WATER VOLUME OF RABBIT SPERMATOZOA MEASURED BY ELECTRON PARAMAGNETIC RESONANCE AT DIFFERENT TEMPERATURES

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Abstract - During cryopreservation cells undergo severe changes due to osmotic pressure of the medium, shrinking to equilibrate their intracellular water to that of the external solution. Therefore, knowledge of cell water content as a function of temperature is a crucial parameter in establishing freezing procedures. In spermatozoa, this information has been obtained by an indirect osmotic procedure which, taking into account water permeability and its activation energy, leads to a theoretical curve connecting intracellular water with temperature. We propose an EPR method which can directly estimate the cell water content at different sub-zero temperatures (-5 °C, -10 °C). The method is based on the use of spin label Tempone and the broadening agent potassium-chromium oxalate. The determination of the intracellular water showed that a maximum of about 44% of the cellular water is involved in cell dehydration during freezing. These results differ substantially from values obtained by others with osmotic procedures. The present findings could help for developing suitable freezing process of rabbit spermatozoa.

INTRODUCTION

During freezing, spermatozoa are subjected to severe osmotic stress caused by water crystallization in the external medium. The increased osmotic pressure in the unfrozen medium induces an egress of intracellular water with a consequent cell shrinkage. The amount of water crystallization during freezing mainly depends on the cooling rate and cryoprotectants added to the medium. The optimum cooling rate is specific for each cell type and depends on water permeability (Lp) and on its activation energy (Ea); cells exhibiting high Lp, permit elevated cooling rates to be used without any risk of intracellular ice formation that can be lethal for the cell. The knowledge of the intracellular water, as a function of temperature, is crucial in order to minimize cell damage which can occur during cryopreservation. The optical measurement of sperm cell volume is however more difficult than for other cell types because of the reduced volume and the non-spherical shape of spermatozoa.

A common procedure to indirectly determine spermatozoa response to deep freezing consists on solving differential equations obtained from the osmotic behaviour of spermatozoa (MAZUR, 1984); such an approach, which requires values for water volume, Lp, Ea and assumes that the cell behaves as an ideal osmometer (water volume varies linearly in accord with the osmolality of external solution) permits to plot a theoretical curve relates cell intracellular water and sub-zero temperatures at different cooling rates.

A different approach, based on the Electron paramagnetic resonance (EPR) technique has been developed to measure spermatozoa water volume of many species (mouse - DU *et al.*, 1994; human - KLEINHANS *et al.*, 1992). This method has been mainly applied to establish the osmotic behaviour of sperm cells.

The aim of the present study is to extend this method to directly determine the water volume of spermatozoa in isosmotic medium at different temperatures. Such an approach has allowed us to directly check the behaviour of sperm cells during the freezing process without any passage for the "osmotic" procedure.

MATERIALS AND METHODS

Preparation of rabbit spermatozoa - Semen samples were collected from 10 rabbit bucks by an artificial vagina and were immediately analyzed to estimate cell concentration (with hemocytometer) and live sperms (tryphan blue staining 0.5 W/v for 20 min). Semen was pooled and released into an isosmotic (286 mOsm) PBS medium (1:1). A swimming-up procedure was performed (37 °C for 1 hour) and spermatozoa, maintained at room temperature, were used within 3 hours. The uppermost 1 ml of buffer, containing motile sperms, was aspirated and diluted in 1 ml of PBS. Two successive experiences were performed with these spermatozoa.

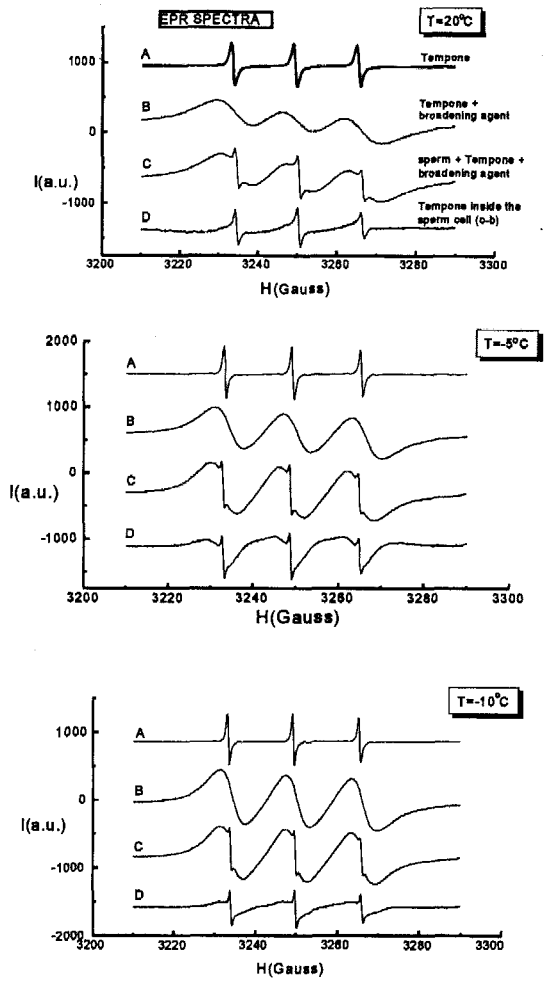
Experiment 1 : Measurement of water content of spermatozoa in isosmotic conditions - Six different spermatozoa series were successively analyzed with EPR.

