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A commercial box for dog semen transport: What happens inside when the environmental temperature is increasing?

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ABSTRACT

Environmental temperatures may influence the temperature inside commercial transport boxes during semen shipment and thereby storage conditions of diluted dog semen. To evaluate the temperature changes inside boxes and their influence on sperm quality, split semen samples ($n = 8$) were placed in Neopor boxes® exposed for 48 h to room temperature (RT) (Box 1), 40 °C for 6 h and then kept at RT (Box 2) or 40 °C (Box 3). A fourth subsample was kept at 4–5 °C in a refrigerator (control). Inside Box 1 temperature initially decreased to <3 °C before it stabilized at 7–8 °C, while in Box 2 no decrease occurred and temperature was at 7–8 °C for 48 h. Temperature inside Box 3 was at 14–15 °C for 24 h and, thereafter, increased to 36.1 °C. Analysis of sperm motility (CASA) and viability (PI and FITC-PNA) after 24 and 48 h revealed marked sensitivity of dog spermatozoa to temperature fluctuations (Box 1). A constant storage temperature of 7–8 °C (Box 2) provided the most desirable semen quality in terms of motility, viability, as well as osmotic resistance when samples were stored for 48 h. Furthermore, results indicate that during 24 h preservation a storage temperature of 14–16 °C may provide optimum conditions for maintenance of sperm viability and function. An increase of the inside temperature to >30 °C (Box 3) resulted in an almost complete loss in sperm integrity. In conclusion, results suggest a revision of current recommendations for storage temperature of diluted dog semen. Boxes for semen transport should be prepared depending on the expected environmental temperatures.

1. Introduction

As in other domestic animal species, cooling to 4–5 °C is an appropriate method for short term preservation of diluted semen in dogs. The biotechnical procedure is a suitable option for semen shipping in connection with artificial insemination solving the problems of transport distance and sanitary borders. Commercial transport

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boxes are routinely used for shipping diluted dog semen (Lopes et al., 2009; Lourenço et al., 2010). The preparation of boxes is proposed to be done at room temperature (Lopes et al., 2009). Milk based diluents are widely used for chilling and transporting semen (Peña et al., 2006 and references therein), and a range of commercial systems for semen preservation and transport are currently available in the market (Linde-Forsberg, 2001; Aurich, 2008; Nunes et al., 2008).

Fertilizing ability of cooled semen may not only depend on fresh semen quality and the used semen extender but also on transport conditions, of which the environmental temperature may be of particular significance (Malmgren, 1998; Brinsko et al., 2000). Purdy et al. (2010) observed that even though boar semen was diluted according to defined instructions and shipping protocol, semen samples arrived in the laboratory at varying temperatures and pH-values. Krakowski et al. (2013) stated that apoptosis and DNA damage in chilled stallion sperm can be induced by many factors. Rapid temperature fluctuation during semen storage is one of the possible problems. Love et al. (2002) demonstrated that stallion sperm DNA denatures at different rates, depending on the storage temperature and the fertility status of the semen donor. Aurich and Spergser (2007) concluded that certain bacteria may have detrimental effects on the quality of chilled stallion semen.

Depending on the geographical region and the distance of semen transport the outside temperature may vary between far less than room temperature and, with respect to the situation in Brazil, up to 40 °C or even more resulting in temperature changes within the transport box and thus in the direct surroundings of the diluted semen. According to the practical aspect of high ambient temperatures, the present study investigated to what extent a short transient or prolonged exposure of commercial transport boxes to increased environmental temperatures affects the inside temperature of the transport system and thereby sperm quality.

2. Materials and methods

2.1. Semen collection and processing

Individual sperm rich fractions from eight mature healthy beagles were extended at room temperature in pre-warmed (37 °C) DogSemenDiluent® (skim-milk based diluent including gentamycin; ref. 13700/0100, Minitube, Tiefenbach, Germany) (pH = 6.85; osmolarity = 306 mOsmol/kg) to a concentration of 50×10^6 sperm/ml and divided in four pairs of split samples. Volumes of all final samples were between 2 and 4 ml. The diluted semen samples were drawn up in sterile plastic syringes provided in the commercial shipping systems and three pairs were placed in three individual shipping boxes (Neopor Box®, ref. 17229/0005, Minitube, Tiefenbach, Germany) together with frozen thermal packs. The thermal packs had been frozen for at least 24 h at −20 °C as advised in the manual (Minitube Manual 17229/0005). One empty vial provided with the commercial shipping systems was placed together with two plastic syringes containing the semen samples in the central compartments of the box. The three individual boxes were designated Box 1, Box 2, and Box 3, respectively. Box 1 was kept at room temperature (RT, i.e. 17.9 ± 1.3 °C in the current experiment) for 48 h. Box 2 was placed in an incubator at 40 °C for 6 h and then kept at RT for further 42 h simulating a short term transport under hot summer conditions. Box 3 was exposed to 40 °C for 48 h, simulating a long-term transport under hot summer conditions. The fourth pair of semen samples (control) was kept at 4–5 °C in the refrigerator, which is the recommended storage temperature for chilled dog semen (Bouchard et al., 1990).

