Article

Clean technique for cryoprotectant-free vitrification of human spermatozoa

Vladimir Isachenko was awarded a PhD in the field of human and animal physiology from Kharkov (in the former USSR). Since 1985, he has been working in the area of reproductive biology. Prior to moving to work with humans in 1990, he worked on the oocytes and embryos of agricultural and laboratory animals. Now his main work in the Department of Obstetrics and Gynaecology of Cologne University (Germany) focuses on the development of new methods of preservation of the genome of cancer patients through vitrification of spermatozoa, oocytes, follicles and ovarian tissue. The specific scientific interest of Dr Isachenko lies in the effect of cold on the genome.

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Abstract

Human spermatozoa can be successfully cryopreserved without the use of cryoprotectants through vitrification at very high warming rates. This is achieved by plunging a small amount of frozen sperm suspension into a warming medium, or a large amount of sperm suspension into an agitated warming medium. The aim of the present study was to compare the motility of human spermatozoa cryopreserved using four different methodologies of cooling and warming: cryoloops, droplets, open-pulled straws and standard open straws. Evaluation of two parameters, motility and viability rate of spermatozoa, suggests that all four methods are suitable for use in assisted reproductive technology. However, only the use of open-pulled straws as well as standard open straws allows the isolation of spermatozoa from liquid nitrogen with low potential risk of microbial contamination during freezing and storage, and is thereby a clean method of vitrification.

Keywords: *clean technology, human spermatozoa, viability, vitrification*

Introduction

A new technique of cryoprotectant-free cryopreservation by direct plunging of a sperm suspension into liquid nitrogen (vitrification) has recently been developed. After storage in liquid nitrogen, warming is achieved by direct dissolving of the vitrified suspension. This freezing/warming method is performed at cooling and warming rates of up to hundreds of thousands of °C/min (Nawroth *et al*., 2002; Isachenko *et al*., 2003, 2004a). Recent findings (Isachenko *et al*., 2004b) suggest that optimal regimes for the cryoprotectant-free cryopreservation of spermatozoa need not be restricted to very fast cooling before storage in liquid nitrogen, and in fact, a wide range of cooling rates was acceptable. This highly efficient combination of 'slow' cooling and 'rapid' warming (Isachenko *et al*., 2004b) was the grounds for testing of four described technologies. The aim of the current investigation was to test different methodologies of cryoprotectant-free vitrification of human spermatozoa, using cryoloops, droplets, open-pulled straws and open-standard straws.

Materials and methods

The study, performed in Italy and Germany, was approved by the University Review Board (Italy) and University Ethics Committees (Germany).

Ejaculates from patients with oligoasthenoteratozoospermia (one ejaculate from one patient) were obtained from 16 men by masturbation after at least 48 h of sexual abstinence. The ejaculates were required to contain <20 × 10^6 motile spermatozoa/ml, <50% progressive sperm motility and <30% morphologically normal spermatozoa. Semen analysis was performed according to published guidelines of the World Health Organization (Nieschlag and Nieschlag, 1999).

Part of each ejaculate (200–500 µl) was prepared by swim-up and divided into five equal parts: for vitrification using cryoloops, droplets, open-pulled straws and open-standard straws, as well as for one fresh (control) group. The controls for all the experimental

groups were swim-up prepared fresh spermatozoa. All manipulations with spermatozoa, including swim-up preparation, cryopreservation and warming, were performed using standard medium for sperm preparation (SpermRinse™; Vitrolife AB, Scandinavian IVF Science, Gothenborg, Sweden) according to World Health Organization instructions (Nieschlag and Nieschlag, 1999). In brief, each ejaculate was washed twice by centrifugation at 380 *g* for 5 min using SpermRinse™. After washing, 300 µl of the medium was pipetted over the pellet. The samples were then incubated for 30 min for swim-up. Each experiment was repeated 3 times.

Cooling in liquid nitrogen vapour using cryoloops

Spermatozoa were cooled according to the described procedure (Isachenko *et al*., 2004b). Cryoloops with a film of spermatozoa suspension were cooled for 3 min in liquid nitrogen vapour at –160°C (**Figure 1**). After 3 min cooling, these cryoloops were placed into cryovials (CryoTubes™, 4.5 ml volume, 92 mm length; Nunc GmbH & Co. KG, Wiesbaden, Germany), which had been pre-cooled in liquid nitrogen for subsequent storage in liquid nitrogen until the time of warming.

