct was dissected free and transected at the point where its diameter became distinctly larger distally. Thereafter a blunted needle was inserted into the vas deferens and 0.5 mL of BilEq used to flush the contents from the epididymal tail into a 5-mL plastic tube. The same was done to the other epididymis of the dog but Andromed was used instead of BilEq. The extenders used to flush the epididymal ducts were at room temperature (about 25 °C). For each dog, epididymides were randomly assigned to Andromed or BilEq. As soon as the spermatozoa were flushed from the epididymal tail, they were further extended to a volume of 4 mL with the same extender as the one originally used, and then the concentration of spermatozoa was determined using a haemacytometer. The content of the tube was then divided into two and the content of one tube further diluted to a final concentration of 50×10^6 mL^{-1} with the same extender as the one that had originally been used, without any prostatic fluid (Fig. 1). The content of the second tube was similarly diluted, except that not only extender but also prostatic fluid was added in such a way that 20% of the final volume consisted of

prostatic fluid (Fig. 1). At this stage, which was about 1 h after castration, a sample from each tube with sperm was taken for the determination of the percentage progressively motile spermatozoa (Prog), as described below. As soon as the sample had been taken for the assessment of motility, cooling of the tube with sperm was started, as described below. This time of estimation of motility was referred to as “at the onset of cooling”.

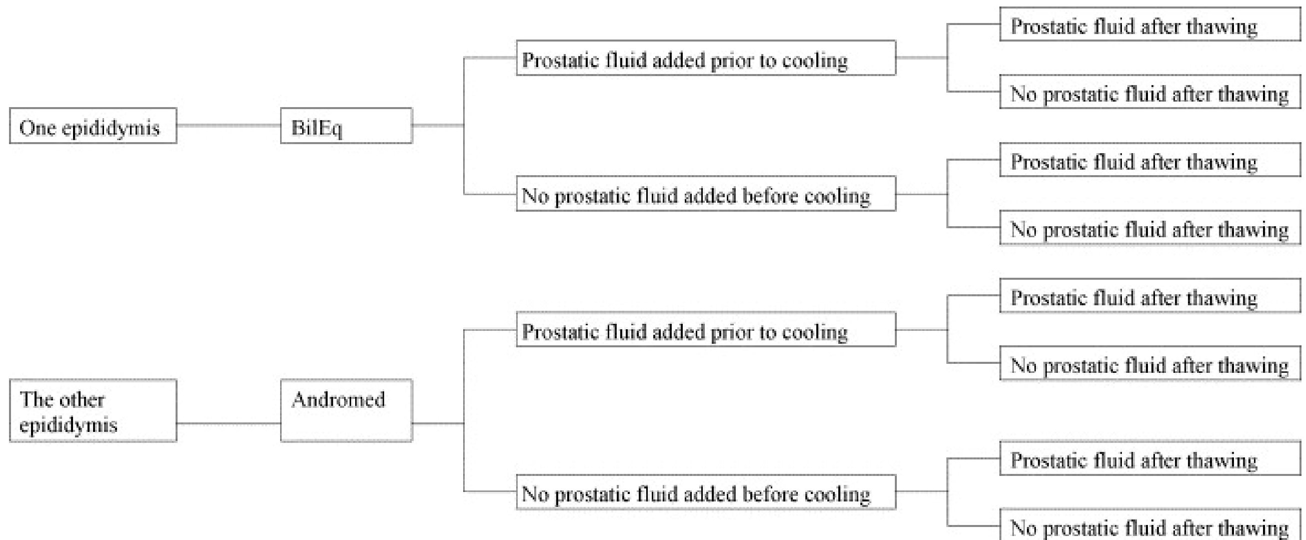

Fig. 1. The allocation of the spermatozoa from each epididymal tail of a dog to a different extender (BilEq or Andromed), thereafter to the addition or not of prostatic fluid prior to cooling and finally, for frozen spermatozoa, to the addition or not of prostatic fluid after thawing (the same process was followed for each of 21 dogs).

2.2. Determination of the percentage progressively motile spermatozoa (Prog) at the onset of cooling

The specimen used for the determination of Prog was prepared by transferring a 7-μL droplet of diluted spermatozoa to the center of a warm 22 mm × 22 mm coverslip, which was inverted and lowered onto a microscope slide on a warm stage set at 37 °C, flattening the droplet into a thin layer covering all of the lower surface of the coverslip without trapping any air bubbles, and without any fluid escaping from underneath the coverslip. The Prog was estimated on each of 10 neighboring microscope fields, using a phase contrast microscope at 200× magnification. The average of the 10 fields was taken as the

Prog for the specimen. One experienced operator, who did not know the treatment that the spermatozoa had been exposed to, did all determinations of Prog.