Semen samples were analyzed immediately after dilution (0 h) and after 24 h and 48 h of cooling (control) and storage in the three boxes, respectively. Boxes were opened briefly after 24 h incubation and then again after 48 h for assessment of split sample 1 and 2, respectively. Samples were warmed for 15 min at 38 °C in an incubator before evaluation.

2.2. Temperature measurements

The internal temperature of the boxes was measured throughout the 48 h test period using a wireless Thermo Station (TFA®, Dostmann GmbH, Wertheim-Reicholtsheim, Germany) for collection of data from three sensors. One sensor was placed inside each box, next to the syringes recording the temperature every 5 min. Temperature data obtained per box and test period were downloaded and evaluated using data recorder software (TFA®, Dostmann GmbH, Wertheim-Reicholtsheim, Germany).

2.3. Assessment of sperm motility

Sperm motility was verified by means of a computer-assisted semen analysis system (CASA) (SpermVision®, Minitube, Tiefenbach, Germany) using two chamber slides (12 μm depth, Leja, Nieuw Vennep, The Netherlands) filled with 3.5 μl of diluted semen. The CASA-system was equipped with a 20-fold objective, a camera adapter (U-PMTVC tv-0.75, Olympus, Hamburg, Germany) and a camera with a resolution of 648 by 484 active pixel (AccuPixel TM6760 CL, JAI A/S, Glostrup, Denmark). The system was operated by SpermVision® software (Version 3.5, Minitube, Tiefenbach, Germany). For each sample, 15 successive fields in the center of a chamber were recorded at a rate of 30 pictures per 0.5 s per field. The percentages of motile sperm (total motility) and of progressively motile sperm (progressive motility) were determined and for the progressively motile sperm the average straight-line velocity (VSL), curved-line velocity (VCL), average path velocity (VAP), linearity (LIN = VSL/VCL), straightness (STR = VSL/VAP), wobble (WOB = VAP/VCL), average amplitude of lateral head-displacement (ALH), and beat cross frequency (BCF) were analyzed. A spermatozoon was considered to be motile if its average head orientation change (AOC) was higher than 9.5, and progressively motile if, in addition, the distance from A to B covered in a straight line (DSL) exceeded 6.0 μm. The motility data evaluated in this study were: percentage of progressively motile spermatozoa (PM), straight-line velocity (VSL), curved-line velocity

(VCL), linearity (LIN), average amplitude of lateral head-displacement (ALH), and beat cross frequency (BCF).

2.4. Assessment of plasma membrane and acrosome integrity

For flow cytometric evaluation of sperm plasma membrane integrity and acrosome integrity an aliquot of the diluted semen sample was re-suspended in isotonic Hepes-buffered saline (isoHBS; 137 mM NaCl; 2.5 mM KOH; 20 mM HEPES; 10 mM glucose, 300 ± 5 mOsmol/kg, pH 7.40 at 20.0 °C) to a concentration of approx. 2.5–5.0 × 10^5 sperm per ml. The diluted aliquot was subsequently stained with propidium iodide (PI; final concentration 2.5 μg/ml) and fluorescein-isothiocyanate–conjugated peanut agglutinin (FITC–PNA; final concentration 3.0 μg/ml) for 10 min at RT. A total of 10,000 events were analyzed by means of a DAKO 'Galaxy' flow cytometer (DAKO Cytomation, Hamburg, Germany) controlled by 'FloMax®' software (version 2.4, Partec, Münster, Germany). The cells were excited at a wavelength of 488 nm by an argon ion laser (20 mW). Fluorescence signals for FITC–PNA were detected using a 537.5/22.5 nm band pass filter and for PI using a 610 nm long pass filter. Signals for forward and side scatter were plotted on linear scales, fluorescence data were plotted on logarithmic scales. The sperm population was identified by characteristic forward and side scatter distribution patterns. The percentages of sperm with intact plasma membrane (PI-negative) and intact acrosomal membrane (FITC–PNA-negative) were determined. The overlap of emission spectra between PI and FITC–PNA was compensated after analysis using 'FloMax®' software for digital compensation. Correction of data for non-DNA containing particles in the samples was performed as proposed by Petrunkina et al. (2010).