Cooling in liquid nitrogen vapour using droplets

Spermatozoa were cooled as shown in **Figure 2**. This method has previously been described by Dinnyes *et al*. (2000) for vitrification of oocytes and embryos; however, in the present experiments, the massive metal block was not used. Aliquots of 40 µl of sperm suspension were located onto aluminium foil previously cooled in liquid nitrogen vapour to –160°C (**Figure 2**). The temperature of the foil was determined using an electrical thermometer (Testo 950; Testo AG, Lenzkirch, Germany). During cooling, the droplet of sperm suspension adopted a spherical form. After 5 min cooling, these solidified droplets of sperm suspension were placed into cryovials (CryoTubes™) that had been pre-cooled in liquid nitrogen, for subsequent storage in liquid nitrogen until the time of warming.

Cooling in liquid nitrogen using open-pulled straws

Aliquots of 5 µl of a sperm suspension were drawn inside the end of open-pulled straws by capillary action (Vajta *et al.,* 1998). Straws were placed inside sterile 90 mm straws, which were prepared from the standard 0.5 ml insemination straws (Medical Technology GmbH, Altdorf, Germany). The 90 mm straw was hermetically closed using a hand-held sealer (Medical Technology GmbH) (**Figure 3**) and plunged into liquid nitrogen. There was no contact between the wall of the 90 mm straw (Medical Technology GmbH) and the suspension of spermatozoa inside the open-pulled straws, due to the presence of a meniscus in the suspension (**Figure 3**).

Cooling in liquid nitrogen using open straws

Using a micropipettor, a 1 µl aliquot of a sperm suspension was deposited inside the end of open-standard (usual) 0.25 ml straws (Medical Technology GmbH). Straws were placed inside sterile 90 mm straws. The 90 mm straw was hermetically closed using a hand-held sealer (Medical Technology GmbH) (**Figure 4**) and plunged into liquid nitrogen.

Warming of cryoloops and droplets

After storage for a minimum of 24 h in cryovials, the samples were thawed by plunging the cryoloops and droplets into a 15 ml tube containing 10 ml SpermRinse™ at 37°C under intense agitation. After warming (five loops in one tube or one droplet in one tube), the tubes were placed in a CO_2 incubator for 5–10 min at 37°C. Next, spermatozoa were concentrated by centrifugation at 380 *g* for 5 min, and the resulting pellet was resuspended in 100 µl of SpermRinse™ and used for further culture and evaluation. After warming, spermatozoa were placed into a Petri dish (Nunc) and were then cultured under mineral oil for an additional 24 h in 5% CO_2 in air at 37°C in SpermRinse™.

Warming of open-pulled straws and open straws

The open-pulled straws, after expelling from the 90 mm straws, were rapidly plunged into 1.5 ml microcentrifuge tubes (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) containing 1 ml SpermRinse™ at 37°C. After warming, spermatozoa were expelled into a Petri dish (Nunc) and were then cultured under mineral oil for an additional 24 h in 5% CO_2 in air at 37°C in SpermRinse™.

Evaluation of motility and viability rate of spermatozoa

Motility is reported as the percentage of total spermatozoa with forward progression (categories a and b), according to World Health Organization guidelines (World Health Organization, 2002). The motility of control (prepared by swim-up, uncooled) spermatozoa was determined in a Makler counting chamber (Sefi Medical Instruments Ltd, Haifa, Israel) immediately after swim-up treatment and after 2, 5 and 24 h. The motility of cryopreserved spermatozoa was assessed immediately after instant thawing by dissolving in warm SpermRinse™ and concentrating the sample by centrifugation as described above (for cryoloops and droplets), or expelling from open-pulled straws and open straws. Motility was estimated under the light microscope at a magnification of ×400. The motility rate of spermatozoa was defined as the percentage of post-thaw motility × 100%, divided by the percentage of motility before cryopreservation. Viability was calculated as the percentage of motile spermatozoa after a certain period of culture, demonstrating the period for which spermatozoa retained motility.

Statistical analysis

Treatment effects on the parameters assessed were evaluated by ANOVA. Data are given as mean values ± SD. The level of statistical significance was set at $P < 0.05$.

Results

The percentages of membrane-intact spermatozoa still retaining progressive motility after cryoprotectant-free cryopreservation using cryoloops, droplets, open-pulled straws and open-standard straws are shown in **Figure 5**. The four regimes of cryopreservation resulted in approximately 40% reduction of sperm motility ($P < 0.05$) in comparison with the swim-up

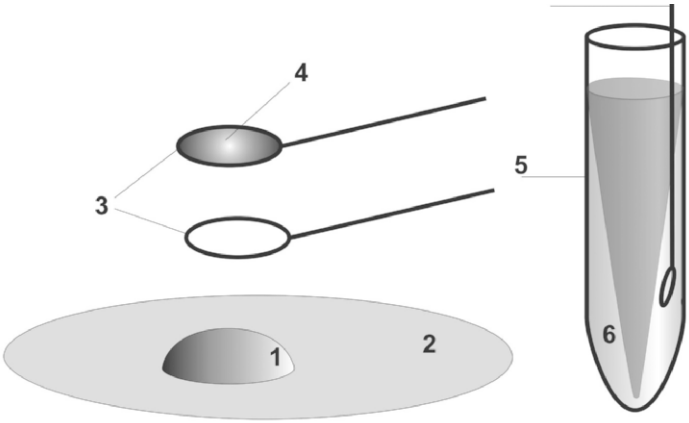

Figure 1. Method for 'cryoloop' vitrification and warming of spermatozoa. (1) Suspension of spermatozoa. (2) Petri dish. (3) Cryoloop. (4) Film of sperm suspension. (5) Tube for warming. (6) Warming medium (agitating).