2.3. Cooling of spermatozoa and the evaluation thereof

One hour after castration, each 5-mL tube with spermatozoa was placed in a 50-mL tube with water at room temperature, which was placed into a 250-mL glass beaker, also with water at room temperature, which was placed in a water bath at 15 °C for 20 min before the beaker with its contents were transferred to a water bath at 5 °C, where it was left for another 3 h and 40 min to give a 4 h cooling period. After the 4-h cooling period, the contents of the tube with diluted spermatozoa were thoroughly mixed before a 0.2-mL aliquot was transferred to a 5-mL plastic tube in a water bath at 37 °C, and the remainder frozen, as described below. Aliquots were taken from the tube as soon it was placed into the water bath at 37 °C (this time is referred to as "at the onset of incubation"), again 2 h later (2 h after onset of incubation) and 4 h later (4 h after onset of incubation), and Prog determined in each of these aliquots, as described for the evaluation at the onset of cooling. Throughout the trial all tubes with spermatozoa were kept closed except when spermatozoa were being removed for evaluation.

2.4. Freezing of the spermatozoa

After the 4 h cooling period, the extended spermatozoa inside the tube were thoroughly mixed, subsequent to which coded 0.25-mL straws were filled, sealed and frozen by horizontally suspending them 4 cm above liquid nitrogen and 1 cm apart in stabilized, clear nitrogen vapor, at a temperature of approximately −140 °C [21] for 20 min before plunging the straws into the liquid nitrogen. All straws containing spermatozoa from the same donor and that had undergone the same treatment prior to freezing were stored together in liquid nitrogen. All straws had been frozen for at least 1 week before they were thawed.

2.5. Evaluation of frozen-thawed spermatozoa

Two straws were thawed in water at 70 °C for 5 s and their contents thereafter transferred to separate vials kept in a water bath at 37 °C. The motility of spermatozoa from each

tube was evaluated and the best tube kept for further treatment and evaluation, while the other tube was discarded. An aliquot was then removed from the tube and used to make an eosin-nigrosin smear. The remainder of the tube's content was then divided into two sub-samples of 90 µL each, which were transferred to separate vials, subsequent to which the content of one tube was diluted with 90 µL of the same extender used initially that was at 37 °C, without adding any prostatic fluid after thawing and the other with 90 µL of prostatic fluid, which had been thawed and warmed to 37 °C (Fig. 1). These tubes were kept in the water bath at 37 °C for the next 2 h and Prog determined as soon as the extender had been added (this time is referred to as "immediately after thawing"), again 1 h later (1 h after thawing) and finally 2 h after thawing.

The percentages of those sperm morphological defects that may have been present prior to flushing the epididymides or may have arisen due to some aspect of the experimental treatment were determined on each smear, using 1000× magnification, oil immersion and phase-contrast. These defects were morphologically altered acrosomes [22], bent midpieces, bent principle pieces of the tails, retained proximal cytoplasmic droplets and retained distal cytoplasmic droplets [23]. In addition, the percentages of spermatozoa with defects considered to have been present prior to flushing were determined on smears from samples to which no prostatic fluid was added. These defects were knobbed acrosomes, abnormally shaped heads, the Daglike defect and distal midpiece reflexes [23].

2.6. Statistical analysis

A repeated measurements analysis of variance (ANOVA) model with dogs as subjects was used to separately analyze the data with respect to Prog, percentages of spermatozoa with normal morphology, abnormally shaped heads, knobbed acrosomes, the Daglike defect, distal midpiece reflexes, altered acrosomes, proximal cytoplasmic droplets, distal cytoplasmic droplets, bent midpieces and bent principle pieces. Prior to analysis, Bartlett's modification of percentages with values of 0 or 100 was done and an arcsine transformation thereafter done on all data [24]. Huynh Feldt's modification to the degrees of freedom was used to derive probability levels [25]. Pairwise comparison of means was done using Bonferroni's method. Probability levels below 0.05 were considered

significant. Although the ANOVA was done on transformed data, non-transformed data are presented in order to facilitate interpretation. Data are presented as least-squares means ± S.E.M.

For Prog of spermatozoa that were extended but not frozen, subject (21 dogs), extender (Andromed or BilEq), the addition or not of prostatic fluid prior to cooling, time of measurement of motility (at onset of cooling, at onset of incubation, 2 h after onset of incubation, 4 h after onset of incubation) and all interactions were included in the model.

For Prog of frozen-thawed spermatozoa, data were first evaluated separately for each extender, with subject, prostatic fluid before cooling and time after thawing, as well as all interactions included in the model. Thereafter, Prog of extenders was compared at each time after thawing separately, by including subject, extender, prostatic fluid before cooling, prostatic fluid after thawing and all interactions in the model. Finally, the effects of extender, prostatic fluid before cooling, prostatic fluid after thawing and the interaction among these factors on Prog were determined over all times combined. Data of 21 dogs were available immediately after thawing, of 20 at 1 h after thawing and of 19 at 2 h after thawing.

For the percentages of spermatozoa with normal morphology, abnormal heads, knobbed acrosomes, Daglike defects or distal midpiece reflexes, subject (21 dogs) and extender were included in the model. For altered acrosomes, proximal cytoplasmic droplets, distal cytoplasmic droplets, bent midpieces and bent principle pieces, subject (21 dogs), extender, prostatic fluid before cooling and the interaction between extender and prostatic fluid before cooling were included in the model.

