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Functional significance of the cell volume for detecting sperm membrane changes and predicting freezability in dog semen

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Abstract

Due to the similarity of plasma membrane changes induced by capacitation and cryopreservation, the parameters describing sperm response to capacitating conditions can be used for evaluating the cryopreservation response in many animal systems. In dog sperm, the response of the total sperm population to ionophore treatment has been shown to be an indication of the freezability of semen samples. Another sperm functional characteristic decisive for cryopreservability is cell volume regulation, due to the generation of essential osmotic gradients across the plasma membrane during the freeze-thaw cycles. In the present study, cryopreservation-induced changes in the membrane functional integrity were examined by monitoring the osmotically induced response of cell volume and the response to an ionophore in live cell populations. Cell volume measurements were performed on Percoll-washed suspensions of freshly diluted and frozen-thawed dog spermatozoa. The proportion of live acrosome-reacted cells was evaluated by flow cytometry after incubation under capacitating conditions in the presence of the calcium ionophore, A23187. During freezing-thawing, significant membrane changes occurred related to the disturbance of volume control ability and the loss of a proportion of live acrosome-reacted cells ($P < 0.05$). There were significant differences between individuals with respect to the degree of functional and structural membrane changes after thawing. Significant correlations were found between acrosomal integrity and functional membrane integrity. When assessed in freshly diluted semen, these parameters correlated with those of frozen-thawed semen samples, pointing to the similarities between mechanisms of cryopreservation-related changes and those mechanisms that mediate changes in membrane permeabilities and in cell volume regulation. The detection of changes in the sperm plasma membrane by monitoring the sperm cell volume represents a simple, rapid and sensitive method to estimate sperm quality after the cryopreservation procedure. The individual variability in response to osmotic stress or to calcium ionophore treatment appears to reflect the subtle differences in the sperm membrane functionality which are crucial for the prediction of cryopreservability.

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Introduction

In many mammalian species, cryopreservation and cooling of spermatozoa induce changes in the plasma membrane which are very similar to changes induced by capacitation (Watson 1995, Maxwell & Johnson 1997, Holt 2000). The understanding and prediction of the sperm functional response to cryopreservation is one of the major questions in sperm cryobiology.

In the last decade, the proportion of acrosome-reacted cells in frozen-thawed sperm has been used as a parameter for estimating sperm function and response to cryopreservation in human, canine, bovine and equine systems (Troup *et al.* 1994, Peña *et al.* 1999, Januskauskas *et al.* 2000, Rathi *et al.* 2001). However, relatively long incubation periods were necessary to record sensitively the changes in the dog sperm plasma membrane (i.e. Peña *et al.* 2001, 2003*a*). Although a more sensitive parameter is the influx of intracellular calcium ions which takes place before the sperm is able to respond to acrosome reaction stimuli (Peña *et al.* 2003*b*), the determination of intracellular calcium ions is a somewhat sophisticated approach not easily established as a routine procedure. Recently, an original approach was suggested by Szasz *et al.* (2000). They observed that the percentage of cells which deteriorated as a result of calcium ionophore treatment in fresh semen correlated with the motility and the percentage of sperm which deteriorated as a result of cryopreservation. Furthermore, the percentage of spermatozoa undergoing the acrosome reaction in response to the ionophore in fresh semen correlated with the percentage of the acrosome-reacted spermatozoa and motility after freezing-thawing. These correlations and the similarities between capacitation and

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cryopreservation-induced changes led to the logical conclusion that the detection of such changes may be a useful approach to predict the quality of frozen-thawed dog semen (Szasz *et al.* 2000).

However, there has not been sufficient emphasis on sensitive evaluation of the differences in response of individual males to cryopreservation and capacitation, which is crucial for sperm function estimation (cf. Petrunkina *et al.* 2003*a*). Although the calcium ionophore test would predict the amount of acrosome reaction induced by cryopreservation, the more subtle prediction of cryopreservability on the impairment of sperm function by freezing-thawing is preferable, especially when there is a narrow range of variation in the levels of acrosome reaction induced by cryopreservation. However, more subtle prediction requires more than the evaluation of conventional parameters, primarily impaired by the cryopreservation, such as loss of acrosomal integrity and of motility. There is a need to establish a functional test using parameters that characterise the response level of a sperm population in the face of secondary stress following cryopreservation. (Such stress factors are exposure to anisotonic or capacitating conditions post-thawing, which are relevant for the fertilising ability of frozen-thawed semen.) The subtle differentiation of responding subpopulations for advanced analysis may also be useful, for example separate analysis of live and dead cells responding to ionophore or osmotically active and inactive subpopulations. The volumetric measurement is an appropriately precise, accurate and informative method for the detection of functional membrane changes in live cell populations. This method can be used particularly in spermatozoa for determining cell osmotic properties, plasma membrane thermodynamic characteristics and for characterising volume regulation processes (Gilmore *et al.* 1995, 1996, 2000, Willoughby *et al.* 1996, Kulkarni *et al.* 1997, Petrounkina *et al.* 2001*a*, 2004*b*). This methodology is characterised by high sensitivity to individual ejaculates (Petrounkina *et al.* 2000). Under *in vivo* conditions, the spermatozoon experiences considerable tonic changes in its environment, most notably during maturation within the epididymis and at ejaculation, resulting, in turn, in dramatic changes in the major intracellular ions (Cooper 1986). It could be expected that during epididymal transit the osmolyte uptake and the regulatory volume increase occur to counter cell dehydration (Cooper & Yeung 2003). In many species osmotic pressure of the epididymal fluid is hyperosmotic with respect to that of seminal plasma and uterine fluid, which are closely isotonic. On deposition in the female tract, the spermatozoon will therefore experience hypotonic stress of between 30 and 120 mosmol/kg (Cooper & Yeung 2003 and references therein). To withstand these changes, spermatozoa developed the mechanisms of the regulatory volume decrease, which allow the adjustment of intracellular ion concentrations, as has been reported for bovine, murine, porcine and human species (Kulkarni *et al.* 1997, Petrunkina *et al.* 2001*a*, Yeung & Cooper 2001, Yeung *et al.* 2002). It has been suggested that there is a functional relationship between the sperm's ability to regulate the cell volume in response to osmotic challenge and fertility (Yeung *et al.* 1999, Petrunkina *et al.* 2001*b*).

Intact volume regulation may also be an important factor with respect to sperm survival during or after cryopreservation. It has long been known that major local osmotic gradients across the sperm's membranes are generated during the freeze-thaw cycle, and a two-factor aspect of cellular damage during cryopreservation occurs (osmotic effects versus intracellular ice formation, Henry *et al.* 1993). However, studies by Gao *et al.* (1993) and Holt and North (1994) have shown that cell death occurs mainly during the thawing process, when the dehydrated sperm after undergoing primary hypertonic shock would be exposed to severely hypo-osmotic conditions (Gilmore *et al.* 1996). Therefore, the inability of spermatozoa to regulate the cell volume already within physiological gradients may result in poor freezing ability of the ejaculate, and monitoring the volume regulation in frozen-thawed samples could help to identify the samples with compromised fertilising potential.

The methodology for recording the cell volume of freshly-ejaculated dog spermatozoa has recently been established (Petrunkina *et al.* 2004*a*). We showed that the volume of canine spermatozoa is controlled by quinine-sensitive channels on the plasma membrane and is dependent on cytoskeletal integrity (Petrunkina *et al.* 2004*a*). In the present study, the volume changes in cryopreserved dog semen samples were recorded for the first time. Then, we investigated the sensitivity of the volume regulatory ability to the cryopreservation procedure and the individual influences on volumetric parameters after freezing-thawing. Further, we recorded the response of freshly diluted sperm cells to capacitating conditions and ionophore treatment in live sperm populations to characterise the sensitivity of this parameter to freezing and to individual influences. Finally, we tested the impact of both modern spermatological techniques (electronic measurement of the osmotic responsiveness and volume regulation, and flow cytometric acrosome reaction test) on predictability of the sperm post-thawing response to osmotic challenge and calcium ionophore.

