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Effects of cryopreservation on progesterone-induced ion fluxes and acrosome reaction in human spermatozoa

M.Rossato, M.Zorzi, A.Ferlin, A.Garolla and C.Foresta[1]

University of Padova, Department of Medical and Surgical Sciences, Padova, Italy

[1]To whom correspondence should be addressed at: University of Padova, Department of Medical and Surgical Sciences, Clinica Medica 3, Via Ospedale 105, 35128 Padova, Italy.
E-mail: forestac@protec.it

The present study evaluated the effects of cryopreservation on progesterone-induced variations of calcium ion concentration $[Ca^{2+}]_i$, plasma membrane potential and acrosome reaction in human spermatozoa. Spermatozoa from 10 fertile donors were divided in two equivalent aliquots, one used as control (fresh spermatozoa) and the other used after freezing–thawing. Measurement of spermatozoa $[Ca^{2+}]_i$ before and after freezing–thawing showed a significant reduction of basal $[Ca^{2+}]_i$ in thawed spermatozoa ($P < 0.01$). Progesterone induced a rise of $[Ca^{2+}]_i$ both in fresh and thawed spermatozoa with a significant reduction after freezing–thawing ($P < 0.01$). The monitoring of sperm plasma membrane potential demonstrated that progesterone induced plasma membrane depolarization in fresh spermatozoa that was absent in thawed spermatozoa. The inhibitory effects of freezing–thawing on progesterone induced $[Ca^{2+}]_i$ and plasma membrane potential variations in human spermatozoa were closely related to the inhibition of the acrosome reaction. In conclusion the present study demonstrates that freezing–thawing procedures reduce the responsiveness of human spermatozoa to progesterone in terms of $[Ca^{2+}]_i$ rise and completely inhibit its effects on plasma membrane potential variations, thus supporting the hypothesis that freezing–thawing procedures may differently modify the plasma membrane receptors for progesterone in human spermatozoa which are known to express at least two receptors for this steroid in their plasma membrane.

Key words: calcium/cryopreservation/membrane potential/progesterone/spermatozoa

Introduction

Frozen–thawed human spermatozoa are routinely used for many assisted insemination and fertilization programmes by donor and by husband (Sherman, 1973, 1980) and for self-preservation programmes for men undergoing vasectomy or therapeutic procedures leading to infertility as a result of surgery or chemo-radiotherapy (Lass *et al*., 1998). The use of frozen–thawed spermatozoa in human reproduction has increased since the introduction of intracytoplasmic sperm injection (ICSI) that allows the use of few spermatozoa obtained by testicular biopsy or needle aspiration for IVF (Al-Hasani *et al*., 1999; Ben-Yosef *et al*., 1999). However lower pregnancy rates have been reported using cryopreserved spermatozoa both in intrauterine inseminations (IUI) and IVF/ICSI programmes with respect to the results obtained with fresh semen (Critser *et al*., 1987; Shenfield *et al*., 1993). These lower pregnancy rates are generally attributed to a reduction of sperm motility and viability induced by cryopreservation procedures (Centola *et al*., 1992; Alvarez and Storey, 1993; Agarwal *et al*., 1995) but damage to plasma membrane structures may explain the lower functionality of frozen–thawed spermatozoa (Barthelemy *et al*., 1990; Check *et al*., 1991). Recently we and others have demonstrated that progesterone, a steroid present at high concentration in follicular fluid and cumulus ooforus at time of ovulation (Osman *et al*., 1989), is a rapid and potent activator of human sperm fertilizing ability (Blackmore *et al*., 1990; Meizel and Turner, 1991; Foresta *et al*., 1993, 1995; Garcia and Meizel, 1996) inducing the opening of at least two ion channels on the plasma membrane permeable to Ca^{2+} and Na^+ leading to cytoplasmic calcium ion concentration ($[Ca^{2+}]_i$) rise and plasma membrane depolarization (Foresta *et al*., 1993; Garcia and Meizel, 1996; Sabeur *et al*., 1996). All these ionic events are followed by the acrosome reaction, a process of primary importance for spermatozoa-egg penetration (Wassarman, 1987).

In this study we have evaluated the effects of freezing and thawing procedures on progesterone-induced $[Ca^{2+}]_i$, plasma membrane potential variations and acrosome reaction induction in human spermatozoa from fertile donors.