In order to further characterize each significant interaction, the transformed data contributing to each interaction cell mean were assigned to a separate variable and a repeated measures ANOVA thereafter used with dogs as subjects and the variable names as levels of a within-subjects factor.

3. Results

3.1. Motility of spermatozoa that were not frozen

Table 1 shows that Prog decreased over time, BilEq resulted in higher Prog than Andromed and adding prostatic fluid resulted in higher Prog than not adding any. The

latter effect was because prostatic fluid increased Prog of sperm extended in BilEq but not of sperm extended in Andromed (Table 2).

Table 1.

Percentages of progressively motile spermatozoa (Prog) from the epididymal tails of 21 dogs were higher after extension in BilEq compared to Andromed®, after prostatic fluid was added compared to when none was added, and decreased over time (mean ± S.E.M.)

	Prog
Extender (*n* = 168 per group)	
BilEq	42.7 ± 1.6 a
Andromed	30.0 ± 1.6 b
Addition of prostatic fluid before cooling (*n* = 168)	
Added	38.4 ± 1.4 a
Not added	34.3 ± 1.4 b
Time (*n* = 84 per group)	
At the onset of cooling (immediately after extension)	61.4 ± 2.5 a
Immediately after onset of post-cooling incubation	52.0 ± 2.5 b
Two hours after onset of post-cooling incubation	27.6 ± 2.5 c
Four hours after onset of post-cooling incubation	4.4 ± 2.5 d

Within main effects, values with different letters differ ($P < 0.05$).

Table 2.

Interaction between extender and prostatic fluid on the percentage progressively motile spermatozoa (mean ± S.E.M.) from the epididymal tails of 21 dogs (extension and addition of prostatic fluid occurred before a 4-h cooling period, which was followed by 4 h of incubation at 37 °C with motility estimated before and after cooling and two-hourly during incubation)

	Extender	
	BilEq	**Andromed**
Prostatic fluid added (n = 84)	47.8 ± 1.7 a	29.0 ± 1.7 bd
No prostatic fluid added (n = 84)	37.6 ± 1.7 bc	31.0 ± 1.7 b

Letter ‘a’ differed from ‘b’ and ‘c’ from ‘d’ ($P < 0.05$).

Extender interacted with time. BilEq resulted in higher Prog than Andromed immediately after extension and after 4 h of cooling, although during the incubation thereafter Prog was similar for the two extenders (Fig. 2).

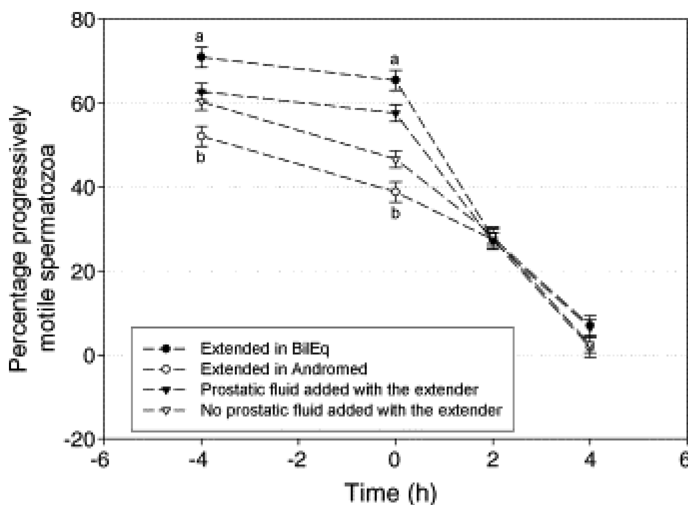

Fig. 2. The interactions between extender (BilEq or Andromed) or prostatic fluid (addition or not prior to cooling), respectively, with time (onset of a 4-h cooling period (time, −4), onset of post-cooling incubation at 37 °C (time, 0), and after 2 or 4 h of post-cooling incubation (times, 2 and 4)) on progressive motility of dog epididymal spermatozoa (42 estimations/group; within a time, a mean marked with (a) differs from that marked with (b) ($P < 0.05$)).

The addition of prostatic fluid before cooling interacted with time. Adding prostatic fluid resulted in a less steep decline in Prog during cooling, a steeper decline during the first 2 h of incubation and a less steep decline during the final 2 h of incubation than not adding it (Fig. 2).

Although Prog generally declined over time for each of the four extenders by prostatic fluid combinations, a striking deviation in this pattern occurred during the first 2 h of incubation with respect to Andromed if no prostatic fluid was added in that Prog did not decline (Fig. 3).