Materials and Methods

Chemicals and solutions

Unless otherwise stated, chemicals were obtained from Merck AG, Darmstadt, Germany, Alexis GmbH, Grünberg, Germany, and Sigma AG (Sigma-Aldrich), Steinheim, Germany.

Fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) was purchased from Vector Laboratories (Burlingame, CA, USA). Calcium ionophore A23187 was obtained from Calciochem (Merck KgaA, Darmstadt, Germany).

A Hepes-buffered Tyrode's medium (HBT) (pH 7.4, 300 mOsm kg^{-1}) consisting of 100 mM NaCl, 3.1 mM KCl, 0.4 mM $MgCl_2$, 25 mM $NaHCO_3$, 0.1 mM $CaCl_2$, 0.3 mM NaH_2PO_4, 1 mM sodium pyruvate, 21.6 mM sodium lactate and 10 mM Hepes (Tyrode's medium; as described by Szasz *et al.* 2000) was used at 38 °C, 5% CO_2 (incubator) for sperm capacitation.

Hepes-buffered saline media without macromolecules (iso- and hypo-HBS; see Petrunkina *et al.* 2001*a*) were used as vehicles for volumetric experimentation. The osmolality of the solutions was measured using a cryo-osmometer (Osmomat 030; Gonotec, Berlin, Germany). To minimise the detection of particular noise during cell volume measurements, the media were passed through a 0.2 μm filter before use.

Tris Egg Yolk Extender Medium consisted of 80 ml Tris stock solution (3.03 g Tris, 1.25 g fructose and 1.8 g citric acid in 100 ml distilled water) and 20 ml egg yolk with the addition of 0.5% Equex STM Paste (Nova Chemicals Sales Inc., Scituate, MA, USA).

Semen collection

Animal housing, care and experimentation (routine semen collection) complied with the animal welfare regulations of Germany. Ejaculates were obtained from eight fertile Beagle dogs (10–20 kg) of the Institute for Reproductive Medicine's colony. Semen collections were performed on a regular basis (generally twice a week) by digital manipulation. Each dog provided two ejaculates, one for testing volume response and one for testing acrosome reaction before freezing (with a total of 8 ejaculates tested for each parameter), and both ejaculates were tested with respect to these parameters after thawing (a total of 16 ejaculates for each parameter) to assess the individual influences; each ejaculate was tested individually. The ejaculates were separated into the different phases (Günzel 1986), and the sperm-rich phases were used for experimentation. The spermatozoa were tested either after dilution, equilibration at room temperature and separation by centrifugation through a two-step gradient of 35% and 70% isotonic Percoll-Saline medium (referred to below as Percoll, Harrison *et al.* 1993), or they were investigated after freezing-thawing (and subsequent washing with Percoll). After removal of the supernatant layers of the gradient, the loose sperm pellet was resuspended in residual 70% Percoll and then diluted in isoHBS. Prior to incubation, semen samples were maintained throughout at room temperature.

Semen processing and cryopreservation

To obtain optimal cryopreservation results, the cryoprotocol described by Linde-Forsberg (1995) was modified as follows.

After dilution of spermatozoa in Tris Egg Yolk Extender Medium containing Equex STM Paste in a proportion of 1:4 and adjustment of the sperm concentration to $300–500 \times 10^6$ cells/ml, the medium was supplemented with 6% glycerol and divided into two aliquots. One aliquot was equilibrated for 15 min at room temperature and used for estimation of motility, cell volume changes in response to osmotic challenge, and induction of the acrosome reaction by calcium ionophore. Another aliquot was packed into a 0.5-ml straw and equilibrated for 90 min at 5 °C before freezing for 10 min in liquid nitrogen vapour. The samples were stored for at least one week in the liquid nitrogen and thawed in a water bath at 38 °C for 30 s prior to use in the experiments.

Experimental design

Samples of freshly diluted and frozen-thawed dog sperm were washed through a Percoll gradient as described above. Assessment of morphology, volumetric and motility analyses were performed immediately after washing and diluting in HBS medium. Subsamples of eight freshly diluted samples and 16 frozen-thawed samples were assessed for volumetric changes as described below. For assessment of acrosome-reacted sperm (8 freshly diluted samples and 16 frozen-thawed samples), the pellets were resuspended in HBT medium (final concentration of sperm about $1–2 \times 10^6$ cells/ml). After 20 min preincubation at 39 °C in humidified air (5% CO_2), calcium ionophore A23187 (2.5 μM) was added to the samples, which were incubated for a further 15 min and assessed for viability and acrosome integrity as described below.

As a control, sperm were incubated in HBT medium without addition of calcium ionophore.

Volumetric measurements

Semen samples washed through Percoll were diluted in an isotonic (300 mOsmol kg^{-1}), hypotonic (180 mOsmol kg^{-1}) or hypertonic (450 mOsmol kg^{-1}) HBS medium at 39 °C to a final concentration of about $1–2 \times 10^5$ cells/ml. After 5 min and 20 min thereafter, two subsamples were taken for analysis of the sperm population volume distributions. The average modal cell volume derived from these two measurements was used in the further analysis. At each examination time, each single sample from each incubated sperm suspension was passed through a CASY 1 cell counter (Schaerfe Systems GmbH, Reutlingen, Germany), which produced cell volume information on the basis of cell frequency distribution. During the measurements, the cells suspended in the electrolyte solution pass the capillary pore. The changes in the electric resistance caused by cells passing the pore lead to voltage changes and are proportional to the cell volume. The measurement signal is registered at the frequency of 1 MHz. The entire signal course is calculated from the single signal measurements by integration (pulse area analysis). This methodology guarantees high accuracy and resolution of measurements (dynamics $>1:32\,000$ in volume); the pulse area values are not dependent on orientation of the sperm cells in the

capillary pore or on the geometrical shape of cells. The recorded electric signals are converted to volume values using a channel analyser with 512 000 measured volume channels (each channel contains the cells which produce the signal with a corresponding pulse area by passing the pore). Further, these original volume signals are re-calculated to a linear distribution within 1024 effective cell size channels. The sample volume setting was 200 μl and the size scale 10 μm; each sampling obtained data from more than 10 000 cells; this resolution corresponds to ~0.1 μm per effective size channel. Effects were largely judged by comparing the modal volumes of such distributions, since modal volume is the most sensitive parameter of the response to osmotic stress (Petrunkina & Töpfer-Petersen 2000). By using modal volumes of different subpopulations assessed under different osmotic conditions, it is possible to differentiate between live, osmotically active sperm and osmotically inactive particles (peak with lower volume contains dead sperm and debris, Petrounkina *et al.* 2000). The high degree of differentiation between peaks related to sperm and debris or non-sperm particles can be improved by Percoll-gradient centrifugation which essentially leads to the elimination of diluent and plasma droplets (Petrunkina *et al.* 2004*a*).

Because the electrical conductivity of hypoHBS or hyperHBS was different from that of isoHBS (due to different concentrations of electrolyte), data recorded from sperm suspensions in the former two media were multiplied by a correction factor. The real volumes were derived by multiplication of the volume equivalents by a calibration factor. The complete multiplication factors were 1.44, 1.2 and 0.98 for data obtained in hypotonic, isotonic and hypertonic solutions respectively, and were obtained according to Petrounkina *et al.* 2000.

Analysis of volumetric data

Unless otherwise stated, the analyses used the modal values of the volume distribution (corrected for the different osmotic conditions). (The modal values were the volume equivalents of the channels detecting local maxima in terms of numbers of particles, and were obtained directly from the cell volume measurements by means of CASY software.)

The relative volume shift, V_r, was used as a measure of the volume response to hypotonic conditions. It was defined in hypotonic solution as $V_r = V_{hypo}/V_{iso}$, where V_{hypo} is the modal value of the hypotonic volume distribution and V_{iso} the modal value of the isotonic volume distribution, V_r5 describes the relative volume increase in the face of hypotonic challenge after 5 min and V_r20 its change (volume decrease) after 20 min. In hypertonic solutions, the reactivity to osmotic stress was expressed as $V_r = V_{hyper}/V_{iso}$, where V_{hyper} is the modal value of the hypertonic volume distribution and V_{iso} the modal value of the isotonic volume distribution, and V_r after 5 and 20 min describes the degree of shrinking and the regulatory volume increase.