Experiment 2 : Analysis of water volume at various temperatures (25, -5, -10 °C) - Three spermatozoa samples, previously selected for high number of live spermatozoa/ml, were successively processed using a cooling rate of -25 °C/min.

10 µl of the same sample were examined to determine cell count and cells with ruptured membranes, at all temperatures.

EPR Protocol - The EPR samples were prepared by mixing 4.8 µl of 50 mmol [^{14}N] Tempone l^{-1}, 52 µl of 115 mmol CrOx l^{-1} and 63.2 µl of packed spermatozoa in PBS. The final solution had a total volume of 120 µl. About 60 µl of each sample were drawn into a 1.5 mm µ capillary tube and sealed, the rest was removed for cell counts. All EPR spectra were recorded by an X-band Varian E109 spectrometer equipped with a variable temperature control (regulated at ± 0.1 °C), which was used to cool the samples in a controlled way. EPR signal was recorded at different temperatures (25, -5, -10 °C) after 10 min of termalization of sample.

EPR water volume measurements - Cell water volume was measured using a procedure described by KLEINHANS *et al.* (1992). In brief, the spin label paramagnetic probe Tempone, was used to label all aqueous regions, intracellular and extracellular (EPR signal at 20 °C is shown in Figure 1-A). A membrane impermeable broadening agent (CrOx; Figure 1-B) was added to mark the EPR Tempone extracellular signal (see Figure 1-C). Analysis of spectra (Figure 1-C) was performed after a digital subtraction of the external water signal. The intensities of the intracellular signal and of the reference standard were estimated by: $I = W^2h$; where: W and h are the peak-to-peak line width and the height of the midfield line respectively. The volume occupied by the cell water is calculated by dividing the intensity of the intracellular signal by a calibration standard with the same spin label concentration. The average water volume of a cell is found by dividing the total water volume by the number of membrane-intact cells. The same procedure was followed for the three different temperatures analyzed (Figures 2, 3).



Statistical procedures - Data were processed using linear models taking into account the effect of any series (experiment 1) and both series and temperature (experiment 2).

RESULTS AND DISCUSSION

Experiment 1 - The water volume in isosmotic conditions showed a rather large variability (Table 1) ranging from 20 to 29.1 µm^3. This variability could be attributed to the different dimensions of spermatozoa and partly, to the effect of sperm concentration which can lightly modify the values obtained. In fact, the accuracy of EPR evaluations depends on the strength of intracellular water signal and therefore on the number of cells: a lower number of live sperms could result in a less accurate estimate of water content. However, a mean of 24.3 µm^3 for the water volume was obtained, which is in accord with the value previously obtained by HAMMERSTEDT *et al.* (1978). The percentage

Table 1 : Water volume of rabbit sperm and percentage of membrane intact cells in isosmotic medium

Sample	Water volume (µm^3)	Intact cells (%)	Live spermatozoa/ml (x10^6/ml)
1	22.4	67	310
2	25.3	78	950
3	29.1	82	430
4	20.5	70	385
5	28.4	75	450
6	20.0	87	420
Average	24.3	76.7	430
SDE	4.39	7.20	230

of cells with intact membranes and the number of live spermatozoa showed standard values.

Experiment 2 - Cells at sub-zero temperatures revealed a progressive dehydration as the temperature decreased (Table 2) although a large variability between samples was observed. At -5° and -10 °C, the spermatozoa water volume was respectively 49.5 and 43.6% of the initial value. A large amount of water was lost between 0° and -5° C (about 50%), while in the successive step (-5° - 10° C), the water volume in the sperms decreased only 6%. This progressive decline of water movement, in connection with the reduction of temperature, is in agreement with other findings which show a relevant effect of temperature on the water permeability of rabbit (CASTELLINI, 1994) and ram spermatozoa (DUNCAN and WATSON, 1992).