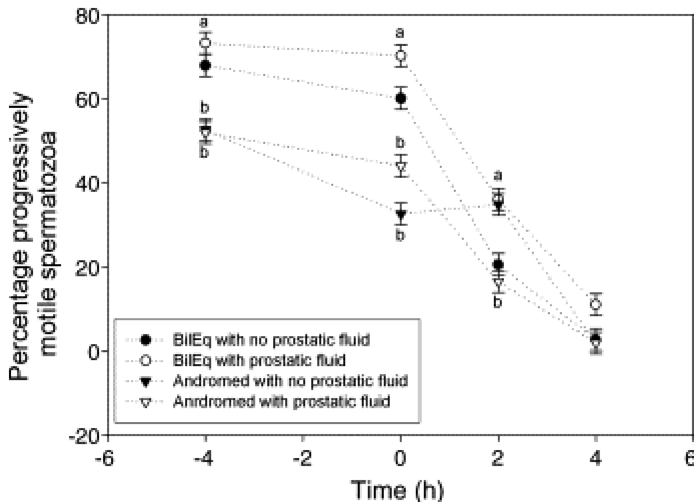

Fig. 3. The interaction among extender (BilEq or Andromed), prostatic fluid (addition or not prior to cooling) and time (onset of 4-h cooling period (time, −4), onset of post-cooling incubation at 37 °C (time, 0), and after 2 or 4 h of post-cooling incubation (times, 2 and 4)) with respect to motility of dog epididymal spermatozoa ($n = 21$ estimations/group; within a time, means marked (a) differ from those marked (b) ($P < 0.05$)).

3.2. Motility of frozen-thawed spermatozoa

The motilities of each of the eight treatment combinations at each of the three times after thawing, as well as the nature of the interactions among extender, the addition of prostatic fluid before cooling and after thawing at each time after thawing, respectively, are shown in Fig. 4.

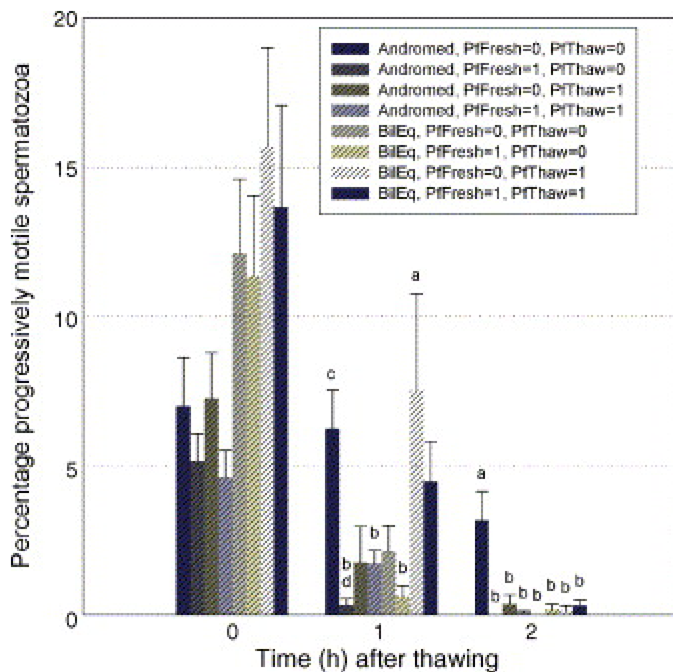

Fig. 4. Interaction among extender (Andromed or BilEq), the addition (PfFresh = 1) or not (PfFresh = 0) of prostatic fluid before cooling and the addition (PfThaw = 1) or not (PfThaw = 0) of prostatic fluid after thawing with respect to motility (mean ± S.E.M.) at each of three times after thawing (post-thaw incubation at 37 °C; n = 19–21 per group; within times, means marked (a) differ from those marked (b) ($P < 0.05$) and the mean marked (c) differs from the one marked (d) ($P < 0.06$)).

3.2.1. Motility of spermatozoa extended and frozen in BilEq

Adding prostatic fluid prior to cooling had no effect on Prog after thawing, whereas adding it after thawing resulted in higher Prog than not adding it (Table 3). Prog decreased during the first hour after thawing and thereafter remained the same (Table 3). The addition of prostatic fluid after thawing interacted with time. Although adding prostatic fluid after thawing resulted in similar Prog at each time after thawing than not adding it (15.3 ± 0.7% versus 12.6 ± 0.7% immediately after thawing, 6.0 ± 0.7% versus 1.4 ± 0.7% at 1 h after thawing and 0.2 ± 0.7% versus 0.1 ± 0.7% at 2 h after thawing (n = 21 per group)), Prog decreased more steeply during the first hour if none was added.

Table 3.

The effects of prostatic fluid and time of incubation at 37 °C on the percentage progressively motile spermatozoa (mean ± S.E.M.) after thawing of epididymal spermatozoa extended in BilEq (21 dogs) or Andromed (19 dogs; data of each extender evaluated separately)

	BilEq	**Andromed**
Prostatic fluid prior to cooling		
None added	6.2 ± 1.1 (n = 126)	4.4 ± 0.5 a (n = 114)
Added	5.6 ± 1.1 (n = 126)	1.9 ± 0.5 b (n = 114)
Prostatic fluid after thawing		
None added	4.7 ± 0.5 a (n = 126)	3.7 ± 0.4 (n = 114)
Added	7.2 ± 0.5 b (n = 126)	2.7 ± 0.4 (n = 114)
Incubation at 37 °C after thawing		
Immediately after thawing	14.0 ± 1.4 a (n = 84)	6.0 ± 0.6 a (n = 76)
One hour after thawing	3.7 ± 1.4 b (n = 84)	2.6 ± 0.6 b (n = 76)
Two hours after thawing	0.2 ± 1.4 b (n = 84)	0.9 ± 0.6 b (n = 76)

Within each main effect means marked with ‘a’ differ from means marked with ‘b’ ($P < 0.05$).