The regulatory volume decrease (RVD) was defined as the recovery of the relative hypotonic volume $RVD = V_r5 - V_r20$; and the regulatory volume increase (RVI) was defined as the recovery of the relative hypertonic volume $RVI = V_r5 - V_r20$.

When several peaks appeared within a volume distribution (see Petrunkina *et al.* 2004*a*), the volume of the first most abundant osmotically active subpopulation peak was used for the analysis. The subpopulation was considered to be osmotically active when the relative volume shift $V_r = V_{hypo}/V_{iso}$ was greater than 1. This reflects the fact that the spermatozoa within this population were able to swell and were alive (Petrounkina *et al.* 2000).

Motility measurements

The motility measurements were performed using a computer-assisted motility analysis system, CASA (Minitüb, Tiefenbach, Germany). Sperm samples (8 μl) were placed in a cell chamber (MTM Mika Chamber, Minitüb, depth 0.015 mm) under a microscope. The tracks of at least 200 sperm were recorded and evaluated. On average, 4 images were evaluated in 32 frames per analysis at 20 ms intervals. The setting for canine sperm was chosen according to Günzel-Apel *et al.* (1993). The Cell Motion Analyser 2.0 software (CMA, Medical Technologies; Montreux, Switzerland) was used to evaluate the percentage of motile sperm and to track velocities: average path velocity, straight line velocity and curvilinear velocity.

Flow cytometric measurements

Sperm samples were labelled with propidium iodide stain (PI, 5 μg/ml final concentration), which binds to DNA in membrane defective cells and can therefore commonly be used for recording membrane integrity (cf. Harrison & Vickers 1990), and FITC-PNA (final concentration 10 μg/ml) for 10 min. Thereafter, the samples were subjected to flow cytometric analysis with a Dako Galaxy flow cytometer (DakoCytomation GmbH, Hamburg, Germany). For each sample, three measurements of FITC-PNA fluorescence were made and the average proportion of the acrosome-reacted spermatozoa was calculated from these three repetitions of the same sample. The cells were excited at 488 nm using an argon laser. PNA-fluorescence was detected using a 520 nm band pass filter (FL-1), and propidium iodide fluorescence was detected using a 610-nm pass filter (FL-3), both on logarithmic scales. The forward and side scatter data (FSC and SSC) were collected in the linear mode.

For each day's series analyses, the control settings were adjusted as follows. An unstained sperm sample was first passed through the instrument. The forward and side scatter settings were adjusted so that the sperm population was presented as an L-shaped profile. The fluorescence

detector voltages were adjusted after measuring the sample stained only with PI so that dead cells were clearly distinguishable from the live (unstained) cells on the Y-axis (FL-3). Unstained cells were detected near the origin with respect to the X-axis (FL-1). An FITC-PNA-stained sperm sample was passed through the flow cytometer, and the setting was adjusted to yield an intensity of the acrosome-intact population in the first decade of the log4 scale, chosen from the preliminary experiments. Data were analysed using Flomax software (version 1997–1999 by Partec, Münster, Germany). Quadrants and ranges were delineated to discriminate between acrosome-intact and acrosome-defective sperm (left and right quadrants respectively) as well as between membrane-intact and membrane-defective spermatozoa (lower and upper quadrants respectively). The FL-1 fluorescence data were also available as one-dimensional histograms for determination of the proportion of responding cells within the live sperm population (live cell populations were selected by gating of low-PI fluorescence in FSC-FL-3 distribution). The response to cryopreservation was determined by comparing quadrant or histogram distributions for the proportion of membrane-intact acrosome-reacted sperm in Ca^{2+}-ionophore-incubated suspensions in freshly diluted and frozen-thawed semen samples respectively.

Statistical analysis

Data were analysed using Excel software, CASY 1 software and the statistics software package SAS (version 7; SAS Inst. Inc., Cary, NC, USA). Non-parametrical ANOVA was used to perform the analysis of variance on the individual differences with respect to sperm responsiveness to incubation in capacitation medium in the presence of calcium ionophore. The analysis of variance was made using the GLM (General Linear Model) procedure to investigate the freezing effect. The Pearson correlation analysis was performed (CORR procedure) to investigate the relationship between different spermatological parameters and between parameters before and after freezing-thawing. Unless otherwise stated, data are presented as means± S.E.M. Differences were considered to be significant if the calculated probability of their occurring by chance was less than 5% ($P < 0.05$).

Results

Computer-assisted motility (CM) and acrosomal alterations

The computer-assisted motility parameters linearity (LIN), average path velocity (VAP), straight velocity (VSL), curvilinear velocity (VCL), and total CM were significantly decreased in frozen-thawed semen as compared with freshly diluted semen ($P < 0.001$); the changes in velocities are shown in Fig. 1.

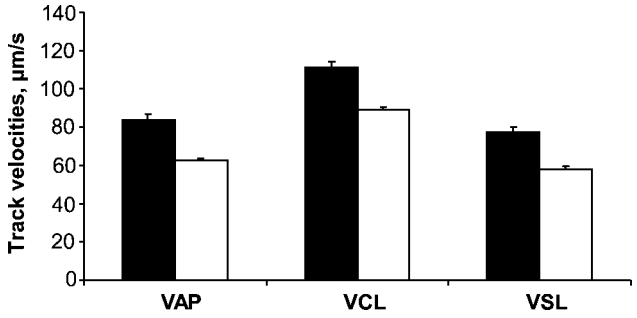

Figure 1 Changes in track velocities after cryopreservation. Data represent the average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL) before (black bars) and after (white bars) freezing-thawing of dog spermatozoa, assessed with CMA. The changes induced by cryopreservation were significant ($P \leq 0.001$) for all track velocities, and for the percentages of the linearly motile cells and total motility (data not shown).

The CM analysis (CMA) was sufficiently sensitive to visualise the differences between individual semen donors after freezing-thawing. There were significant differences in linearity, the percentages of linearly motile sperm, straightness and straight line velocity ($P < 0.05$). No significant individual differences after freezing-thawing were observed with respect to the percentage of the spermatozoa with acrosomal alterations ($P > 0.05$).

Changes in spermatozoal volume after cryopreservation

In frozen-thawed spermatozoa, the major peak of sperm volume occurred in the range between 2 and 7 fl in several samples. This peak usually contains dead or deteriorated sperm, cell debris and 'mess' (Petrunkina *et al.* 2004*a*). This peak was observed only sporadically or not at all in freshly diluted sperm.

In both freshly diluted and frozen-thawed sperm, the sperm volume of the osmotically active subpopulation after a short incubation time was changed as a reciprocal of osmolality ($P < 0.05$; Fig. 2), demonstrating that dog sperm behaves as a perfect osmometer in both situations.

Freezing-thawing led to increased hypertonic and isotonic volumes as compared with the volumes of freshly diluted sperm ($V_{450} = 11.9 \pm 1.2$ fl in frozen-thawed vs $V_{450} = 10.0 \pm 0.5$ fl in freshly diluted semen, $P < 0.05$ for the effect on hypertonic volume; and $V_{300} = 16.3 \pm 0.6$ fl in frozen-thawed vs $V_{300} = 14.9 \pm 0.7$ fl in freshly diluted semen, $P < 0.09$ for the effect on isotonic volume; other parameters did not reach the significance level due to high variation in the frozen-thawed sperm). Volume regulatory ability after freezing-thawing was diminished: RVD $= 0.06 \pm 0.23$ vs -0.19 ± 0.14 in freshly diluted and frozen-thawed sperm respectively, and RVI $=$ 0.06 ± 0.05 vs 0.006 ± 0.07 in freshly diluted and frozen-thawed sperm respectively, but these tendencies were not significant. However, the nominal reduction of RVI ability was observed in 5 out of 8 dog semen donors, and