Table 2 : Cell water volume and live spermatozoa at different temperatures

Sample	Water volume μm^3, (%)			Live spermatozoa ($x10^6$/ml) and % of survival		
	25 °C	-5 °C	-10 °C	25 °C	-5 °C	-10 °C
1	21.2	10.2	9.2	620	559	519
		(48.1)	*(43.3)*		*(90.1)*	*(83.8)*
2	25.3	13.3	9.5	1,250	1,152	926
		(52.5)	*(37.5)*		*(92.2)*	*(74.1)*
3	27.4	11.5	12.1	980	866	774
		(48.1)	*(50.1)*		*(88.3)*	*(78.7)*
mean	23.2	11.6	10.2	980	859	740
		(49.5)	*(43.6)*		*(90.2)*	*(78.8)*

A comparison between our results and those obtained with the "osmotic" method (Table 3), shows a significant discrepancy in the percentage of water loss. In particular, intracellular water at -10 °C, estimated by EPR (cooling rate of -25 °C/min) is about 8 times larger than that determined by the theoretical equation (cooling rate between -5 and -50 °C/min).

Table 3 : Comparison of cell water volume (%) at different temperatures obtained with EPR and osmotic procedure (from CASTELLINI, 1994 modified)

	Water volume (%) Osmotic procedure				EPR
Cooling rate (°C/min)	-5	-50	-100	-500	-25
Temperature (°C)					
25	100	100	100	100	100
-5	10	17	56	83	49.5
-10	4.7	5	6.5	35	43.6

The trial shows that a large quantity of intracellular water (about 44%) still remains in the cell at -10 °C. At the same temperature, osmotic procedures estimate a water volume of about 5%; only when the cooling rate is very high (-500 °C/min) the value is above 30%. Different hypothesis can be suggested to explain these discrepancies:

1) water permeability of spermatozoa at sub-zero temperatures is much lower than estimated with osmotic procedures;

2) the osmotic inactive water (not exchangeable) is higher than the postulated values.

3) Boyle van't Hoff law (relating osmotic pressure and cell volume) is not perfectly respected by spermatozoa (HAMMERSTEDT, 1978).

It should be noted that the present findings are in agreement with the experimental results obtained with the freezing-thawing process. In fact, while the theoretical curve shows that a sufficient shrinkage of sperm is also possible with very high cooling rates, the operative results were disastrous when these cooling rates (CASTELLINI, 1994, -560 °C/min) were applied. Another possible explanation of this large disparity consists in the method used to predict the intracellular water during freezing. The dehydration of spermatozoa during freezing was estimated in simple solution without any cryoprotectants which are always used in the extenders for semen freezing. The use of these additives could reduce the water permeability of spermatozoa (DUNCAN and WATSON, 1992).

In conclusion, EPR has been shown to be a tool for measuring the intracellular water of spermatozoa during freezing. In connection with other techniques (cryo-microscopy, osmotic procedure, etc) this technique could help to improve the procedure of freezing live cells. More analysis of other sub-zero temperatures and the effect of induced seeding on EPR signal are necessary. Moreover other investigations to explain the differences between spermatozoa samples must be executed.

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Stima del volume idrico degli spermatozoi di coniglio a diverse temperature mediante risonanza elettronica paramagnetica - Durante la crioconservazione il mestruo diluitore cambia profondamente la propria pressione osmotica e impone alle cellule una disidratazione che permette all'acqua intracellulare di equilibrarsi con quella esterna. Perciò la conoscenza del contenuto di acqua a varie temperature è un parametro molto importante per stabilire le procedure di congelamento di materiale biologico. Il comportamento degli spermatozoi durante il congelamento viene studiato con una procedura osmotica indiretta che, stimando la permeabilità idrica e la sua energia di attivazione, costruisce una curva teorica che collega % di acqua intracellulare e temperatura. Il presente lavoro propone un metodo EPR che è in grado di stimare direttamente la percentuale di acqua a diverse temperature sotto zero (-5, -10 °C). Il metodo è basato sull'uso di uno spin label (tempone) e di un agente allargante (ossalato di cromo e potassio). La determinazione dell'acqua intracellulare durante il congelamento ha permesso di evidenziare che solamente il 44% dell'acqua cellulare è interessata alla disidratazione cellulare. Questi risultati differiscono sostanzialmente da quanto ottenuto da altri autori con procedure osmotiche. Le presenti indicazioni potrebbero modificare sostanzialmente le procedure di congelamento utilizzate per gli spermatozoi di coniglio.