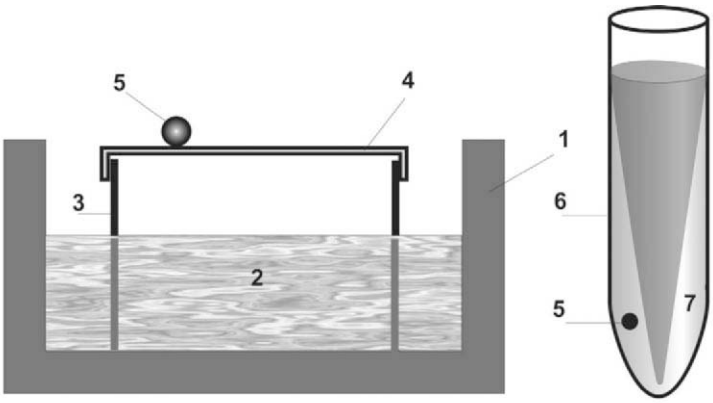

Figure 2. Method for 'droplet' vitrification and warming of spermatozoa. (1) Foam box. (2) Liquid nitrogen. (3) Foot for aluminium foil. (4) Aluminium foil. (5) Suspension of spermatozoa. (6) Tube for warming. (7) Warming medium (agitating).

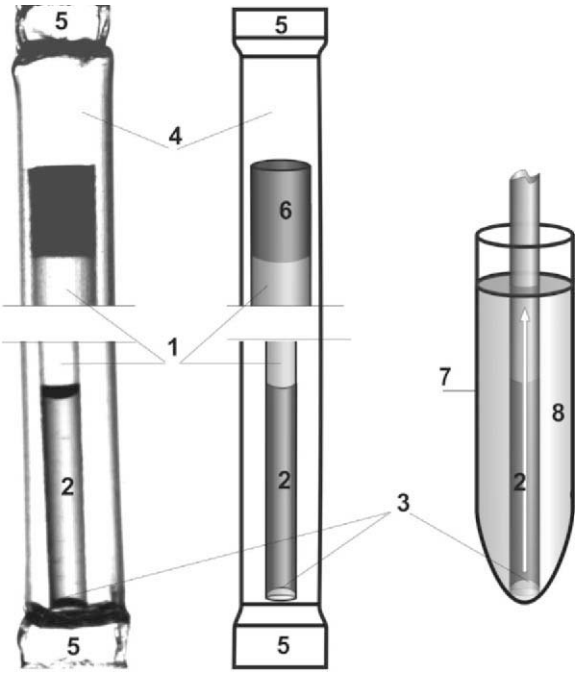

Figure 3. Photograph of container and method for 'open-pulled straw' vitrification and warming of spermatozoa. (1) Open-pulled straw. (2) Suspension of spermatozoa. (3) Meniscus of suspension. (4) 90 mm straw. (5) Heat sealed end of 90 mm straw. (6) Marked end of open-pulled straw. (7) Tube for warming. (8) Warming medium. White arrow indicates the direction of thawing and swim-up of a sperm suspension.

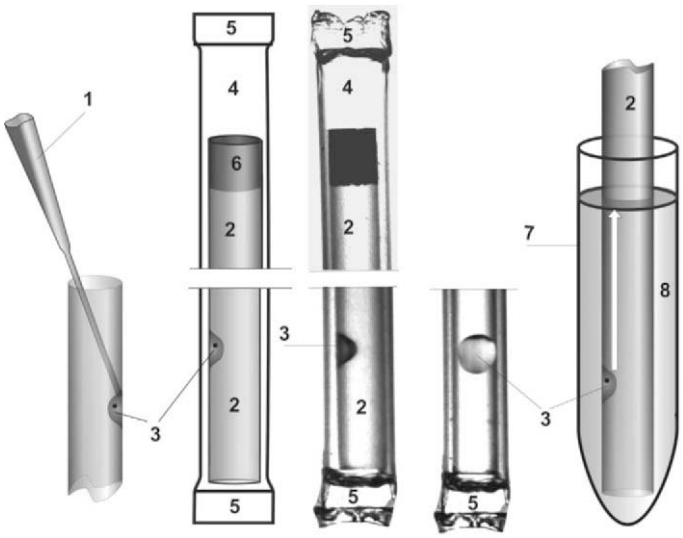

Figure 4. Photograph of container and method for 'open straw' vitrification and warming of spermatozoa. (1) Tip of pipettor. (2) Open straw. (3) Drop of spermatozoa. (4) 90 mm straw. (5) Heat sealed end of 90 mm straw. (6) Marked end of open straw. (7) Tube for warming. (8) Warming medium. White arrow indicates the direction of thawing and swim-up of a sperm suspension.