2.5. Modified osmotic resistance test

A modified osmotic resistance test (mORT) was performed, to test the functional integrity of the plasma membrane in stored spermatozoa. Semen samples (final concentration approx. 2.5–5.0 × 10^5 sperm per ml) were incubated for 5 and 20 min in an isotonic and hypotonic Hepes-buffered saline at 37 °C (isoHBS: 300 ± 5 mOsmol/kg, hypoHBS: 180 ± 5 mOsmol/kg, both: pH 7.40 ± 0.05 at 38.0 °C). The hypoHBS was essentially composed as the isoHBS, only the content of NaCl was lowered to 76 mM. Five minutes before the end of an incubation time, PI and FITC–PNA were added to the samples as described above. Analysis of the samples was conducted in the same way as for regular assessment of plasma membrane and acrosome integrity.

2.6. Statistics

Data were statistically evaluated using Wilcoxon's signed rank test (SAS Version 9.2; SAS Inst. Inc., USA). Differences were considered to be significant when their probability of occurring by chance was less than 5% ($P \leq 0.05$).

3. Results

After 1 h incubation of the boxes at RT (Box 1) and at 40 °C (Box 2 and 3) the mean internal box temperatures were 2.2, 7.6, and 8.3 °C, respectively. Individual samples in Box 1 were cooled to temperatures as low as 0.5 °C within the first hours. After 6 h, the respective temperatures were 7.3, 12.8 and 13.3 °C. In Box 1, the temperature increased slightly from 6.5 to 7.6 °C between 6 and 48 h. In Box 2, the temperature decreased from 12.8 to 8.0 °C within 1 h 20 min after turning the box over to RT and then remained between 7.0 and 8.0 °C until 48 h. In Box 3 temperature was relatively constant at 14–16 °C until 24 h, and increased to 36.1 °C within the following 24 h. Slight oscillations observed after 24 h of incubation were related to opening of boxes to remove the split sample for evaluation (Fig. 1).

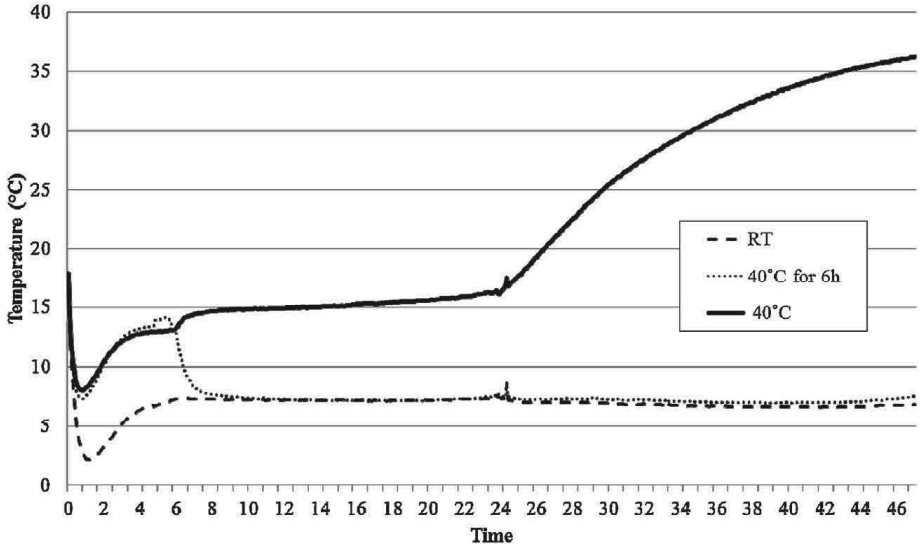

Fig. 1. Mean temperature curves inside commercial semen transport boxes kept at room temperature (RT, Box 1), at 40 °C for 6 h and at RT for 42 h (Box 2), and at 40 °C for 48 h (Box 3).