Materials and methods

Semen collection

From October 1999 to January 2000 we evaluated semen samples of volunteer fertile sperm donors. Semen was produced by masturbation into sterile containers from 13 healthy, fertile donors after 3 days of sexual abstinence. After liquefaction at room temperature for 30 min (mean room temperature 20–22°C), all samples were evaluated for semen volume, sperm concentration, percentage motility and normal morphology, percentage viable spermatozoa following the indications of the World Health Organization (WHO, 1992). Samples were not used unless there were at least 40×10^6 spermatozoa/ml with 50% motility (grade a and b) and 40% normal morphology (these parameters are the mean of seminal standard parameters of sperm donors referred to our laboratory and who are of proven fertility). Following these criteria, sperm samples from 10 donors were used in this study. After semen analysis (about 30 min) each sperm sample was divided in

two equivalent parts: an aliquot as control and an aliquot to be frozen and thawed.

Sperm cryopreservation

Sperm aliquots to be frozen were diluted (1:1, v:v) with human sperm cryopreservation medium (Irvine Scientific, Santa Ana, CA, USA). The diluted aliquots were drawn into 0.25 ml sterile plastic straws (130×2 mm, CryoBiosystem, L'Aigle Cedex, France) and placed in the chamber of a programmable biological freezer (Biomed Kryo 10/1.7 III, Planer Bloxed, Sunbury, UK) and cooled with the freezing programme described previously (Serafini and Marrs, 1986). In brief: from room temperature to 5°C at a rate of –0.5°C/min; from 5–4°C at a rate of –1°C/min; from 4–3°C at a rate of –2°C/min; from 3–2°C at a rate of –4°C/min; from 2–1°C at a rate of 8°C/min; from 1°C to –80°C at a rate of –10°C/min. Sperm straws were then maintained at this temperature for 10 min and then plunged into liquid nitrogen (–196°C) for 60 min.

Thawing procedure

Sperm samples were maintained in liquid nitrogen for 60 min and then thawed by placement of the straws upright on a rack at room temperature until all visible ice was gone.

Sperm preparation

The non-frozen sperm aliquot from each donor remained in seminal plasma at room temperature until the frozen aliquot was placed into liquid nitrogen. After this time and after thawing of frozen spermatozoa, motile cells from each aliquot were isolated by the swim-up technique as previously described (Foresta *et al*., 1992). Before and after isolation, motile spermatozoa were washed in Biggers–Whitten–Whittingham (BWW) medium containing (in mmol/l): 95 NaCl, 4.8 KCl, 1.7 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 20 HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid), 5.6 fructose, 0.25 sodium pyruvate, 3.7 ml/l sodium lactate syrup (60%) to pH 7.4 and resuspended at a concentration of 10×10^6 spermatozoa/ml. Aliquots of fresh and thawed spermatozoa isolated as above were incubated in the absence and presence of progesterone (1.0 μg/ml) for 120 min at 37°C in a controlled atmosphere (5% CO_2 and 95% O_2). After incubation sperm samples were evaluated for motility, viability and acrosome reaction.

Acrosome reaction evaluation

Sperm aliquots from fresh and thawed sperm suspensions were retrieved before and after incubation with progesterone in the different experimental conditions as described above. After fixation with formaldehyde the percentage of acrosome reacted spermatozoa was assayed using an indirect fluorescence technique with FITC-conjugated lectins from pisum sativum which selectively binds to intact acrosome (Morales and Cross, 1989). Then non-fluorescent spermatozoa were scored as acrosome reacted while fluorescent spermatozoa were scored as acrosome intact. Two hundred spermatozoa were scored in each sample to evaluate the percentage of acrosome reacted spermatozoa.

Measurement of spermatozoa $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured utilizing the fluorescent probe fura-2/AM (Foresta *et al*., 1996): spermatozoa isolated as above were incubated for 30 min at 37°C in the presence of fura-2/AM (2 μmol/l). After loading, spermatozoa were washed to remove extracellular fura-2/AM by centrifugation at 800 *g* for 10 min, resuspended in BWW and maintained at room temperature until used. One ml sperm aliquots were used to measure $[Ca^{2+}]$ in a LS50B Perkin Elmer fluorometer equipped with a thermostat and magnetically-stirred cuvette holder (Perkin Elmer, Norwalk, CT, USA). The excitation wavelength was alternated between 350 and 380 nm and emission fluorescence was continuously monitored at 505 nm.