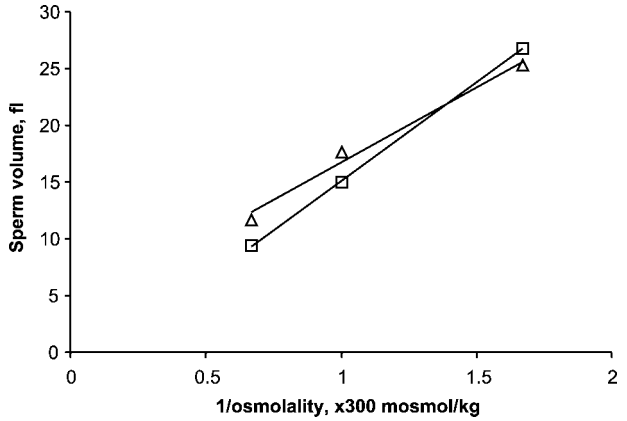

Figure 2 Osmotic behaviour of freshly diluted and cryopreserved dog spermatozoa. Dog spermatozoa behave as linear osmometers in the range of 180 to 450 mosmol/kg before and after freezing-thawing, demonstrating a closely similar response pattern in this range. Sperm volume (V) changed linearly, reciprocal to osmolality both before and after cryopreservation: $V = 17.4x–2.33$ and $V = 13.3x + 3.4$ respectively; $R^2 = 0.99$; x is reciprocal to osmolality. Open squares, freshly diluted sperm samples; open triangles, frozen-thawed sperm samples.

reduction of RVD ability was observed in 6 out of 8 dog semen donors.

The volumetric parameters demonstrated high sensitivity with respect to differences between individual samples of the frozen-thawed sperm; this was observed for hypertonic and isotonic cell volume, relative volume decrease, and regulatory volume increase ($P < 0.05$). Some ejaculates contained cells responding strongly to hypotonic and hypertonic conditions as well as showing an increase in isotonic volume after cryopreservation, while other ejaculates contained dominant subpopulations of weakly responding but osmotically active cells (Fig. 3). Individual volumetric data for hypertonic and isotonic volume for four selected dogs are presented in Fig. 4.

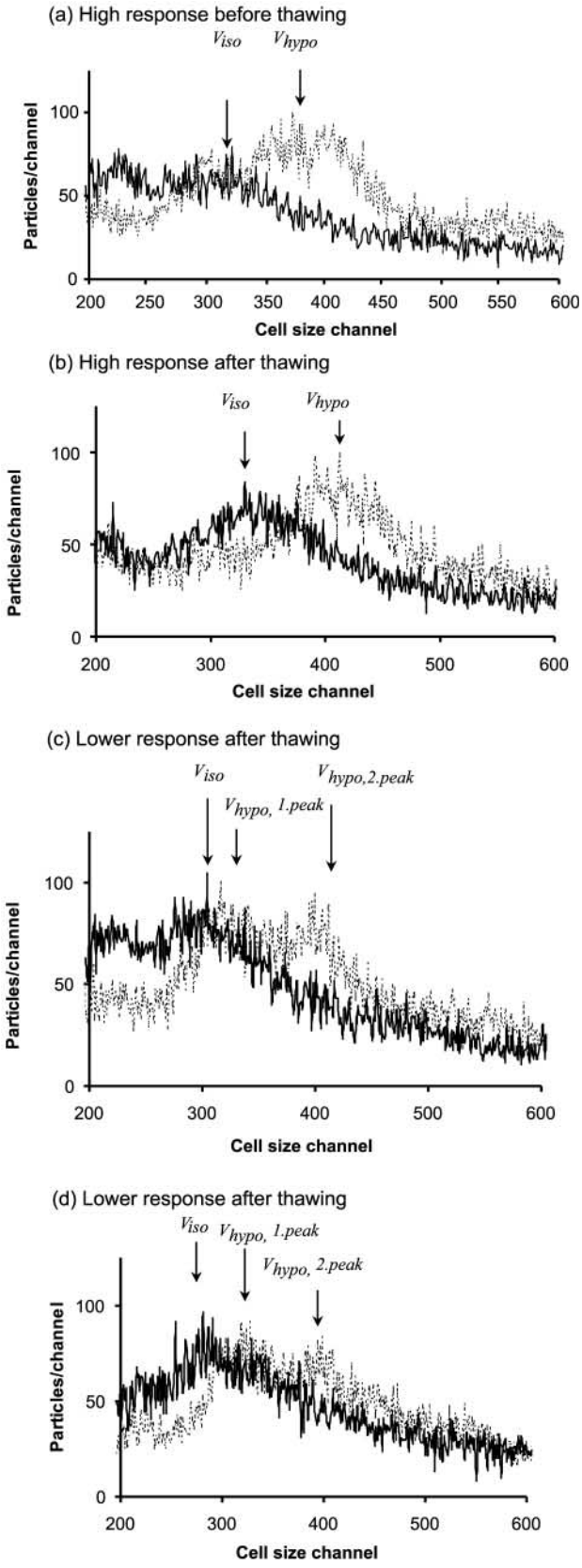

Figure 3 Volume distributions in individual dog sperm samples before and after freezing, under isotonic conditions and after hypotonic shock. (a and b) Histograms representing volume response patterns from the dog sperm donors containing cells which were highly responsive with respect to osmotic challenge. This semen sample showed a higher level of isotonic volume and higher response to hypotonic challenge before and after cryopreservation: $V_{iso} = 16.2$ fl, $V_{hypo} = 25.2$ fl before freezing, and $V_{iso} = 17.5$ fl and $V_{hypo} = 34.0$ fl after freezing-thawing; $V_r = 1.71$ and 2.14 respectively. (c and d) Histograms representing the response patterns from a dog ejaculate containing cells that showed a low isotonic cell volume and poor reaction to osmotic challenge both before and after cryopreservation (first osmotically active peak): $V_{iso} = 13.6$ fl, $V_{hypo} = 19.0$ fl before freezing-thawing, and $V_{iso} = 10.8$ fl and $V_{hypo} = 17.0$ fl after freezing-thawing; $V_r = 1.53$ and 1.73 respectively. The responsiveness of these samples to hypertonic conditions was similar.

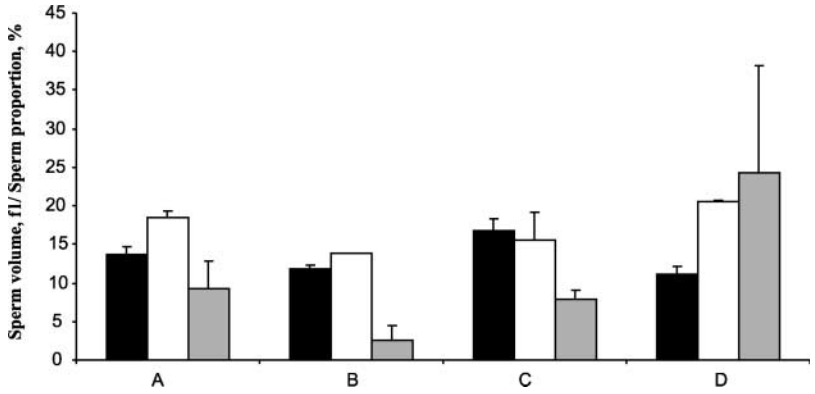

Figure 4 Individual differences in the proportion of live acrosome-reacted spermatozoa and isotonic and hypertonic volume in dog spermatozoa. Data are presented for four selected semen donors (A, B, C, D) which showed considerable differences in volumetric behaviour and responsiveness to capacitating conditions after freezing-thawing. Black bars represent the hypertonic cell volume after 20 min of incubation, white bars represent the isotonic cell volume, and grey bars represent the the proportion of the acrosome-reacted and live cells.

Changes in the proportion of live acrosome-reacted spermatozoa in sperm incubated under capacitating conditions with calcium ionophore

The percentage of sperm responding to ionophore in live cell populations was reduced from 14.8 ± 6.1% before freezing to 9.0 ± 1.9% after freezing-thawing ($P < 0.05$). The levels of acrosome-reacted live sperm detected in sperm samples incubated without addition of calcium ionophore were very low (1.5 ± 0.1% post-thawing, $n = 32$); therefore the comparison between the proportions of live acrosome-reacted spermatozoa in freshly diluted and frozen-thawed samples refers to data produced in samples treated with calcium ionophore.

