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ARTICLE

Efficiency of aseptic open vitrification and hermetical cryostorage of human oocytes

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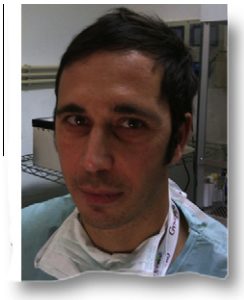

Lodovico Parmegiani obtained his degree in biology in 1996 at the University of Bologna, Italy and his post-graduate specialization in biochemistry and clinical chemistry in 2000 at the University of Modena and Reggio Emilia, Italy. He trained as a clinical embryologist at the Reproductive Endocrinology Centre, S Orsola Hospital in Bologna. Since 2002 he has been laboratory director at the Reproductive Medicine Unit, GynePro Medical Centres in Bologna. In 2008 he received certification as senior clinical embryologist from the European Society for Human Reproduction and Embryology. His current research interests are cryobiology, gamete selection and micromanipulation.

Abstract The present study reports, as far as is known for the first time, the safety of UV sterilization of liquid nitrogen and hermetical cryostorage of human oocytes by comparing the efficiency of fresh and vitrified sibling oocytes of infertile patients. A prospective randomized study on sibling oocytes of 31 patients was carried out. Metaphase-II oocytes were randomized for intracytoplasmic sperm injection and the supernumerary sibling oocytes were vitrified using a novel Cryotop aseptic procedure (UV liquid nitrogen sterilization and hermetical cryostorage). After unsuccessful attempts with fresh oocytes, vitrified sibling oocytes were injected. Mean outcome measures observed were fertilization, cleavage and top-quality embryo rates. No significant differences were observed between the fresh and vitrified–warmed sibling oocytes: oocyte fertilization was 88.3% versus 84.9%; cleavage 72.6% versus 71.0%; top-quality embryos 33.8% versus 26.3% and mean number of transferred embryos 2.6 ± 0.1 versus 2.5 ± 0.1, respectively. Clinical pregnancy rate per cycle with vitrified–warmed oocytes was 35.5% (implantation rate 17.1%) and seven healthy babies were born. This study demonstrated that UV liquid nitrogen sterilization and hermetical cryostorage does not adversely affect the developmental competence of vitrified oocytes, allowing safe aseptic open vitrification applicable under strict directives on tissue manipulation.

KEYWORDS: Cryotop, hermetical cryostorage, liquid nitrogen, oocyte vitrification, ultraviolet sterilization

Introduction

Vitrification is a cryopreservation technique that leads to a glass-like solidification with rapid cooling of cells or tissues by direct contact with liquid nitrogen (LN_2) without ice crystal formation in the solution containing the biological sample (Vajta et al., 2009). Nowadays vitrification is seen as the future of cryopreservation of human embryos, oocytes and

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ovarian tissue due to improved survival rates and clinical outcomes (Al-Hasani et al., 2007; Cobo et al., 2008; Xiao et al., 2010).

Particularly when specific 'open carriers' for ultra-rapid cooling are used (Almodin et al., 2010; Bernal et al., 2009; Chian et al., 2009; Kuwayama et al., 2005a; Lane et al., 1999; Sugiyama et al., 2010; Vajta et al., 1998; Vandervorst et al., 2001), there is the risk of disease transmission during the vitrification procedure (**Figures 1—3**) if the cells are directly plunged into accidentally contaminated LN_2 (Bielanski et al., 2000, 2003). Even though this risk has never been clearly demonstrated in reproductive tissues (Pomeroy et al., 2010), the need to guarantee the absolute sterility of LN_2 for vitrification purposes is therefore felt as a critical problem due to the new directives on tissue manipulation (The Commission of the European Communities, 2006).