Preservation of dog semen for 24 h at 4–5 °C or 7–8 °C tended ($P = 0.1094$ and $P = 0.0547$, respectively) to result in decreased progressively motile spermatozoa when compared to fresh diluted samples (Table 1). A statistically significant ($P < 0.05$) decrease was noted in samples which were stored at 7–8 °C with an average decrease in temperature to 2 °C during the cooling phase (Box 1). Semen storage for 24 h at 14–16 °C (Box 3) resulted in more progressively motile sperm than storage at 4–5 °C or storage at 7–8 °C with a transient decrease in temperature to 2 °C during cooling ($P < 0.05$; control and Box 1). A similar trend ($P = 0.0547$) was observed in comparison to samples stored at 7–8 °C (Box 2). After 48 h of storage, progressive motility was less ($P < 0.05$) in samples stored at 4–5 °C (control) and in samples whose temperature increased to 36 °C (Box 3) compared to those stored at 7–8 °C (Boxes 1 and 2).

After 24 and 48 h of storage a marked increase in VCL with a concomitant decrease in VSL was observed when compared to fresh diluted samples ($P < 0.05$, Table 1). Consequently, this resulted in a decrease ($P < 0.05$) in linearity (VCL/VSL) with storage time indicating a more circular movement pattern of spermatozoa after cooling and storage. Concomitantly, the ALH was greater in samples stored for 24 and 48 h than for the fresh samples ($P < 0.05$) irrespective of the temperature inside the boxes.

The results for intact spermatozoa, i.e. PI and FITC–PNA negative cells, demonstrate that a loss ($P < 0.05$) in the amount of membrane intact cells occurs during cooling and subsequent storage in a temperature range between 4 and 15 °C (Table 2). Furthermore, storage for 24 h at 14–16 °C results in less ($P < 0.05$) membrane damaged spermatozoa compared to a temperature of 4–5 °C or 7–8 °C (control, Boxes 1 and 2; Table 2). After 48 h, the greatest ($P < 0.05$) amount of intact spermatozoa was assessed in samples that were stored at 7 °C (Box 2) or stored at 7–8 °C with an initial decrease to approximately 2 °C (Box 1). Storage at 4–5 °C resulted in significantly lesser values ($P < 0.05$; Table 2).

The results after 24 h showed that incubation under iso- or hypo-tonic conditions is tolerated to the greatest extent by sperm stored at 7–8 °C (Box 2) or 14–16 °C (Box 3). Samples that were stored at 4–5 °C or where temperatures decreased to 2 °C contained less intact spermatozoa after only 5 min under isotonic conditions ($P < 0.05$; Table 2). Remarkably, sperm stored for 24 h at 14–16 °C responded to hypotonic conditions for 5 or 20 min similar to fresh semen samples ($P > 0.05$). After 48 h storage, samples constantly stored at 7–8 °C (Box 2) contained more ($P < 0.05$) intact spermatozoa after a hypotonic challenge than samples that were either stored at 4–5 °C (control) or where there was a decrease to 2 °C (Box 1).

4. Discussion

Data from the present study demonstrate that the temperature inside semen transport boxes is clearly influenced by the environmental temperature. Temperature fluctuations may occur inside the boxes which can be deleterious for spermatozoa (Love et al., 2002; Purdy et al., 2010; Krakowski et al., 2013). In sequential temperature measurements in the present study it was observed that, dependent on the outside temperature, individual samples

Table 1
Sperm motility parameters (mean ± SD) in diluted semen immediately after dilution (0 h) and after 24 and 48 h storage at 4–5 °C or in a commercial transport box kept at room temperature (RT, Box 1), at 40 °C for 6 h and at RT for 42 h (Box 2), and at 40 °C for 48 h (Box 3).