3.2.2. Motility of spermatozoa extended and frozen in Andromed

Adding prostatic fluid prior to cooling resulted in lower Prog than not adding it; adding prostatic fluid after thawing had no effect and, the same as for sperm frozen in BilEq, Prog decreased during the first hour after thawing thereafter remained the same (Table 3). There was an interaction among the addition of prostatic fluid prior to cooling, the addition thereof after thawing and time. Irrespective of when it was added, prostatic fluid had no effect on Prog immediately after thawing and by 2 h after thawing whereas, 1 h after thawing, Prog was higher for samples that received no prostatic fluid at all (mean

6.4 ± 0.6%) than the 0.3 ± 0.6%, 1.8 ± 0.6% or 1.7 ± 0.6%, for samples that received no prostatic fluid and those that received prostatic fluid before cooling, after thawing or at both stages, respectively (*n* = 19 per group). Prog remained similar over the 2 h after thawing for sperm to which no prostatic fluid was added at all (7.1 ± 0.9%, 6.4 ± 0.9% and 3.2 ± 0.9% immediately, 1 or 2 h after thawing, respectively (*n* = 19 per group)).

3.2.3. Motility immediately after thawing

BilEq resulted in higher Prog than Andromed (Table 4). Prostatic fluid had no effect on Prog but interacted with extender in such a way that the addition of prostatic fluid after thawing increased Prog of sperm frozen in BilEq by 2.7% and decreased Prog of sperm frozen in Andromed by 0.9% (Table 4).

Table 4.

The effects of extender (BilEq and Andromed) and prostatic fluid, as well as the significant interactions between extender and the addition of prostatic fluid after thawing, on the percentage progressively motile spermatozoa (Prog) from the epididymides of 21 dogs immediately after thawing and 20 dogs 1 h after thawing

	Means for extenders (±S.E.M.)		
	BilEq	**Andromed**	**Combined**
Immediately after thawing			
Overall mean per extender (*n* = 84)	14.0 ± 1.4 a	6.7 ± 1.4 b	
No prostatic fluid added before cooling (*n* = 84)			10.8 ± 1.3
Prostatic fluid added before cooling (*n* = 84)			9.9 ± 1.3
No prostatic fluid added after thawing (*n* = 42)	12.6 ± 0.7 c	7.2 ± 0.7 d	9.9 ± 0.5
Prostatic fluid added after thawing (*n* = 42)	15.3 ± 0.7 c	6.3 ± 0.7 d	10.8 ± 0.5
One hour after thawing			
Overall mean per extender (*n* = 80)	3.7 ± 0.9	2.5 ± 0.9	
No prostatic fluid added before cooling (*n* = 40)			4.4 ± 0.7 a

	Means for extenders (±S.E.M.)		
	BilEq	**Andromed**	**Combined**
Prostatic fluid added before cooling (*n* = 84)			1.8 ± 0.7 b
No prostatic fluid added after thawing (*n* = 42)	1.4 ± 1.0 d	3.3 ± 1.0	2.3 ± 0.5
Prostatic fluid added after thawing (*n* = 42)	6.0 ± 1.0 c	1.7 ± 1.0 d	3.9 ± 0.5

Within a time, the mean marked with 'a' differs from that marked with 'b' and any mean marked with 'c' differs from any marked with 'd' ($P < 0.05$). The interactions between addition of prostatic fluid prior to cooling and extender are not significant ($P > 0.05$) and the data are not shown.

3.2.4. Motility 1 h after thawing

The data of one dog were incomplete and were removed from the analysis.
Although the addition of prostatic fluid prior to cooling had no effect on Prog immediately after thawing, it resulted in lower Prog by 1 h after thawing (Table 4). Similar to immediately after thawing, the addition of prostatic fluid after thawing resulted in higher Prog with BilEq, although such addition had no effect with Andromed (Table 4). The second-order interaction among extender, the addition of prostatic fluid prior to cooling, and the addition of prostatic fluid after thawing is complex and essentially follows the above effects (Fig. 4).

3.2.5. Motility 2 h after thawing

The data of two dogs were incomplete and those dogs were removed from the analysis. Similar to 1 h after thawing, Prog of sperm frozen in Andromed was higher if no prostatic fluid was added at all (Fig. 4). Prog for this combination was 3.2 ± 0.4%, which was higher than those of all other combinations among extenders and prostatic fluid (Fig. 4).