When analysed pairwise, significant differences between individual samples after freezing-thawing could be detected with flow cytometric parameters ($P < 0.05$). The elevated levels of the acrosome reaction were observed in some individual dogs, for which the deviation in volumetric parameters have already been shown (Fig. 4). Samples from some dogs showed a high level of live acrosome-reacted cells after incubation under capacitating conditions in the presence of the ionophore, while samples from other dogs contained cells which responded

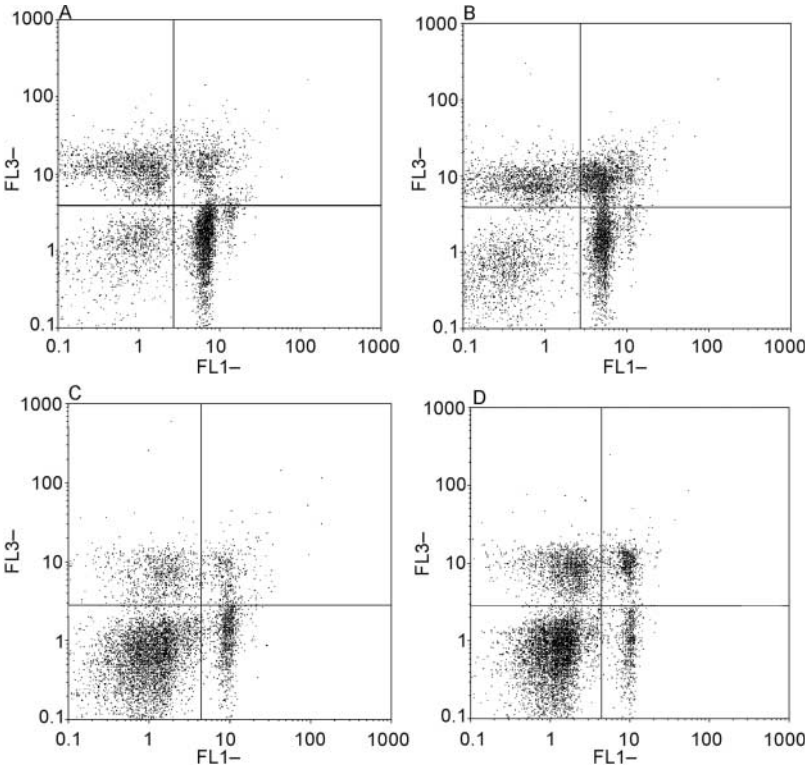

Figure 5 Flow cytometric dot plots, showing staining patterns of dog spermatozoa labelled with FITC-PNA and propidium iodide after calcium ionophore treatment before (left) and after (right) cryopreservation. (A and B) Dot plots representing staining patterns in semen samples containing cells which were highly responsive with respect to loss of acrosomal integrity in the presence of ionophore. The semen of this individual showed higher levels of live acrosome-reacted cells both before and after cryopreservation, 55% and 36% respectively. (C and D) Dot plots representing staining patterns for a dog ejaculate containing cells that showed a low proportion of acrosome reaction after incubation in HBT medium containing ionophore, 15% and 9.0% of live acrosome-reacted cells before and after cryopreservation respectively.

Table 1 Correlation between different spermatological parameters in frozen-thawed sperm samples ($n = 8$).

	MAA	CMA	SMA	VAP	VCL	VSL	ARL
V_{hyper},5	−0.13	−0.31	−0.54	0.10	0.52	0.07	0.69†
V_{hyper},20	−0.91**	0.35	0.09	0.18	0.59	0.24	0.04
V_{iso},5	−0.30	0.13	0.67†	0.91**	0.90*	0.95*	0.15
V_{iso},20	0.02	−0.44	0.04	0.56†	0.72*	0.48	0.88*
V_{hypo},5	−0.19	−0.32	−0.20	0.40	0.71*	0.32	0.91*
V_{hypo},20	0.05	−0.27	0.17	0.71*	0.67†	0.61	0.74*
$V_{r,hyper}$,5	0.33	−0.30	−0.90*	−0.83*	−0.65†	−0.86*	0.14
$V_{r,hyper}$,20	−0.84*	0.56	−0.01	−0.19	0.13	−0.09	−0.43
$V_{r,hypo}$,5	0.27	−0.39	−0.84*	−0.67†	−0.48	−0.75*	0.50
$V_{r,hypo}$,20	0.07	0.07	−0.23	−0.45	0.19	0.39	0.14
RVD	0.17	−0.33	−0.74*	−0.73*	−0.45	−0.76*	0.31
RVI	−0.79*	0.58	−0.62†	0.45	0.54	0.54	−0.38

*$P < 0.05$; **$P < 0.001$; †$P < 0.10$.
MAA, morphological alterations of acrosome; CMA, computer-assisted motility; SMA, subjectively assessed motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; ARL, acrosome-reacted live cells after incubation in HBT medium and addition of calcium ionophore.
V_{hyper},5 or V_{hyper},20, cell volume under hypertonic conditions after 5 or 20 min of incubation; V_{hypo},5 or V_{hypo},20, cell volume under hypotonic conditions after 5 or 20 min of incubation; V_{iso},5 or V_{iso},20, cell volume under isotonic conditions after 5 or 20 min of incubation; $V_{r,hyper}$,5 or $V_{r,hyper}$,20, relative volume increase in response to hypertonic conditions after 5 or 20 min of incubation, $V_{r,hyper} = V_{r,hyper}/ V_{iso}$; $V_{r,hypo}$,5 or $V_{r,\ hypo}$,20, relative volume decrease in response to hypotonic conditions after 5 or 20 min of incubation, $V_{r,hypo} = V_{hypo}/V_{iso}$.
RVD, regulatory volume decrease, RVD = $V_{r,\ hypo}$,5 − $V_{r,hypo}$,20; RVI, regulatory volume increase, RVI = $V_{r,\ hyper}$,5 − $V_{r,hyper}$,20.

weakly to capacitating conditions as determined by the levels of acrosome-reacted live cells (Fig. 5).

Correlation analysis between sperm parameters in frozen-thawed dog semen samples

Parameters of volume regulation and of motility, and membrane and acrosomal integrity were correlated in frozen-thawed semen samples (Table 1). There were positive significant correlations particularly between hypertonic and isotonic sperm volumes and track velocities: VAP, VCL and VSL ($P < 0.05$). Moreover, the isotonic volume correlated significantly positively with the percentage of the live acrosome-reacted cells ($r = 0.88$, $P < 0.0003$), indicating that destabilisation of the volume regulation in the sperm cell occurred simultaneously with cryopreservation-induced damage. The ability to regulate the cell volume after hypertonically induced changes (RVI) was negatively correlated with the percentage of the sperm showing morphological alterations ($P < 0.05$); the ability to regulate the cell volume after induction of hypotonic stress was negatively correlated with the path velocities. No pairwise significant correlations were observed between acrosomal integrity of the frozen-thawed sperm and motility parameters after thawing (data not shown).

Prediction of the changes in frozen-thawed dog semen samples based on the membrane changes in freshly diluted sperm

Highly significant correlations were found between hypertonic volumes of freshly diluted sperm and frozen-thawed sperm after 5 and 20 min of exposure to anisotonic conditions (negative and positive correlations respectively; Fig. 6a and b). The 5 min response to hypotonic challenge before freezing had a poor regression relationship with these characteristics after thawing (Fig. 6c); the 20 min response correlated positively with initial ($r = 0.70$, $P < 0.05$) and 20 min volume after thawing ($r = 0.68$, $P < 0.06$, Fig. 6d).

No significant correlations were found between the percentage of the induced acrosome reactions (AR) in live cell populations in freshly diluted sperm and any of the motility parameters, or between the proportion of cells with acrosomal alterations induced by cryopreservation immediately after thawing (data not shown). However, significant correlations were observed between the proportion of live acrosome-reacted cells in freshly diluted and frozen-thawed sperm samples. Moreover, the percentage of live acrosome-reacted sperm after addition of calcium ionophore in freshly diluted samples showed highly significant positive correlations with absolute isotonic and hypotonic volume, but not with hypertonic volume, in frozen-thawed samples (Fig. 7a and b; $P < 0.01$).