Storage (h)	Treatment	Average temperature of semen sample in past 18/24 h (°C)*	PM (%)	VCL (μm/s)	VSL (μm/s)	LIN (VSL/VCL)	ALH (μm)	BCF (Hz)
24	Fresh diluted		$86.2 \pm 7.5^{A,C}$	181.5 ± 17.9^{A}	114.8 ± 11^{A}	0.64 ± 0.10^{A}	4.10 ± 0.79^{A}	30.1 ± 2.1^{A}
	Control	4–5	$74.2 \pm 13.8^{A,B,E,F}$	220.0 ± 22.6^{B}	80.1 ± 11.1^{B}	$0.36 \pm 0.04^{B,D,F}$	$6.93 \pm 0.93^{B,D}$	22.3 ± 2.0^{B}
	Box 1	7.3 ± 0.1	$76.6 \pm 7.7^{B,F}$	$219.5 \pm 19.9^{B,C}$	81.2 ± 7.1^{B}	$0.37 \pm 0.04^{B,D}$	$6.79 \pm 0.88^{B,D}$	$22.3 \pm 1.9^{B,D}$
	Box 2	7.5 ± 0.7	$78.5 \pm 4.0^{A,B,C,E}$	221.0 ± 12.1^{C}	83.9 ± 9.1^{B}	0.37 ± 0.03^{B}	6.61 ± 0.66^{B}	$23.4 \pm 1.6^{B,C}$
	Box 3	15.2 ± 0.5	83.5 ± 5.1^{C}	$211.9 \pm 14.2^{B,C}$	90.2 ± 9.0^{B}	0.42 ± 0.03^{C}	5.89 ± 0.59^{C}	24.8 ± 1.8^{C}
48	Control	4–5	58.1 ± 20.1^{D}	$203.7 \pm 25.5^{B,C}$	68.7 ± 9.8^{C}	$0.34 \pm 0.03^{D,E}$	$6.93 \pm 0.97^{B,D}$	$20.5 \pm 1.8^{E,F}$
	Box 1	6.8 ± 0.2	$68.3 \pm 16.6^{E,F}$	$219.3 \pm 15.6^{B,C}$	71.9 ± 6.5^{C}	0.32 ± 0.04^{E}	7.21 ± 0.84^{D}	20.3 ± 1.9^{E}
	Box 2	7.2 ± 0.2	70.2 ± 7.2^{F}	215.3 ± 14.7^{B}	73.1 ± 8.7^{C}	$0.33 \pm 0.04^{E,F}$	$6.95 \pm 0.86^{B,D}$	$21.0 \pm 1.9^{D,F}$
	Box 3	29.2 ± 5.8	1.4 ± 1.5^{G}	51.3 ± 24.5^{D}	29.2 ± 15.3^{D}	$0.50 \pm 0.24^{A,B,C,D,E}$	3.45 ± 1.91^{A}	11.0 ± 9.1^{G}

PM: progressively motile spermatozoa; VCL: curved-line velocity; VSL: straight-line velocity; LIN: linearity of sperm movement; ALH: amplitude of lateral head-displacement; BCF: beat cross frequency
* Average temperature was calculated for hours 6–24 on day 1 and for hours 24–48 on day 2 of storage. Different superscripts within a column indicate differences ($P < 0.05$).

Table 2
Sperm motility and membrane integrity (mean ± SD) in diluted semen immediately after dilution (0 h) and after 24 and 48 h storage at 4–5 °C (control) or in a commercial transport box kept at room temperature (RT, Box 1), at 40 °C for 6 h and at RT for 42 h (Box 2), and at 40 °C for 48 h (Box 3).

Storage (h)	Treatment	Average temperature of semen sample in past 18/24 h (°C)*	IntSp (%)	Membrane intact sperm in mORT (%)			
				isoHBS 5 min	isoHBS 20 min	hypoHBS 5 min	hypoHBS 20 min
0	Fresh diluted		91.9 ± 2.2^{A}	89.8 ± 5.5^{A}	82.1 ± 9.8^{A}	89.1 ± 2.9^{A}	84.0 ± 9.0^{A}
24	Control	4–5	81.9 ± 4.5^{B}	73.9 ± 4.4^{B}	64.4 ± 9.4^{B}	73.6 ± 3.9^{B}	70.3 ± 6.1^{B}
	Box 1	7.3 ± 0.1	84.1 ± 3.5^{B}	72.4 ± 11.7^{B,C,E}	59.9 ± 15.4^{B,C}	69.6 ± 12.0^{B,D}	65.6 ± 13.8^{B,D}
	Box 2	7.5 ± 0.7	83.1 ± 4.2^{B}	77.8 ± 5.1^{C}	71.6 ± 6.7^{A}	79.3 ± 4.8^{C}	76.2 ± 6.5^{C}
	Box 3	15.2 ± 0.5	89.2 ± 2.6^{C}	86.0 ± 2.9^{D}	78.7 ± 6.0^{A}	85.9 ± 2.5^{A,C}	84.1 ± 3.4^{A,C}
48	Control	4–5	67.9 ± 7.9^{D}	63.4 ± 10.8^{E}	54.7 ± 11.3^{C}	57.2 ± 8.5^{D}	53.5 ± 8.8^{D}
	Box 1	6.8 ± 0.2	70.9 ± 11.6^{D,E}	65.3 ± 10.7^{E}	58.1 ± 13.4^{C,D}	58.2 ± 12.0^{D}	54.0 ± 12.6^{D}
	Box 2	7.2 ± 0.2	75.2 ± 7.0^{E}	71.8 ± 8.3^{B}	64.3 ± 10.4^{B,D}	69.7 ± 7.9^{B}	65.8 ± 9.9^{B}
	Box 3	29.2 ± 5.8	11.1 ± 5.7^{F}	16.2 ± 12.6^{F}	14.2 ± 12.8^{E}	7.7 ± 6.0^{E}	6.6 ± 6.0^{E}