3.3. Effects of extender and prostatic fluid on Prog for all times combined

BilEq resulted in 5.9 ± 0.7% progressively motile spermatozoa, which was higher than the 3.2 ± 0.7 of Andromed ($n = 228$ and $P < 0.05$). The addition of prostatic fluid prior to cooling resulted in lower Prog than not adding any (5.4 ± 0.6% versus 3.6 ± 0.6%,

$n = 228$ and $P < 0.05$), whereas the addition after thawing resulted in higher Prog than not adding any (4.9 ± 0.3% versus 4.1 ± 0.3%, $n = 228$ and $P < 0.05$). The addition of prostatic fluid after thawing interacted with extender because such addition increased Prog from 4.5 ± 0.5% to 7.2 ± 0.5% for BilEq but decreased Prog from 3.7 ± 0.5% to 2.7 ± 0.5% for Andromed ($n = 114$ and $P < 0.05$).

3.4. Morphology of frozen-thawed spermatozoa

Morphology of sperm frozen in BilEq and Andromed was similar, except that BilEq resulted in more bent principle pieces (Table 5).

Table 5.

Effect of Extender (BilEq or Andromed) and the addition of prostatic fluid prior to freezing on morphology after thawing of epididymal spermatozoa from 21 dogs (values are mean percentages ± S.E.M)

	Extender		**Addition of prostatic fluid**	
	BilEq	**Andromed**	**Added**	**Not added**
Normal spermatozoa ($n = 21$)	10.4 ± 1.4	10.4 ± 1.4		
Spermatozoa with head defects ($n = 21$)	3.6 ± 0.4	3.0 ± 0.4		
Spermatozoa with knobbed acrosomes ($n = 21$)	2.7 ± 0.5	1.8 ± 0.5		
Daglike defects ($n = 21$)	1.2 ± 0.3	0.7 ± 0.3		
Distal midpiece reflex ($n = 21$)	1.0 ± 0.4	1.0 ± 0.4		
Morphologically altered acrosomes ($n = 42$)	34.8 ± 2.3	40.8 ± 2.3	36.4 ± 1.4	39.2 ± 1.4
Proximal cytoplasmic droplets ($n = 42$)	8.1 ± 0.6	6.4 ± 0.6	6.0 ± 0.6 c	8.5 ± 0.6 d
Distal cytoplasmic droplets ($n = 42$)	49.5 ± 1.7	51.4 ± 1.7	44.2 ± 1.4 c	56.7 ± 1.4 d
Simple bent midpiece ($n = 42$)	1.4 ± 0.4	1.6 ± 0.4	2.0 ± 0.3 c	1.0 ± 0.3 d
Bent principle piece ($n = 42$)	23.0 ± 1.5 a	9.9 ± 1.5 b	12.3 ± 1.1 c	20.6 ± 1.1 d

The mean marked ‘a’ differs from the one marked ‘b’ and, within rows, means marked ‘c’ differ from those marked ‘d’ ($P < 0.05$).

Adding prostatic fluid prior to cooling had no effect on acrosomal morphology, slightly increased the mean percentage of bent midpieces and resulted in lower mean percentages of cytoplasmic droplets and bent principle pieces (Table 5). These latter two effects were mainly because failure to add prostatic fluid prior to cooling resulted in a higher prevalence of distal cytoplasmic droplets in spermatozoa extended in Andromed and a higher prevalence of bent principle pieces in spermatozoa extended in BilEq (Fig. 5).

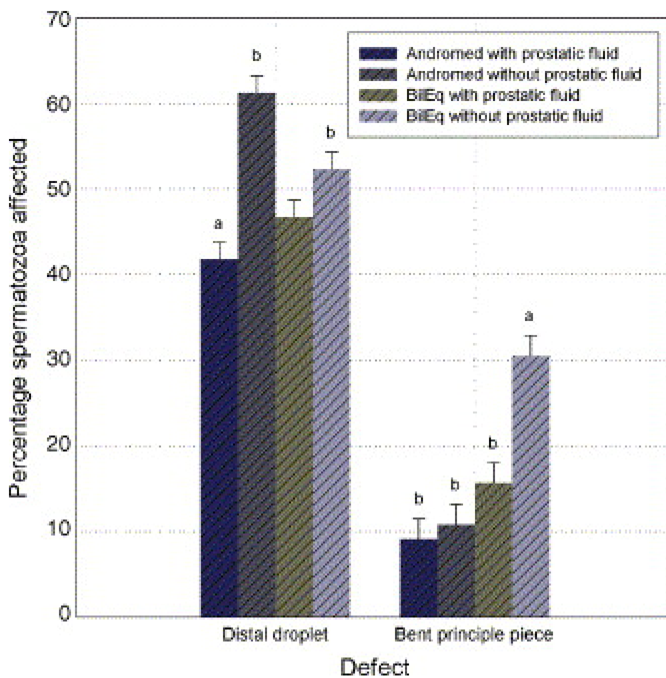

Fig. 5. The interaction between extender (Andromed or BilEq) and prostatic fluid (added prior to cooling or not) and the prevalence (mean ± S.E.M.) of distal cytoplasmic droplets and bent principle pieces in frozen-thawed dog epididymal sperm (n = 21 per group; within a defect, means marked with (a) differ from those marked with (b)).