Regarding sterilization, some authors reported the purification or filtration of LN_2 (Cobo et al., 2008; Kuwayama et al., 2005a; Sugiyama et al., 2010; Vajta et al., 2009). However, at the present time, only one filter is specifically designed for LN_2 (Ceralin; Air Liquide, Spain; a ceramic filter with pore size of 0.1 μm; A Cobo, personal communication). Given that this device filters up to 0.1 μm, it is obviously inadequate for smaller micro-organisms, such as viruses. Other authors have postulated the possibility of sterilizing liquid nitrogen with UV (Eum et al., 2009; Vajta et al., 2009) and it was recently demonstrated that UV radiation can sterilize a small volume of LN_2 from bacteria, viruses and fungi (Parmegiani et al., 2009, 2010). The sterilization method is based on administering a UV dose of 660,000 $\mu Ws/cm^2$, which de-activates all kinds of known micro-organisms, thus easily obtaining sterile LN_2 for an aseptic vitrification procedure. Nevertheless, until recently this method had never been applied to vitrification of human or mammalian cells.

Some embryologists have postulated that UV radiation might be dangerous for oocytes and embryos, due to the formation of ozone during the sterilization process. Ozone is a strong oxidant which can be harmful to human cells. This molecule is produced when UV rays collide with oxygen molecules, with carbon monoxide or with nitrogen oxides (NO_2, N_2O) and will persist for up to 30 min, before reverting to oxygen. However, during the sterilization process, the formation of ozone is negligible given that the environment is virtually free from oxygen, because it is completely saturated by nitrogen, both in the liquid and vapour phase (see Materials and methods). Thus, the objective of the present study was to demonstrate that UV sterilization of LN_2 is not detrimental and this method can be routinely used for aseptic open vitrification of human oocytes. In addition, this study also describes, as far as known for the first time, an efficient system to avoid contamination during cryostorage thus satisfying even the most stringent present and future directives.

In order to assess the safety of this kind of vitrification/storage procedure, this study performed a randomized comparison between fresh and vitrified—warmed sibling oocytes for infertile couples coming to the study centre for intracytoplasmic sperm injection (ICSI) treatment. After unsuccessful attempts with fresh oocytes, patients returned for a ICSI cycle with their vitrified sibling oocytes. At the beginning of this study, it was decided to perform at least 30 warming cycles before analysing the results. The outcome measures observed were fertilization, cleavage and top-quality embryo rates.

Materials and methods

Patient selection

Since April 2004, all patients undergoing an IVF treatment in GynePro Medical Centres Bologna with more than five metaphase II (MII) oocytes at oocyte recovery have been included

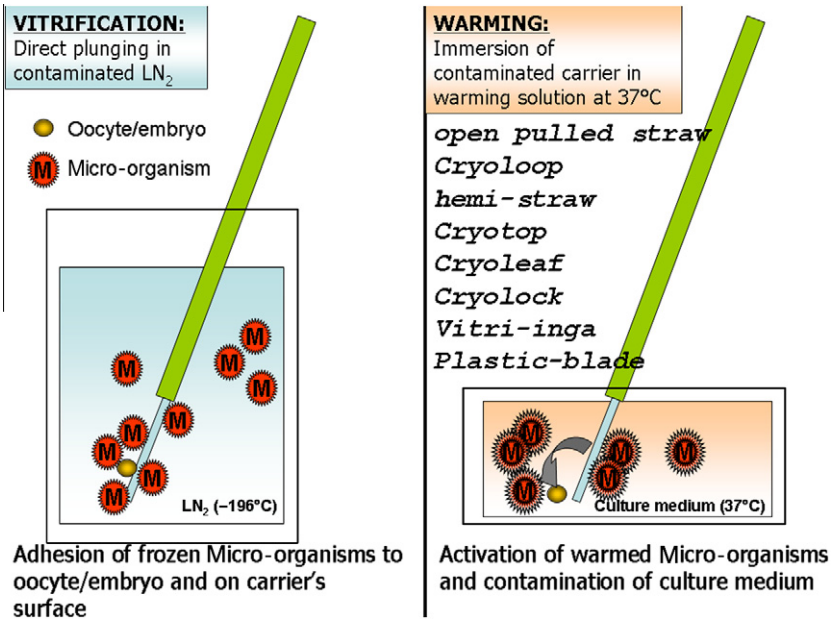

Figure 1 Risk of contamination with open carrier. Frozen micro-organisms in LN_2 (liquid nitrogen) adhere to the vitrified cells and to the carrier surface. At warming, the re-activated micro-organisms contaminate the culture medium and they may attach themselves to the oocyte/embryo zona pellucida if this is cracked.