Table I. Functional sperm parameters before and after selection by swim-up in fresh and frozen–thawed spermatozoa

	Fresh		Frozen–thawed	
	Basal	Swim-up	Basal	Swim-up
Viability (%)	83.6 ± 8.9	98.1 ± 1.8	61.4 ± 7.6	98.4 ± 1.0
Forward motility grade a–b (%)	66.1 ± 8.9	92.1 ± 1.2	40.5 ± 8.6	90.4 ± 1.8
Normal morphology (%)	66.3 ± 5.2	78.1 ± 4.3	59.9 ± 5.8	73.4 ± 3.9

Evaluation of sperm plasma membrane potential changes

Sperm plasma membrane changes were monitored utilizing the potential sensitive fluorescent dye bis-oxonol as previously described (Foresta *et al*., 1996). Briefly, 1.5×10^6 spermatozoa were placed in a cuvette containing the bis-oxonol solution (200 nmol/l) in BWW at 37°C. After stabilization of the fluorescent signal, progesterone was added to sperm suspension at the final concentration of 1.0 μg/ml. Excitation and emission wavelengths were 540 and 580 nm respectively.

Statistical analysis

Experimental data were analysed using the Stat View II (Abacus Concepts, Berkeley, CA, USA) statistical package. Data are reported as mean ± SD of 10 separate experiments performed in triplicate. Statistical analysis was carried out using analysis of variance (ANOVA) and Student's *t*-test. A P value of < 0.05 was chosen as the limit for statistical significance.

Results

Effects of cryopreservation on sperm motility and viability

Freezing–thawing procedures induced a reduction of viability and motility when compared with those of fresh semen (Table I). However, after sperm isolation by the swim-up technique, the viability and motility of fresh and selected-thawed sperm aliquots were similar (Table I), enabling the results obtained in all experiments utilizing fresh and frozen–thawed spermatozoa to be compared.

Evaluation of spermatozoa $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ in sperm aliquots before and after freezing and thawing showed a significant reduction of basal levels of $[Ca^{2+}]_i$ in thawed spermatozoa with respect to those found in fresh spermatozoa (77.8 ± 17.6 versus 113.4 ± 13.1 nmol/l, $P < 0.01$). Progesterone (1.0 μg/ml) induced a prompt and rapid rise of $[Ca^{2+}]_i$ in fresh spermatozoa followed by a sustained plateau phase (Figure 1, trace a) that was completely dependent on an influx of Ca^{2+} from the external medium since it was completely absent in Ca^{2+}-free medium (not shown), as previously described (Foresta *et al*., 1993). This steroid induced similar effects on $[Ca^{2+}]_i$ also in spermatozoa isolated after freezing and thawing but the increase of $[Ca^{2+}]_i$ above basal levels was significantly reduced when compared

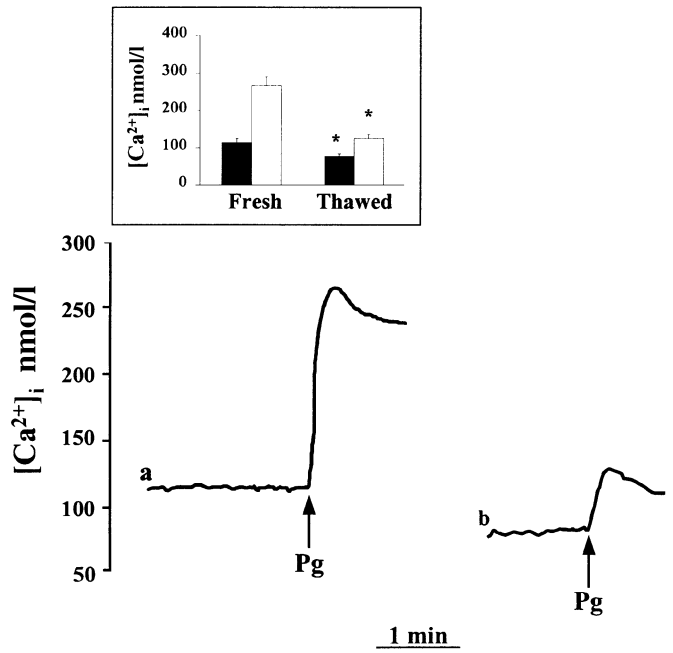

Figure 1. Effects of freezing and thawing procedures on progesterone-induced $[Ca^{2+}]_i$ rise. Trace a shows $[Ca^{2+}]_i$ variations induced by progesterone (Pg, 1.0 μg/ml) in fresh spermatozoa isolated by swim-up technique. Trace b shows progesterone-induced $[Ca^{2+}]_i$ variations in spermatozoa after freezing and thawing. Inset: basal (filled columns) and progesterone-induced peak (open columns) intracellular calcium concentrations ($[Ca^{2+}]_i$) in fresh and thawed spermatozoa. Traces represent a typical experiment from 10 similarly performed in triplicate. $*P < 0.01$ versus fresh spermatozoa.

with those observed in fresh spermatozoa (Figure 1, trace b and inset).