4. Discussion

4.1. Main findings

In this study the effects of two extenders and the addition of homologous prostatic fluid prior to cooling or after thawing on motility before or after cooling, after thawing and

longevity were compared. Furthermore, the effects of the same two extenders and the addition of prostatic fluid prior to cooling on sperm morphology after thawing were also compared.

The current study shows that BilEq is more suitable than Andromed to flush and extend spermatozoa from the epididymides of dogs because it results in a higher percentage of progressively motile spermatozoa (Prog) immediately after flushing and after cooling, and that prostatic fluid should be added to the BilEq prior to extension because this combination results in the highest Prog after flushing and cooling.

Prog was higher after thawing when BilEq was used compared to Andromed, when no prostatic fluid was added prior to cooling compared to when it was, and when prostatic fluid was added after thawing compared to when not. BilEq resulted in a higher mean percentage of spermatozoa with bent principle pieces than Andromed and the addition of prostatic fluid prior to cooling resulted in lower mean percentages of cytoplasmic droplets and bent principle pieces than when none was added. There were, however, various interactions among the extender used, the addition of prostatic fluid prior to cooling, the addition of prostatic fluid after thawing and time of incubation with respect to motility and longevity after thawing. There were also various interactions between extender used and the addition of prostatic fluid prior to cooling with respect to sperm morphology after thawing. These interactions suggest a need to identify that combination among the various treatments that has optimal effects on motility, longevity and morphology after thawing. This study suggests that the optimal combination for the freezing of dog epididymal sperm is the use of BilEq together with prostatic fluid added prior to cooling (in order to inhibit the development of bent principle pieces) and prostatic fluid added after thawing (in order to achieve maximal motility until 1 h after thawing).

4.2. Poor freezability of epididymal spermatozoa of dogs

In the current study Prog immediately after cooling was best for sperm extended in BilEq and this Prog was similar to that of freshly ejaculated spermatozoa of dogs extended in the same [21] or a similar [26] extender. Yet, the mean Prog immediately after thawing in the current study was only 12.1 ± 0.7 for epididymal sperm extended in BilEq, which

appears lower than the 37.6 ± 1.6 for spermatozoa in the sperm-rich fractions of the ejaculates of eight other dogs frozen, thawed and evaluated by the same method [21] or the 53.4 (S.D., 12.6) found when the sperm-rich fractions of 54 ejaculates from five dogs were extended in a similar extender and frozen and thawed in the same way as used in the current study [26]. These comparisons suggest that the freezability of epididymal spermatozoa is lower than that of ejaculated spermatozoa. Similar to the current study, the findings of Hewitt et al. [27], and Hori et al. [28] also suggest poor freezability of epididymal sperm. Hewitt et al. reported mean values for Prog after thawing of 6.2 (S.D., 7.5) to 17.5 (S.D., 10.4) for epididymal sperm frozen in a Tris–egg yolk extender, whereas Hori et al., who used a similar extender to BilEq, reported a Prog of 12.5 ± 2.0 after thawing.

4.3. The effect of prostatic fluid

If, instead of directly extending and freezing epididymal spermatozoa in an extender containing Tris, egg yolk and Equex STM paste, the spermatozoa are temporarily exposed to prostatic fluid before such extension and freezing, Hori et al. found that Prog after thawing more than doubles to 32.0 ± 1.4 [28]. This motility seems comparable to that obtained with frozen-thawed ejaculated spermatozoa [21], suggesting that an important, if not the only reason for the poor freezability of dog epididymal sperm may be a lack of exposure to prostatic fluid. In contrast, the current study shows that, with epididymal spermatozoa extended in BilEq, which is similar to the extender Hori et al. used, the addition of prostatic fluid prior to freezing the spermatozoa had no effect on Prog after thawing.

Prostatic fluid that is present with ejaculated spermatozoa during freezing results in lower Prog after thawing [13]. One reason why, in the current study, the addition of prostatic fluid prior to freezing of epididymal spermatozoa extended in BilEq had no effect on Prog after thawing may therefore be that the prostatic fluid was not removed prior to freezing.

The prostate is the sole accessory sex gland in the dog [29]. The concentration of spermatozoa in the duct of the epididymal tails of dogs is unknown but the mean concentration of spermatozoa in the ejaculates of dogs in which prostatic secretion had

been severely depressed by the long-term treatment with finasteride was approximately 1750×10^6 mL^{-1} (S.D., approximately 250×10^6) [30]. This concentration appears higher than the means of 483×10^6 mL^{-1} (S.D., 266×10^6) and 300×10^6 mL^{-1} (S.D., 128×10^6) for proficiently collected sperm-rich fractions of dogs' ejaculates [26] and [31] suggesting that, on average, the sperm-rich fractions of dogs' ejaculates may consist of epididymal contents diluted with as much as 260–480% (v/v) of prostatic fluid. Assuming, as suggested in an earlier paragraph, that spermatozoa from the sperm-rich fraction of the ejaculate result in better motility after thawing than epididymal spermatozoa, the fact that Hori et al. [28] exposed epididymal sperm to pure prostatic fluid may have resembled the situation in the sperm-rich fraction of the ejaculate more closely than in the current study, where the epididymal spermatozoa were exposed to a fluid containing only 20% (v/v) of prostatic fluid. The reason why the addition of prostatic fluid to epididymal spermatozoa prior to freezing improved Prog in the study by Hori et al., but not in the current study may be that the concentration of prostatic fluid in the current study was too low.