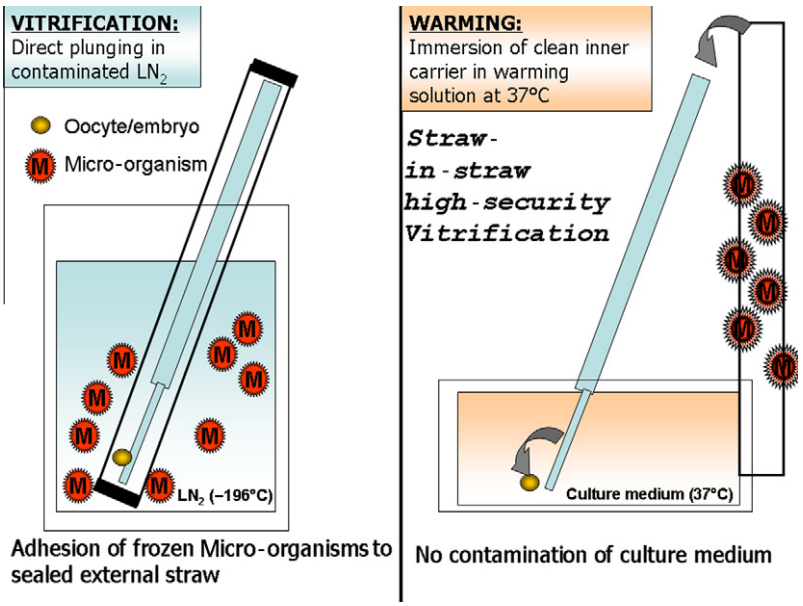

Figure 2 Absence of contamination with straw-in-straw closed carrier. This vitrification system avoids the direct contact between cells and LN_2 (liquid nitrogen) and also any risk of contamination. Frozen micro-organisms in LN_2 adhere to the external straw, which is hermetically sealed. At warming the inner straw is extracted from the external straw and immersed in the culture medium with no risk of contamination.

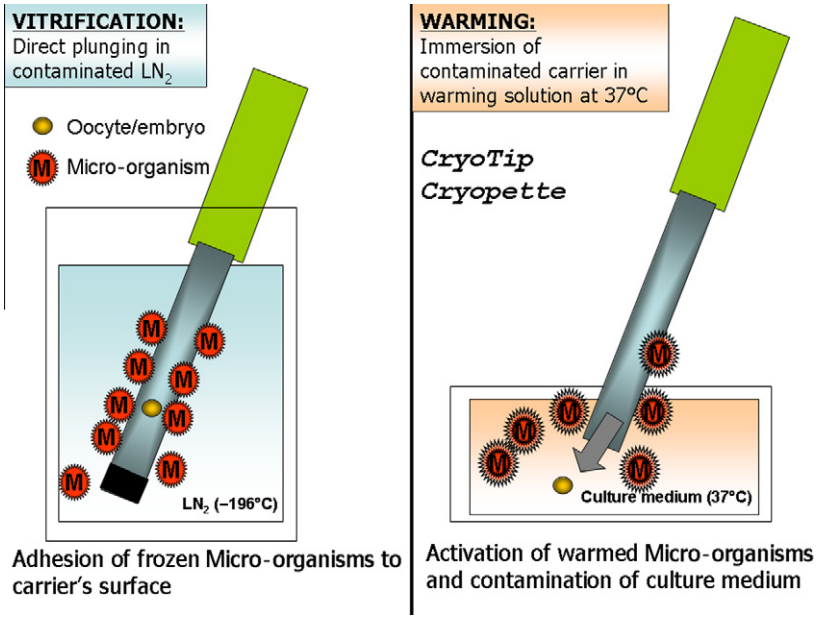

Figure 3 Risk of contamination with 'CryoTip Cryopette' closed carrier. This vitrification system avoids the direct contact between cells and LN_2 (liquid nitrogen) but not the risk of contamination. Frozen micro-organisms in LN_2 adhere to the carrier's external surface. At warming the re-activated micro-organisms contaminate the culture medium and may attach themselves to the oocyte/embryo zona pellucida if this is cracked.