% IntSp: spermatozoa with intact plasma membrane and acrosome (PI and FITC–PNA negative); isoHBS 5 min/isoHBS 20 min = values after 5 or 20 min incubation in isotonic Hepes-buffered saline (300 mOsmol/kg); hypoHBS 5 min/hypoHBS 20 min = values after 5 and 20 min incubation in hypotonic Hepes-buffered saline (180 mOsmol/kg).

* Average temperature was calculated for hours 6–24 on day 1 and for hours 24–48 on day 2 of storage. Different superscripts within a column indicate differences ($P < 0.05$).

were cooled to temperatures as low as 0.5 °C (transport box at room temperature, Box 1) and other samples were heated to 37 °C during 48 h of storage (transport box at 40 °C, Box 3).

The results of semen analysis after 24 h of storage indicate that a rapid decrease in temperature inside boxes to values of less than 4 °C during the cooling phase should be avoided for dog semen to yield optimum semen quality. Therefore, the use of less or smaller frozen thermal packs might be considered to optimize the temperature profile inside the shipping boxes that are handled at environmental temperatures of about 20 °C.

When considering the aspect of greater environmental temperatures, a constant exposure of the transport boxes to 40 °C was clearly the maximal insulating capacity under these conditions. The constant increase in temperature after 24 h resulted in a drastic decrease in progressive motility. With respect to this, it should be added that opening the boxes resulted in a full exchange of air inside the boxes which may have accelerated the increase in the temperature inside the boxes kept under greater temperature until 48 h.

Not only had the amount of progressively motile sperm decreased with time and cooling but the motility pattern also changed. After storage in the present study, it was noted that a more circular movement with greater lateral head displacement occurred, resembling a motility pattern associated with hyper-activation (Mortimer and Maxwell, 1999; Suarez, 2008). Such alterations in the motility pattern may indicate capacitation-like changes at low storage temperatures. Similarly, capacitation-like changes in calcium influx and tyrosine phosphorylation patterns have been associated with cooling of sperm during chilling and freezing procedures (e.g. Rota et al., 1999; Green and Watson, 2001).

Besides maintaining sperm motility semen shipment in transport boxes must consider protection of plasma and acrosomal membrane integrity. Results of the present study regarding spermatozoa with intact membranes (PI and FITC–PNA negative spermatozoa) demonstrate that a loss of membrane intact cells may occur during cooling and subsequent storage at temperature ranges between 4 and 15 °C. These results are similar to observations from Love et al. (2012), who studied the effect of storage on chilled stallion semen. In this previous study, there was a reduction in available energy substrates or an increase in metabolic waste products in the stored semen that could have contributed to a decrease in sperm quality.

Detection of bacteria in semen samples does not necessarily indicate an infection, but may rather represent contamination with the typical bacterial flora of the male genital tract during semen collection (Aurich and Spergser, 2007; Martín et al., 2010; Abeysundara et al., 2013). Bouchard et al. (1990) indicated bacterial growth was common in dog semen kept at 22 and 35 °C but at the lesser storage temperature (4 °C) the bacterial growth was only modest. It was emphasized that this phenomenon may explain some of the differences in sperm quality at greater storage temperatures.