Some significant correlations were observed between volumetric parameters before freezing-thawing and acrosome reaction and motility after thawing. The cell volume after 20 min of exposure to hypertonic conditions in freshly-diluted sperm correlated negatively with the percentage of the acrosome-reacted sperm after thawing ($r = -0.80$, $P < 0.02$; Fig. 8a). The recovery of hypotonic volume after 20 min correlated significantly positively with the percentage of live acrosome-reacted cells ($r = 0.72$, $P < 0.05$; Fig. 8b). Only the isotonic volume of the freshly diluted sperm showed significantly positive correlations with the initial sperm track velocities after thawing ($r = 0.71$, $P < 0.05$ for both VAP and VSL). The total motility was negatively correlated with the hypotonic volume of the freshly diluted sperm after 20 min of incubation ($r = -0.64$, $P < 0.09$) and with the relative volume decrease in response to hypertonic conditions ($r = 0.69$, $P < 0.06$). Additionally, motility of cryopreserved sperm showed a non-linear regression with the ability of freshly diluted sperm to recover after hypertonic stress (Fig. 8c and d).

Discussion

Unlike semen of other domestic species, dog spermatozoa respond to cryopreservation in a very specific way, demonstrating broad variability in loss of progressive motility and acrosomal integrity and viability (Rodriguez-Martinez *et al.* 1993). The variability in the ability to tolerate

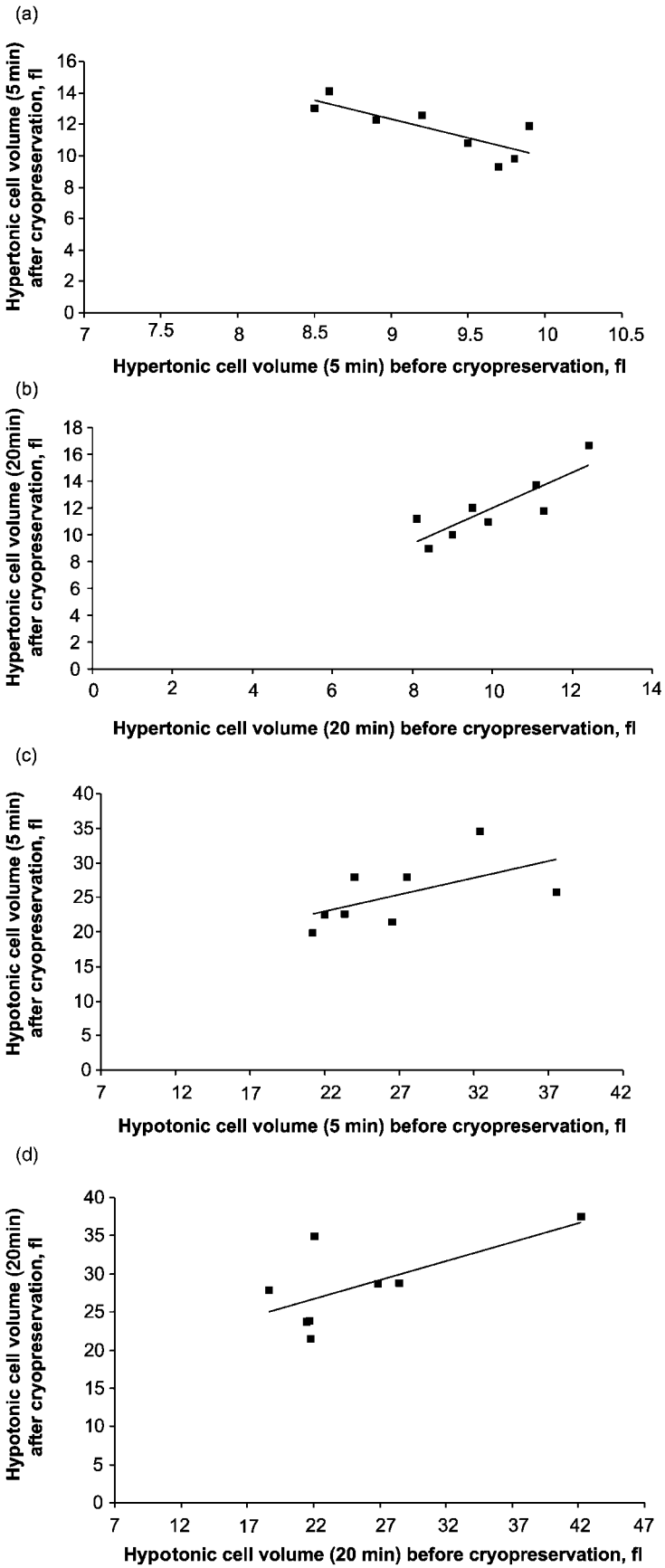

Figure 6 Relation between anisotonic sperm volume before and after cryopreservation. (a) Relation between initial hypertonic volume (5 min exposure) before and after cryopreservation. Significant negative correlations were observed between initial hypertonic volume before (x) and after (y) cryopreservation. Samples with amplified degrees of initial shrinking (lower than values expected according to the perfect osmometer model) demonstrated higher hypertonic volumes after cryopreservation, i.e. a reduced ability to shrink. $R^2 = 0.64$, $P < 0.05$, $y = -2.39x + 33.8$. (b) Relation between cell volume after 20 min of exposure to hypertonic conditions before and after thawing. Significant positive correlations were observed between hypertonic volume before (x) and after (y) cryopreservation. Samples of freshly diluted semen with unimpaired/normal ability to recover after hypertonic stress (higher volumes) demonstrated higher hypertonic volumes after cryopreservation. $R^2 = 0.71$, $P < 0.05$, $y = 1.32x - 1.26$. (c) Relation between initial hypotonic volume before and after cryopreservation. Non-linear regression was observed between initial hypotonic volume before (x) and (y) after cryopreservation. Samples of freshly diluted semen with increased ability to swell in response to hypotonic challenge showed a higher responsiveness level after cryopreservation (nearly linear behaviour in the normal response range of 20–30 fl). However, it is likely that the highly amplified degree of initial swelling (much higher than values expected according to the perfect osmometer model) reflects membrane instability which could lead to loss of membrane integrity after cryopreservation. $R^2 = 0.55$, $P < 0.13$, $y = -0.08x^2 + 5.6x - 60.0$. (d) Relation between cell volume after 20 min of exposure to hypotonic conditions before and after thawing. Significant positive correlations were observed between hypotonic volume before (x) and after (y) cryopreservation. Samples of freshly diluted semen with unimpaired/normal ability to recover after hypotonic stress (lower volumes) demonstrated lower hypotonic volumes after cryopreservation. $R^2 = 0.46$, $P < 0.06$, $y = 0.5x + 15.7$.