in the oocyte cryopreservation programme. In fact, the Italian IVF law introduced at that time set a limit of insemination of three oocytes but allowed cryopreservation of surplus oocytes (Benagiano and Gianaroli, 2004). The study centre's oocyte vitrification programme started in April 2008. From that date, the cryopreservation procedure for oocyte slow freezing has been randomized, using sealed envelopes (Parmegiani et al., 2008) or vitrification.

The study included all women not older than 41 years with at least six MII oocytes at retrieval, who were undergoing ICSI with ejaculated spermatozoa and randomized for vitrification. Patients with male partners with testicular spermatozoa or severe oligoastenoteratozoospermia (motile sperm count $\leq$500.000/ml after sperm preparation) were not included. This study compares the outcome of 31 warmed ICSI cycles performed from January 2009 to May

2010 with the outcome of fresh sibling oocytes ICSI performed from April 2008 to March 2010. The results were analysed at the end of May 2010, after the final warming cycle. All women in the cryopreservation programme were informed about the procedure and written consent was obtained from each. The study was approved by the Institutional Review Board of the clinic.

Ovarian stimulation, oocyte retrieval and selection, and study design

Ovarian stimulation, transvaginal ultrasound-guided oocyte retrieval, oocyte decumulation and quality evaluation were performed as previously described (Filicori and Cognigni, 2001; Parmegiani et al., 2008). Immediately after decumulation and quality evaluation, the high-quality mature metaphase II (MII) oocytes were put in progressively numbered culture droplets and randomized for ICSI; the supernumerary sibling MII oocytes were vitrified. At the beginning of this study, the Italian IVF law allowed the injection of a maximum of three oocytes (Benagiano and Gianaroli, 2004), so between April 2008 and 8 April 2009 three MII oocytes were randomized for ICSI and the supernumerary sibling oocytes were vitrified. Randomization was performed by a different embryologist to the operator who performed oocyte denudation using a specific software tool (http://www.randomizer.org). Since 9 April 2009, due to changes in the Italian law (Benagiano and Gianaroli, 2010), the number of oocytes to randomize for ICSI or vitrification has been defined following the study centre's guidelines based on female age at oocyte recovery and semen parameters, but generally not more than six oocytes are injected.

Only the first warming cycle per patient was included in the study: from the beginning of the study to 8 April 2009, a maximum of three random warmed oocytes were injected by ICSI; from 9 April 2009 the number of warmed oocytes to inject has been defined following the centre's guidelines. The clinical trial design is available at http://www.controlled-trials.com (registration number: ISRCTN51912603).

LN_2 sterilization, vitrification and hermetical storage

Before LN_2 sterilization the presence of oxygen in the environment at 15 cm from LN_2 surface was checked by using a portable oxygen detector (Pac 5000; Dräger, Lübeck, Germany). The nitrogen vapour phase resulted virtually free from oxygen (less than 0.2%) at every check, demonstrating that the environment was completely saturated by nitrogen and the formation of ozone during LN_2 sterilization process was negligible (**Figure 4**). LN_2 sterilization via UV irradiation was performed as described elsewhere (Parmegiani et al., 2009). Briefly, a stainless steel container filled with 500 ml of LN_2 was irradiated with UV rays at 253.7 nm by a UV lamp placed at 15 cm from the LN_2 surface. A temperature probe was placed over the bulb wall to control the efficiency of the lamp and to calculate the relative UV intensity. In fact, the relative UV output decreases with low lamp temperatures and it is maximum at around 40°C. The UV dose is defined as the smallest amount of UV radiation able to guarantee the complete death of a given antigen UV dose = UV intensity

Figure 4 Oxygen check. Before LN_2 (liquid nitrogen) sterilization the presence of oxygen in the environment at 15 cm from LN_2 surface was checked by using a portable oxygen detector (Pac 5000; Dräger, Lübeck, Germany). The nitrogen vapour phase resulted virtually free from oxygen (less than 0.2%) at every check.