Of those ejaculated spermatozoa frozen in BilEq that are progressively motile after thawing, 34% retain their progressive motility during the first hour of post-thaw incubation [21], compared to only 11.1% of epididymal spermatozoa frozen, thawed and evaluated by the same method in the current study. If prostatic fluid is added to the epididymal sperm after thawing, an average of 39% of progressively motile spermatozoa retain their progressive motility during the first hour of incubation, implying that the addition of prostatic fluid to frozen-thawed epididymal sperm improves the longevity of spermatozoa to a level similar to that achieved with ejaculated spermatozoa over the same period.

Similar to the study by Hori et al. [28], the current study also shows that the addition of prostatic fluid prior to freezing decreases the prevalence of retained cytoplasmic droplets after thawing. In contrast to the study by Hori et al., where prostatic fluid decreased the average prevalence of retained cytoplasmic droplets from 61% to 4%, the current study resulted in a much smaller reduction from 65% to 54%. Another difference in the findings of the study by Hori et al., and the current study is that the former showed that contact with prostatic fluid prior to freezing decreased the prevalence of abnormal

acrosomes after thawing, whereas the current study showed no effect. These differences may be because Hori et al. exposed spermatozoa to a higher concentration of prostatic fluid than the one used in the current study.

BilEq resulted in a high prevalence of bent principle pieces after thawing. Although exposure of spermatozoa to a hypotonic medium may induce swelling of spermatozoa with normal membranes with resultant flexion of the axial filaments of the tail [11] and [32] such a cause seems unlikely in the current study as BilEq is not hypotonic but has an osmolality of 1.4805 osmol/kg. Although the mechanism is unknown, the addition of prostatic fluid prior to freezing largely prevented bending of the tails.

The current study shows that the effects of prostatic fluid differ for BilEq and Andromed with respect to motility, as well as the prevalence of spermatozoa with distal cytoplasmic droplets and bent principle pieces after thawing. It is not known whether these differences in effect may be due to interactions between prostatic fluid and Equex STM paste or egg yolk, both of which occur in BilEq but not in Andromed, or some other difference between the extenders.

Various studies showed that the suspension of ejaculated spermatozoa in pure prostatic fluid reduces their motility and longevity [10], [11] and [12]. In contrast, the current study shows that the suspension of epididymal spermatozoa in a mixture of BilEq and prostatic fluid improves their longevity. Similar to the current study, Hori et al. [28] showed that exposing epididymal spermatozoa to prostatic fluid before extending and freezing them in an extender similar to BilEq also increased their longevity after thawing. The reason for the difference in effect of prostatic fluid in the studies involving ejaculated spermatozoa and those involving epididymal spermatozoa may be that, in the latter studies, the extender in which the spermatozoa were suspended allowed the beneficial effects of prostatic fluid to become manifest.

The current study as well as that of Hori et al. [28] shows that prostatic fluid improves the motility, longevity and morphology of frozen-thawed dog epididymal spermatozoa. Hori et al. also showed that prostatic fluid improves the fertility of frozen-thawed dog epididymal spermatozoa inseminated into the uterus. Vaginal insemination is easier than uterine insemination and, seeing that prostatic fluid improves the fertility of vaginally inseminated frozen-thawed ejaculated spermatozoa of dogs [7] and [9], there is a need to

determine whether prostatic fluid will also improve the fertility of vaginally inseminated frozen-thawed epididymal spermatozoa of dogs.

4.4. Andromed as an extender for dog spermatozoa

The use of Andromed would avoid any risk of transmitting pathogens via egg yolk. The findings of the current study, however, suggest that Andromed is a less suitable extender than BilEq for the freezing of dog epididymal spermatozoa. Two of the reasons for this unsuitability are that prostatic fluid added prior to and remaining present during freezing results in poor post-thaw motility and longevity and the non-addition thereof results in a high prevalence of retained distal cytoplasmic droplets. The latter reason to add prostatic fluid does not apply to ejaculated spermatozoa as such spermatozoa (even those of carefully collected sperm-rich fractions) have a low prevalence of retained distal cytoplasmic droplets [31]. Although Prog was very poor for all treatment combinations by 2 h after thawing, it was best for sperm frozen in Andromed and to which no prostatic fluid was added at any stage. These observations, together with the impression that the freezability of epididymal spermatozoa seems to differ from that of ejaculated spermatozoa suggests a need to investigate the suitability of Andromed for ejaculated dog spermatozoa, especially if any prostatic fluid that may be present is removed by centrifugation prior to freezing.

5. Conclusion

BilEq seems more suitable as a freezing extender for dog epididymal spermatozoa than Andromed. Prostatic fluid should be added prior to freezing of epididymal spermatozoa extended in BilEq, as well as after thawing, because such addition results in better motility, longevity and sperm morphology.

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