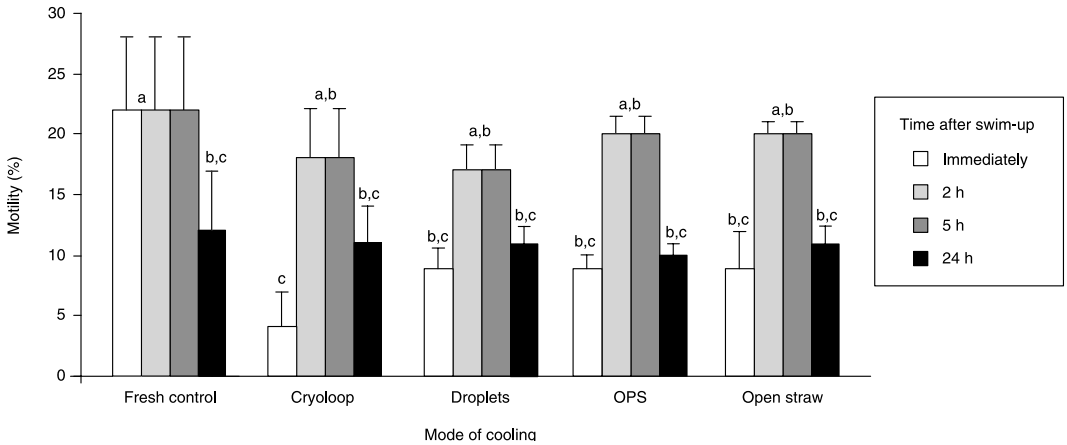

Figure 5. Motility of human spermatozoa after cryoprotectant-free vitrification using different modes of cooling and warming. Different superscripts indicate significant differences ($P < 0.05$).

control. Whilst progressive motility was much reduced just after thawing, motility of spermatozoa increased dramatically 2 and 5 h later, but decreased again after 24 h of culture. No statistically significant difference was found in this parameter among the droplet, open-pulled straw and open-standard straw regimes of cryopreservation (**Figure 5**). Thus, there were no differences in motility rate of spermatozoa vitrified using all four methods during culture periods of 2, 5 and 24 h (**Figure 5**). However, the motility rate of the cryoloop vitrified spermatozoa (first method) just after warming was significantly lower ($P < 0.05$) than that measured 2 and 5 h after warming. After 2 h culture, restoration of sperm activity in this group was observed.

Discussion

Many years ago, spermatozoa of many mammalian specimens were successfully cryopreserved. In 1937, Bernstein and Petropavlovski used 0.5–3 mol/l glycerol for freezing of bull, ram, stallion, boar and rabbit spermatozoa to a temperature of –21°C. They reported obtaining the best results at 0.5–2 mol/l glycerol. The observations of Jahnel (1938), who had cryopreserved human spermatozoa in liquid nitrogen and liquid helium without cryoprotectants, and Parkes in 1945, on freezing of human spermatozoa also without cryoprotectants, were published. The work of Polge *et al.* (1949) is now considered as a milestone of modern cryobiology. Hoagland and Pincus, in 1942, described the freezing of human and rabbit spermatozoa using a bacteriological loop to cool small specimens rapidly. These authors obtained up to 40% of viable human spermatozoa after cooling of a sperm suspension or sperm film in liquid nitrogen followed by quick warming of these microvolumes.

The aim of the present study was to compare the motility of human spermatozoa cryopreserved using four different methodologies of vitrification/warming. These involved a high speed of warming due to the use of small amounts of

cooling/warming medium or due to the use of a combination of relatively large volumes of cooled suspension and fast warming rates in agitated medium. In order to achieve the above-mentioned vitrification regimes without cryoprotectants, the film of sperm suspension and droplets was cooled in liquid nitrogen vapour, in addition to a small amount of the sperm suspension being located in hermetically sealed inner straws in the open-pulled and open-standard straw methods. All four methods of warming permitted rapid devitrification.

In conclusion, evaluation of two parameters, motility and long-term survival of spermatozoa, suggests that all four methods are suitable for use in assisted reproductive technology. However, the open-pulled straw method of vitrification is preferable because it allows isolation of the spermatozoa from liquid nitrogen, with a maximum reduction of the potential risk of microbial contamination.

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Received 19 November 2004; refereed 9 December 2004; accepted 25 January 2005.