(I) × residence time (T). Thus T = UV dose/I.The irradiation was suspended after the administration of 660,000 μWs/cm^2, which de-activates all kinds of known micro-organisms (http://www.ultraviolet.com/microorgan.htm) and is double the minimum UV dose to de-activate the most UV-resistant micro-organism ever found in LN_2 (*Aspergillus niger*; Bielanski et al., 2003; Parmegiani et al., 2009). For vitrification–warming procedures performed after January 2010, the prototype of a specifically designed device for UV LN_2 sterilization was used (Nterilizer; **Figure 5A**); this device ensures and certifies a UV dose of 660,000 μWs/cm^2 per each sterilization cycle (International patent pending, application no. PCT/IB2009/007801, L Parmegiani).

Cryotop (Kitazato BioPharma, Fuji-Shizuoka, Japan) oocyte vitrification was performed at room temperature in a solution comprising 15% dimethylsulphoxide (DMSO; Sigma–Aldrich, Steinheim, Germany), 15% ethylene glycol (EG; Sigma–Aldrich) and 0.5 mol/l sucrose (Sigma–Aldrich), after a gradual initial equilibration of 15 min in a solution comprising 7.5% DMSO and 7.5% EG (Kuwayama et al., 2005a; Rienzi et al., 2010). For the ultra-rapid cooling, the Cryotops, containing 1–2 oocytes, were plunged into UV-sterilized LN_2 and closed with their plastic caps. Subsequently, each patient's Cryotops were enclosed in home-made hermetical aluminium cylindrical containers (high-security goblets; **Figure 5B**), which can contain up to six Cryotops each. These goblets had been previously submerged vertically in LN_2 in order to avoid the infiltration of LN_2 and checked for an inner temperature of −196°C at the end of the UV sterilization process. The Cryotops were inserted into the high-security goblets, taking care to keep the Cryotop strip containing the oocyte in the nitrogen vapour phase above the LN_2. Finally, the goblets were hermetically closed with sterilized caps and polypropylene adhesive tape (Scotch; 3M Italia, Pioltello, Italy).

Warming procedure

At warming, the hermetical goblets containing the Cryotops were opened into the UV-sterilized LN_2 and the Cryotops were immediately transferred from the goblet to the

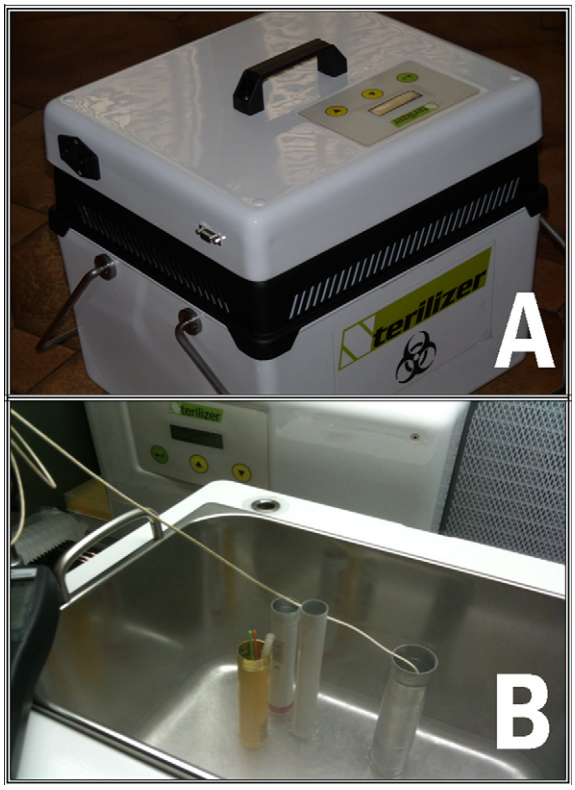

Figure 5 Ultraviolet sterilization and hermetical cryostorage. Prototype of a specifically designed device for UV LN_2 (liquid nitrogen) sterilization (Nterilizer; **A**). Cryotops are enclosed in home-made hermetical aluminium cylindrical containers (high-security goblets; **B**). The goblets are submerged vertically in LN_2 in order to avoid the infiltration of LN_2 and checked for an inner temperature of −196°C at the end of the UV sterilization process.