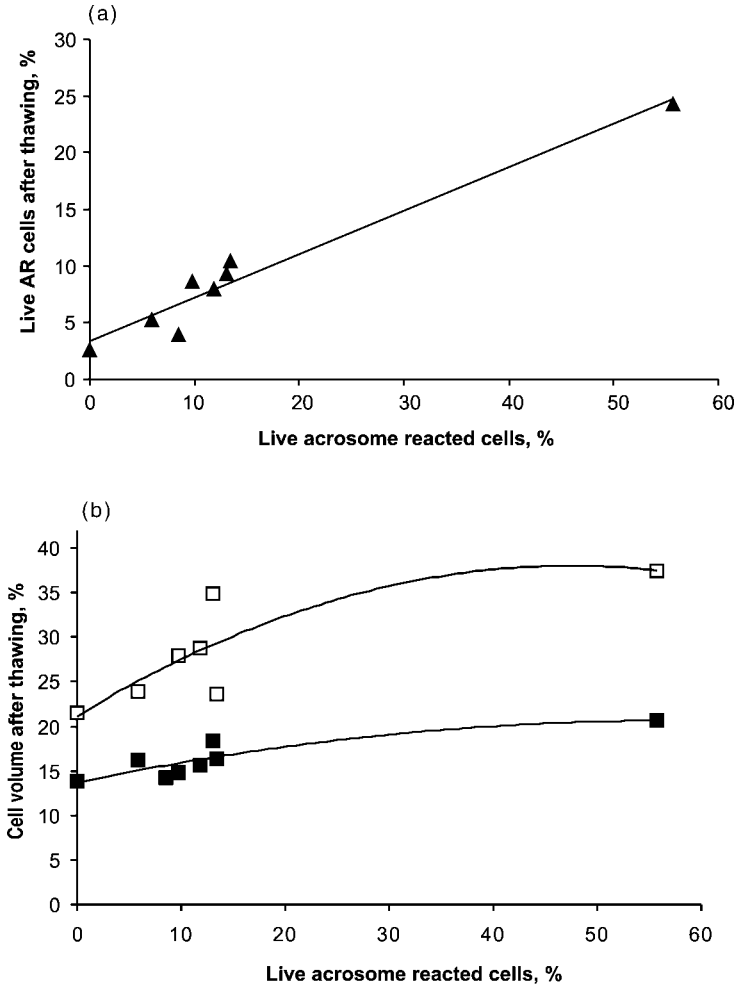

Figure 7 Relation between different membrane parameters in live populations before and after cryopreservation. (a) Proportion of live acrosome-reacted cells in the presence of ionophore in freshly-diluted semen (x) vs cryopreserved sperm (y). A strong positive correlation was found between these two parameters: $R^2 = 0.95$, $P < 0.05$, $y = 0.39x + 3.35$. (b) Proportion of live acrosome-reacted cells in the presence of ionophore in freshly diluted semen (x) vs isotonic (closed squares, y_1) and hypotonic (open squares, y_2) volume in cryopreserved semen. Significant positive correlations were found between these parameters: $r = 0.86$, $P < 0.01$ for y_1, and x; $r = 0.77$, $P < 0.03$ for y_2 and x.

freezing between individual semen donors is one of the sources of variability in canine pregnancy rates, which vary between 40% and 60% (Linde-Forsberg 1995).

During cryopreservation, essential changes take place in the major osmotic gradients across the membrane. Therefore, the responsiveness of spermatozoa to osmotic challenge and their ability to regulate cell volume is a characteristic closely related to cryopreservability. Electronic measurement of cell volume is a computer-assisted method for detection and quantification of the membrane changes in live sperm cell populations characterised by high sensitivity to individual ejaculates (Petrounkina *et al.* 2000, Petrunkina *et al.* 2004*a*). The response of the cell volume of spermatozoa in different species to anisotonic conditions reflects the profound changes in the sperm plasma membrane, and this response is related not only to the membrane's permeability to the major intracellular ions, but also to the functionality of the ion channels and to cytoskeletal integrity (Petrunkina *et al.* 2001*a*, Petrunkina *et al.* 2004*a*,*b*).

Absolute volumes under different osmotic conditions and the volumetric parameters derived here underscored the differences between individual samples after thawing, indicating that this parameter fulfils the requirement of sensitivity. The response of the dog sperm volume to anisotonic conditions was found to be reciprocally proportional to the osmolality, confirming that dog sperm, like bull and boar sperm, act as a perfect osmometer (Drevius 1972, Petrunkina & Töpfer-Petersen 2000). The slope of the linear response remained almost unchanged after the cryopreservation procedure, demonstrating that dog sperm are relatively robust in terms of their initial osmotic behaviour.

Changes induced by cryopreservation resulted in markedly increased isotonic and hypertonic volumes; this indicates that the regulation mechanisms such as chloride channels, quinidine-sensitive cation channels or protein kinase-dependent mechanisms responsible for changes in intracellular ion concentrations (Petrunkina *et al.* 2004, Petrunkina *et al.* 2003*b*) seem to be affected by the damage induced by cryopreservation. As there was less effect on the hypotonic volume, the related mechanisms, i.e. quinine-sensitive channels, thus appear to be more stable in the face of cryoconservation. In other species, there have been reports of the negative effect of cryopreservation on the outcomes of the hypo-osmotic swelling test (HOST) (Hauser *et al.* 1992, Ponce *et al.* 1998).