Evaluation of sperm plasma membrane potential

The monitoring of sperm plasma membrane potential variations induced by progesterone showed that this steroid induced a rapid and long lasting plasma membrane depolarization in fresh spermatozoa (Figure 2, trace a) that was observable both in the presence and in the absence of Ca^{2+} in the external medium (not shown) as previously demonstrated (Foresta *et al*., 1993). All fresh sperm samples showed a depolarization of the plasma membrane after progesterone addition while this steroid did not induce any plasma membrane potential variations in any of the frozen–thawed sperm samples (Figure 2, trace b). Addition of gramicidin D, a Na^+ ionophore, induced a significant plasma membrane depolarization to demonstrate that sperm membrane potential was not collapsed after freezing and thawing.

Effects of progesterone on acrosome reaction in fresh and frozen–thawed spermatozoa

Figure 3 shows acrosome reaction percentages in fresh and frozen–thawed spermatozoa after incubation with progesterone. This steroid induced a rise of acrosome reacted spermatozoa in all sperm samples although at a significant lower percentages in frozen–thawed with respect to fresh spermatozoa ($P < 0.01$).

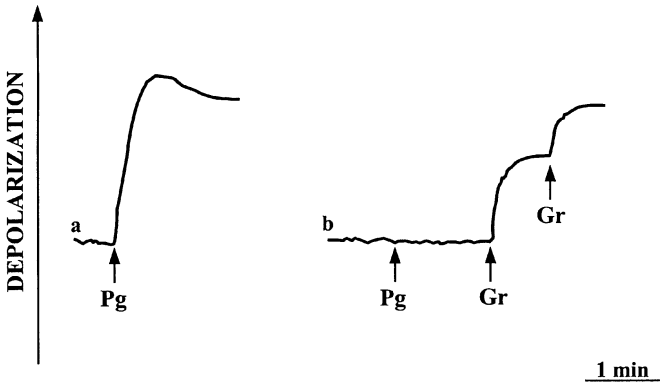

Figure 2. Effects of freezing and thawing procedures on plasma membrane potential variations induced by progesterone. Trace a shows the depolarizing effects of progesterone (Pg, 1.0 μg/ml) in fresh spermatozoa isolated by swim-up. Trace b shows the effects of progesterone on plasma membrane potential in thawed spermatozoa isolated by swim-up. Arrows indicate where progesterone (Pg, 1.0 μg/ml) and gramicidin D (Gr, 0.1 μg/ml) were added. Traces represent a typical experiment from 10 similarly performed in triplicate.

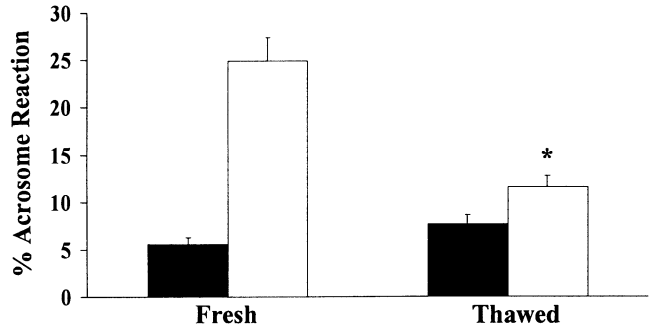

Figure 3. Percentages of acrosome reaction induced by progesterone in spermatozoa before and after freezing and thawing. Basal (filled columns) and progesterone-stimulated (open columns) acrosome reaction percentages are shown. Data are expressed as mean ± SD of 10 experiments performed in triplicate. $*P < 0.01$ versus fresh spermatozoa.