warming solution without having contact with the LN_2 in which the goblet is plunged. Each Cryotop was submerged in 1 ml of warming solution containing 1 mol/l sucrose at 37°C. Then, the oocytes were incubated at room temperature for 3 min first in 0.5 mol/l and subsequently in 0.25 mol/l and finally washed for 4 min in basic medium (phosphate-buffered saline; Sigma—Aldrich) supplemented with 20% Enhance hyaluronidase (Conception Technologies, San Diego, USA) before culture. Warmed oocytes were considered to have survived in absence of negative characteristics: dark or contracted ooplasm, vacuolization, cytoplasmic leakage, abnormal perivitelline space and cracked zona pellucida. After 1—2 h of post-warm culture, the surviving oocytes were checked again and inseminated by ICSI. Fertilization and embryo development were examined by inverted microscope. Embryos were graded 1—5 (1 best, 5 worst), with grade 1 assigned to the best-quality embryos containing equally sized symmetrical blastomeres with no fragmentation, according to the criteria previously described by Veeck (1999).

Endometrial preparation and embryo transfer

Preparation of endometrium for the embryo transfer was performed as described elsewhere (Parmegiani et al., 2008). Embryo transfer was carried out on days 2 or 3 after oocyte warming and ICSI. Clinical pregnancy was defined as the presence of a gestational sac with or without a fetal heart beat at ultrasound examination 2 weeks after a positive test for human chorionic gonadotrophin.

Statistical analysis

Continuous variables are presented as mean and standard error (SE). Categorical variables are presented as percentage. Normality of distribution of continuous variables was assessed with a Kolmogorov—Smirnov test with Lillefor correction. Between-group differences of normally distributed continuous variables were assessed with parametric statistic (Student's t-test), whereas non-parametric statistics (Mann—Whitney rank sum test) were employed when the normality test was not passed. Between-group differences in non-continuous variables were assessed using the chi-squared method with Yates correction if needed. Difference was considered significant when a *P*-value was <0.05.

Results

Mean female age at oocyte retrieval was 35.0 ± 0.8 years.

Clinical outcome: fresh oocytes

Four clinical pregnancies were obtained after 31 cycles (12.9% and 13.3% pregnancy rate per cycle and per transfer, respectively) in 31 patients. In one patient, the embryo transfer was not performed due to the risk of ovarian hyperstimulation syndrome. Two pregnancies ended in abortion before week 8 of gestation. One pregnancy was ectopic and the fourth pregnancy was an empty sac without fetal heartbeat. After these unsuccessful attempts with fresh oocytes, these patients returned for an ICSI cycle with their vitrified sibling oocytes.

Laboratory results: fresh versus vitrified—warmed oocytes

This study was performed on oocytes cryostored between 2 and 11 months (mean ± standard error 5.3 ± 0.5, median 5.0). A total of 168 oocytes were warmed (5.4 ± 0.3 per patient): 151 oocytes survived (survival rate 89.9%), 126 were injected and 25 were discarded (oocytes not injected 14.9%) after morphological selection immediately before ICSI. The mean number of injected oocytes was comparable between the fresh and the vitrified—warmed oocyte group (3.9 ± 0.2 versus 4.1 ± 0.2, respectively). Fertilization and cleavage rates and mean number of transferred embryos were comparable in the two groups: no significant differences on main outcome measures were observed between the fresh and vitrified—warmed sibling oocytes (**Table 1**).