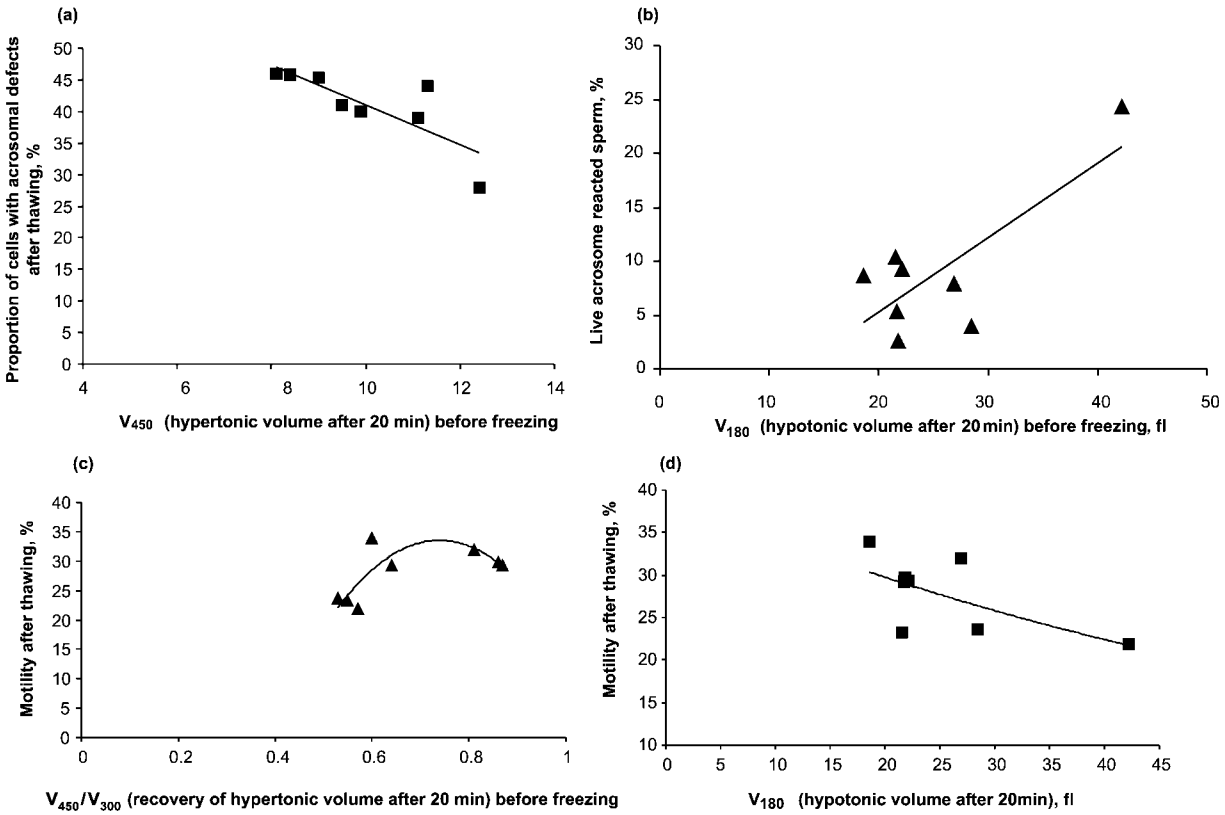

Figure 8 Relation between hypertonic and hypotonic volume recovery, acrosome reaction and motility. (a) Relation between ability to recover after hypertonic stress (V_{450}, hypertonic volume after 20 min) in freshly-diluted sperm (x) vs increase in loss of acrosomal integrity after cryopreservation (y). Ejaculates containing cells with a low degree of recovery (reduced hypertonic volumes) demonstrated a higher degree of acrosomal loss after freezing-thawing as evaluated by light microscopy; freshly diluted ejaculates with a higher recovery ability maintained a low level of acrosome defective cells after freezing-thawing: $y = -3.2x + 72.6$, $R^2 = 0.64$, $P < 0.05$. (b) Relation between ability to recover hypotonic volume (V_{180}, 20 min) in freshly diluted sperm (x) vs the proportion of live acrosome-reacted cells in the presence of ionophore after thawing (y). Ejaculates containing cells with a low degree of volume recovery (higher hypotonic volumes) demonstrated a higher proportion of live acrosome-reacted cells after freezing-thawing; freshly diluted ejaculates with higher recovery ability maintained a low level of acrosome-defective cells after incubation of cryopreserved sperm samples in HBT medium containing calcium ionophore: $y = 0.69x - 8.6$, $R^2 = 0.59$, $P < 0.03$. (c) Relation between the ability to recover after hypertonic stress (expressed by the relative volume shift V_r) in freshly-diluted sperm (x) vs increase in loss of motility after thawing (y). Ejaculates containing cells with a low degree of recovery demonstrated higher loss of motility after freezing-thawing; freshly diluted ejaculates with higher recovery ability maintained a relatively stable motility level after thawing: $y = -260.6x^2 + 385.1x - 108.8$, $R^2 = 0.61$, $P < 0.09$. (d) Relation between the ability to recover hypotonic volume after 20 min (V_{180}, 20 min) in freshly diluted sperm (x) vs increase in loss of motility after thawing (y). Ejaculates containing cells with a low degree of recovery demonstrated higher loss of motility after freezing-thawing; freshly diluted ejaculates with a higher recovery ability maintained a relatively stable motility level after thawing: $y = 39.5e^{-0.01x}$, $R^2 = 0.41$, $P < 0.09$.

However, there are conflicting reports on the reliability of HOST for evaluation of functionality and fertilising competence of sperm after freezing-thawing. Whereas in some species (rainbow trout and humans) the outcome of the HOST test does not correlate with functional and structural integrity of the sperm plasma membrane or with fertility (Hauser *et al.* 1992, Cabrita *et al.* 1999), the reliability of this test has been confirmed in other species (bovine, Brito *et al.* 2003). Although no significant correlations were found in this study for dog sperm between morphological integrity of plasma membrane (PI-negative cells) and functional integrity (relative volume increase to hypotonic conditions), there were significant positive correlations between absolute isotonic and hypotonic volumes and between the percentage of live acrosome-reacted sperm after freezing-thawing, indicating the functional relationship between these two parameters. Moreover, there was a relationship between sperm track velocities and volumetric parameters of frozen-thawed sperm. Increased sperm velocities, characteristic of sperm undergoing hyperactivation (as an indication of cryopreservation-induced cell destabilisation), were also observed in frozen-thawed samples with elevated isotonic and hypotonic volumes and lowered RVD.

Significant correlations were found between the ability to respond to hypotonic and hypertonic challenge in freshly diluted semen in comparison with the osmotic volume response in thawed semen. The freshly diluted samples demonstrating functional disturbance of volume regulatory ability (higher isotonic volumes and a high

degree of initial shrinking and/or swelling before freezing-thawing indicating that functionality of the ion channels and/or major intracellular ion concentrations were affected in these samples) also exhibited the characteristics commensurate with instability of cellular volume regulation after cryopreservation. In contrast, samples with higher hypertonic volumes and lower hypotonic volumes after 20 min exposure before thawing maintained a sufficient level of volume regulatory ability after cryopreservation. This suggests that the cell population reacts to cryopreservation conditions in a similar manner as to osmotic stress in terms of the volumetric response. This indicates that at least a part of the functional instability induced by the cryopreservation (i.e, loss of membrane integrity, the ability to respond to stress or to maintain the constant level of the isotonic volume) is mediated by changes in intracellularly osmotically active components (ion concentrations) and changes in functionality of volume regulatory mechanisms during freezing-thawing.

Although the level of acrosome-reacted cells after cryopreservation is a well-accepted parameter for estimating sperm function and response to cryopreservation, relatively long incubation periods are necessary to record changes in the plasma membrane sensitively (i.e. Peña *et al.* 2001). A more specific instrument with a high level of predictability was provided by the approach demonstrating that the changes in the sperm membrane in response to ionophore treatment (the percentages of deteriorated and acrosome-reacted cells) are related to acrosome reaction induced by cryopreservation and motility after thawing (Szasz *et al.* 2000). In the present study the differentiation was made between live and dead acrosome-reacted cells after incubation in HBT medium containing the calcium ionophore. Such specification may not be necessary if the cryopreservation-induced damage results in a low proportion of live acrosome-intact and a relatively high proportion of dead cells (as reported by Szasz *et al.* 2000, Peña *et al.* 2003*a*). However, if the frozen-thawed samples show a relatively high level of viable sperm, and the proportion of acrosome alterations remains at a relatively moderate level after cryopreservation (29–54% in this study), additional differentiation is required between live and dead acrosome-reacted cells. We were unable to see any correlation between the percentage of live acrosome-reacted cells in freshly diluted sperm exposed to ionophore under capacitating conditions and the proportion of morphological alterations induced by cryopreservation, unlike Szasz *et al.* (2000) who observed such a relationship for the total percentage of sperm responding to ionophore. These differences could be caused by different cryopreservation and evaluation procedures and by the fact that the range of cryopreservation-induced acrosome reaction was narrower in our study than in Szasz *et al.* (2000). However, the levels of live cells responding to ionophore treatment were highly sensitive to the freezing-thawing process, differed between individual animals, and correlated with levels of live cells responding to calcium ionophore after thawing. Therefore, more sensitive differentiation between live- and dead-responding cells may be advantageous for sperm quality estimation and subtle predictability of cryopreservability in terms of responsiveness to a secondary stress.

Some cross-relations were observed with respect to predictability of both membrane parameters (live acrosome-reacted sperm and cell volume response). This indicates a relationship between structural membrane changes, allowing PNA to bind to the outer acrosomal membrane (characterised by the response to ionophore and resulting in Ca^{2+} entry in the apical sperm head region), and the functional membrane changes (characterised by the response in cell volume and occurring mainly in the tail region). It has already been reported for bull semen in terms of cell population proportions that the poor response to HOST may be associated with spontaneous acrosome reaction (Thundathil *et al.* 2002). The quantitative analysis of sperm cell volume performed in the present study suggests that sperm that maintain high swelling levels instead of recovering have impaired membrane function, which predisposes them, after thawing, to undergo acrosome reaction in response to capacitating conditions and calcium ionophore. Moreover, lack of RVD or RVI (expressed in increased hypotonic volumes after longer exposition times or in a delayed or missing increase after initial shrinking after exposure to hypertonic conditions) resulted in compromised motility after thawing, confirming that the ability to regulate volume is related to sperm motility (cf. Yeung & Cooper 2001). Similarly, increased sperm velocities immediately after thawing, which indicate the onset of capacitation-like changes, were observed in samples whose isotonic volume was already high before cryopreservation. However, neither parameter describing functional membrane changes (volume response and acrosome reaction in live cell populations) could completely predict the functional response in the other parameter after cryopreservation. It was possible to predict the post-thaw volume behaviour under iso- and hypotonic conditions, but not hypertonic volume, by estimating membrane changes induced by ionophore in freshly diluted samples. Indirectly, that indicates that the modification in the regulatory mechanisms involved in the control of hypotonic and isotonic volume and subsequent ionic changes may be calcium dependent. Similarly, only the hypotonic and hypertonic volume, but not the isotonic volume, of freshly diluted sperm were related to the acrosome reaction levels after cryopreservation. As a single sperm function test usually examines only a narrow range of the sperm attributes required for fertilising an oocyte *in vivo*, such tests are therefore more useful for identifying specific causes of function failure. As combining the results of the various tests appears to improve the reliability of sperm function estimation, both these parameters should be assessed to permit subtle prediction of sperm function parameters

after cryopreservation and to estimate the expected cryopreservation-induced damage.

In conclusion, the use of electronic volume analysis and flow cytometry represents a simple, rapid and sensitive method to estimate sperm quality or to improve the cryopreservation procedure by detecting changes in the sperm plasma membrane by monitoring the sperm cell volume and ionophore-induced changes in the live cell population. The variability in response to osmotic stress or calcium ionophore treatment under capacitating conditions may reflect the subtle differences in sperm membrane functionality which are crucial for the cryopreservability of the ejaculates. For best results, both procedures have to be performed together. The financial support of the Deutsche Forschungsgemeinschaft (DFG) is gratefully acknowledged.

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