Discussion

Although a number of different procedures utilizing different freezing protocols and cryoprotectants have been proposed, motility and fertilizing ability of mammalian and human spermatozoa are greatly reduced after freezing and thawing procedures (Centola *et al*., 1992; Alvarez and Storey, 1993; Agarwal *et al*., 1995). These observations could be the reasons why clinical pregnancy rates with frozen–thawed spermatozoa are reported to be lower than those observed utilizing fresh semen (Keel and Black, 1980; Cohen *et al*., 1981; Critser *et al*., 1987; Shenfield *et al*., 1993) but the actual alterations induced by cryopreservation in human spermatozoa are not fully understood. The effects of freezing procedures on those cellular events considered fundamental for the occurrence of acrosome reaction and oocyte fertilization have not been fully evaluated. Progesterone, a steroid present in high concentration in follicular fluid and in the cumulus oophorus at the time of ovulation (Osman *et al*., 1989), has been shown to increase spermatozoa $[Ca^{2+}]_i$ and to induce the acrosome reaction in

human spermatozoa (Blackmore *et al*., 1990; Meizel and Turner, 1991; Foresta *et al*., 1993). On this basis progesterone is considered as a possible physiological inducer of the sperm acrosome reaction *in vivo*.

In this study we have evaluated the effects of freezing–thawing procedures on the progesterone-induced effects in human spermatozoa. The results of this study demonstrated that human sperm cryopreservation modified the ability of spermatozoa to respond to progesterone in terms of $[Ca^{2+}]_i$ increase, plasma membrane depolarization and acrosome reaction induction. In fact in frozen–thawed spermatozoa, the $[Ca^{2+}]_i$ rise induced by this steroid was significantly lower than that observed in fresh spermatozoa. Furthermore freezing–thawing procedures deeply modified the action of progesterone on plasma membrane potential variations blunting the depolarizing effects of this steroid in all thawed sperm samples. The lack of any depolarizing effect of progesterone in human spermatozoa after freezing and thawing procedures was not due to sperm plasma membrane collapse since gramicidin D was promptly able to depolarize it. Cryopreservation induced also a reduction of the percentages of sperm acrosome reaction induced by progesterone, probably due to a reduction of the effects on $[Ca^{2+}]_i$ rise and plasma membrane depolarization observed in frozen–thawed spermatozoa after progesterone addition. These results are in agreement with that previously demonstrated, i.e. that both $[Ca^{2+}]_i$ rise and Na^+-dependent plasma membrane depolarization are important events in the biological pathway leading to acrosome reaction induced by progesterone in human spermatozoa (Foresta *et al*., 1993; Garcia and Meizel, 1996). The reasons for these effects are not known but it is possible that the freezing–thawing procedures may have induced damage to the sperm plasma membrane leading to a reduction of Ca^{2+} influx and the inhibition of Na^+ influx followed by plasma membrane depolarization. Thus, in agreement with the existence of at least two progesterone receptors on human sperm plasma membrane, one coupled to Ca^{2+} influx and $[Ca^{2+}]_i$ rise and the other coupled to Na^+ influx and plasma membrane depolarization (Foresta *et al*., 1993, 1995; Sabeur *et al*., 1996), it could be postulated that the Na^+ channel or the progesterone-receptor coupled to its activation is more sensitive to freezing–thawing procedures than that coupled to Ca^{2+} influx activation. The small increase in acrosome reaction percentages induced by progesterone in frozen–thawed spermatozoa (although not significantly different from controls) may be due to the small rise of $[Ca^{2+}]_i$ induced by the steroid, and that might have been sufficient (Foresta *et al*., 1993) for acrosome reaction induction in a small number of spermatozoa. Another possibility is that, in a small number of spermatozoa, progesterone may also have induced a plasma membrane depolarization that could not be detected by our system.

The reduction of progesterone induced $[Ca^{2+}]_i$ rise observed in spermatozoa after freezing–thawing may be due (i) to the lack of voltage-operated calcium channels (VOCC) activation induced by progesterone-dependent plasma membrane depolarization that can be observed only in fresh spermatozoa and is completely absent in thawed spermatozoa or (ii) to the lack of Ca^{2+} influx through a Na^+-permeable channel that is not functional in thawed spermatozoa and that is permeable also to Ca^{2+} (Foresta *et al*., 1993). Further studies are needed to confirm these hypotheses.

All these data put together demonstrate that cryopreservation modifies the responsiveness of human spermatozoa to putative physiological sperm activators such as progesterone. These results could explain the reduced fertilizing potential of frozen–thawed spermatozoa. At present it is difficult to understand what the precise effects of freezing–thawing procedures on human spermatozoa are but it is possible to postulate that cryopreservation alters plasma membrane receptors for progesterone thus modifying sperm responsiveness to this steroid. Further studies will be necessary to evaluate the effects of cryopreservation on human sperm functions and to develop new strategies for freezing and thawing that preserve the functional integrity of spermatozoa.

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