Regarding the addition of antibiotics to semen extenders used for storage of chilled semen, Aurich and Spergser (2007) pointed out that primarily Gram-negative bacteria may act on sperm function through toxic components at the outer membranes and in this way might contribute to an increased production of reactive oxygen species (ROS) in stored semen. It was also indicated that antibiotics are generally effective at temperatures above 15 °C. In the present study the amount of membrane damaged spermatozoa was less in semen stored for 24 h at 14–16 °C compared to 4–5 °C or 7–8 °C (control, Box 1 and Box 2; Table 2), nevertheless, after a longer incubation time (48 h) the damage in membranes of samples stored at greater temperatures (Box 3; Table 2) is highly significant. Based on these findings, it was speculated that if semen temperature increases to ≥15 °C, as was the case in Box 3 kept at 40 °C, antibiotics may increasingly display the antibacterial action and by this generate an increase in ROS causing a massive decrease in sperm quality. To our knowledge, only the effects on

sperm motility have been systematically evaluated for dog semen stored at 22 and 35 °C for 6, 12, 24, 96 and 120 h (Bouchard et al., 1990). Observations in the present study enlighten aspects on storage temperatures for chilled dog semen and indicate the need for further studies to assess the impacts of addition of antibiotics on sperm stability and effectiveness at greater temperatures (above 15 °C) for more efficient ROS scavenger mechanisms.

The resistance of sperm to osmotic stress is a functional sperm variable that has been positively correlated with sperm quality in dogs (Kumi-Diaka, 1993; Rodriguez-Gil et al., 1994) and with fertility in other species (Men: Check et al., 1988; Boar: Petrunkina et al., 2004; Bull: Khalil et al., 2006). For testing this feature in the present study, a flow cytometric approach was used which evaluated the integrity of the plasma membrane and the acrosomal membrane after incubation in isotonic and hypotonic media at 38 °C. The results obtained after 24 h show that incubation under iso- or hypo-tonic conditions is tolerated to the greatest extent by sperm stored at 7–8 °C (Box 2) or 14–16 °C (Box 3). Samples which were stored at 4–5 °C or where there was a temperature decrease to 2 °C had less membrane intact spermatozoa after only 5 min incubation under isotonic conditions. It is likely that sub-lethal damage known to be associated with chilling has occurred (Love et al., 2002; Krakowski et al., 2013). This damage may have been due to adversely low storage temperatures (cold injury) or less than desirable increased cooling rates (cold shock; reviewed in Mazur et al., 2008). Sperm stored for 24 h at 14–16 °C responded to storage conditions in a similar way as fresh semen samples when exposed to hypotonic conditions for 5 or 20 min (Table 2). This may substantiate the previous concentration about critically reviewing current concepts of chilled dog semen preservation. In summary, results from the hypo-osmotic swelling test after 24 and 48 h clearly indicate that function and probably also fertilizing ability of dog sperm is more ideally maintained at storage under constant temperatures above 7 °C.

From the spermatozoa data of the present study, three suggestions can be derived regarding sample temperatures during storage or shipment of liquid preserved dog spermatozoa. First, to achieve a stable semen quality for 48 h a constant storage temperature of approximately 7–8 °C is desirable. Second, even a short term decrease in semen temperature below 4 °C should be avoided to prevent sub-lethal sperm damage. Third, regarding sperm survival a storage temperature of 14–16 °C may be advantageous over lesser temperatures. However, under this storage condition efficiency of antibiotic inclusion in extenders regarding protection against bacterial growth and of impacts on scavenger mechanisms need to be considered.

In practical terms, boxes should be individually prepared depending on environmental temperature. In case of longer periods with outside temperatures above 30 °C mechanisms to keep the temperature inside the box constant for >24 h should be improved. Semen shipment should occur as rapidly as possible and the boxes should be kept tightly closed to avoid an accelerated and sperm damaging increase in semen temperature.

5. Conclusion

Dog spermatozoa are sensitive to an initial temperature decrease occurring inside a commercial transport box kept at environmental temperatures of about 20 °C (RT). Keeping transport boxes at 40 °C for 6 h results in a constant storage temperature of 7–8 °C inside the box. However, an increase of the environmental temperature to 40 °C for more than 24 h results in a temperature of >30 °C inside the transport box and concomitant severe sperm damage. A constant storage temperature of 7–8 °C inside the transport box may provide for enhanced semen quality in terms of sperm motility, integrity of plasma and acrosomal membrane as well as osmotic resistance when samples are stored for 48 h. A temperature of 14–16 °C may provide optimum conditions for maintenance of sperm viability and function during 24 h of storage of diluted dog semen.

Conflict of interest

The authors have no conflicts of interest that biased or influenced this work.

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