Clinical outcome: warmed oocytes

Eleven clinical pregnancies were obtained after 31 embryo transfers (35.5% pregnancy rate per cycle and per transfer) with 13 embryos implanted out of 76 transferred (17.1%

Table 1 Laboratory results: fresh versus sibling vitrified–warmed oocytes.

Variable	*Fresh oocytes*	*Vitrified–warmed oocytes*
No. of injected oocytes	3.9 ± 0.2	4.1 ± 0.2
Fertilization rate	106/120 (88.3)	107/126 (84.9)
Cleavage rate	77/106 (72.6)	76/107 (71.0)
Grade-1 embryo rate	26/77 (33.8)	20/76 (26.3)
No. of transferred embryos	2.6 ± 0.1	2.5 ± 0.1

Values are mean ± standard error or *n*/total (%).
No statistically significant differences were found between the two groups.

implantation rate). The abortion rate was 18.2% (2/11). Two patients had a gestational sac without the fetal heartbeat. Seven healthy babies were born (seven singletons).

Discussion

Vitrification is a cryopreservation technique which is increasingly preferred in clinical practice for human reproductive cells. During vitrification, the oocytes (or cleavage-stage embryos, blastocysts, cells, ovarian tissue, etc.) need to be cooled in LN_2 at an extremely rapid rate to obtain a good survival rate at warming (Vajta et al., 2009). The best option for performing an ultra-rapid cooling is to use specific 'open carriers' such as the open pulled straw (Vajta et al., 1998), Cryoloop (Lane et al., 1999), hemi-straw (Vandervorst et al., 2001), Cryotop (Kuwayama et al., 2005a), Cryoleaf (Chian et al., 2009), Cryolock (Bernal et al., 2009), Vitri-inga (Almodin et al., 2010) and Plastic-blade (Sugiyama et al., 2010). Showing high efficiency with human oocytes, these open carriers are generally preferred for clinical use (Antinori et al., 2007; Cobo et al., 2008; García et al., 2011; Rienzi et al., 2010; Ubaldi et al., 2010). However, these systems cannot avoid the risk of micro-organism contamination during the vitrification procedure if the LN_2 is accidentally contaminated (Bielanski et al., 2000, 2003) (**Figure 1**). Another option for vitrification is the closed carrier based on the straw-in-straw model (high-security vitrification), which is designed to isolate the inner carrier containing the oocytes/embryos against LN_2 during vitrification by using a sealed external straw (Isachenko et al., 2005; Kuleshova and Shaw, 2000). This vitrification system avoids the direct contact between cells and LN_2 and also any risk of contamination (**Figure 2**). Although it allows good results with zygotes, cleaved embryos, blastocysts and ovarian tissue (Isachenko et al., 2005, 2010; Liebermann, 2009), this sealed system determines a reduction in the rate of cooling and is therefore not preferred in clinical oocyte cryopreservation.

Recently, new closed systems have been developed which allow faster rates of cooling, such as CryoTip (Kuwayama et al., 2005b) or Cryopette (Keskintepe et al., 2009). These closed carriers consist of a very thin straw specifically designed to load oocytes or embryos with minimum volume of cryoprotectant solution and to be hermetically sealed with a heat (or ultrasound) sealer. These vitrification systems avoid direct contact between cells and LN_2. Unfortunately, because of their design, these systems cannot avoid the transmission of micro-organisms in the culture medium during the warming procedure due to the previous direct contact during vitrification between the external surface of the carrier and the LN_2 (**Figure 3**). In fact, it is conceivable that the contamination of cells would occur at 37°C, when any cryopreserved micro-organism found in the LN_2 would reactivate after thawing in the culture medium. It could be argued that IVF culture media can be supplemented with antibiotics, but some micro-organisms (resistant bacteria or viruses) may resist the antibiotic (usually gentamicin) in the medium and infect the culture. In these circumstances, the bacterial or viral particles released in the culture medium may attach themselves to the oocyte/embryo zona pellucida if this is cracked (Bielanski et al., 2000; Tedder et al., 1995). Another procedure is to quickly wipe the carriers with 70% ethanol for disinfection at warming (Kuwayama et al., 2005b). However, the de-activation of all micro-organisms can be obtained only by 5-min contact between ethanol and carrier (Sopwith et al., 2002); this prolonged contact time can damage human cells, which remain inside the carrier in the warmed vitrification solution rich in potentially toxic cryoprotectants (Fahy et al., 1990).

This hypothetical cell contamination by LN_2 is the reason for the need to guarantee the absolute sterility of LN_2 for vitrification purposes, particularly in Europe due to the directives on tissue manipulation (European Union Tissues and Cells Directive EUTCD: 2004/23/EC, 2006/17/EC and 2006/86/EC). These directives have been issued by the European Parliament in order to increase the safety and quality of tissues, including reproductive cells, processed for human re-implantation through the control of equipment, devices and environment. Similar regulations will probably be introduced by the Food and Drug Administration (FDA) for Assisted Reproductive Centres in the USA (Pomeroy et al., 2010). Thus, both in Europe and potentially in the USA, human reproductive cells are treated in the same way as other non-reproductive tissues. For this reason, even though Pomeroy et al. (2010) considered the cross-contamination of infectious agents a negligible risk and the majority of cryobiologists and embryologists maintains that vitrification with open systems using non-sterile LN_2 is in practice safe, international regulations require specific procedures in embryo/oocyte/ovarian tissue cryopreservation in order to avoid any contamination of human cells due to direct contact with accidentally contaminated

LN_2. Any techniques preventing contamination must be welcome; an oocyte vitrification system which avoids any risk of contamination is urgently needed. In the future, this vitrification system may be useful not only for human oocytes but also for other human cells or tissues, or indeed whole organs. This study demonstrates to the legislator that an aseptic vitrification is feasible also when using open carriers and at the same time emphasizes that not every closed system is completely safe against contamination by LN_2 (**Figure 3**).

The only way to perform an aseptic vitrification procedure with all kinds of open carrier or with closed systems such as CryoTip and Cryopette is by sterilizing the LN_2 used during cooling and warming procedures. Furthermore, after vitrification, to guarantee an aseptic cryostorage, these carriers must be enclosed in hermetical containers (Parmegiani et al., 2009; Vajta et al., 2009).

For these reasons, subsequently to the first studies on LN_2 sterilization (Parmegiani et al., 2010), UV LN_2 sterilization was introduced as part of the clinical vitrification programme. Cryotops are used because of the high success rate with human oocytes with this open carrier reported in the literature (Antinori et al., 2007; Kuwayama et al., 2005a). For many years now, some authors (Liebermann and Tucker, 2002; Vajta et al., 1998, 2009; Vandervorst et al., 2001) have been proposing the enclosure of open carriers in pre-cooled hermetical containers. Due to the absence of specific hermetical containers for Cryotops at the time of the current study, home-made aluminium high-security goblets were used (**Figure 5B**) which reach a temperature of −196°C during the UV sterilization procedure and can contain up to six Cryotops. These goblets can be used also for other open or closed carriers requiring hermetical cryostorage, such as open pulled straws, Cryoleaf, Cryolock, Vitri-inga, CryoTip and Cryopette. Two other open carriers, Cryoloop and Plastic-blade, are already designed to be hermetically enclosed in a serum cryotube; unfortunately most of these serum cryotubes are not specifically designed for immersion in LN_2. In fact, the best known producer of these devices (Nunc, Roskilde, Denmark) recommend the difficult procedure of heat-sealing the cryotube inside another specific plastic tube (CryoFlex tubing; Nunc).

As an alternative to hermetical storage in LN_2, cryostorage contamination might be avoided by storing the carrier containing the vitrified oocytes in LN_2 vapour (Cobo et al., 2010a; Eum et al., 2009). However, Grout and Morris (2009) maintain that storage in the vapour phase of LN_2 still carries a risk of sample contamination.

The clinical results obtained in the current study on aseptic human oocyte open vitrification are comparable to the results obtained by other authors with infertile patients (Antinori et al., 2007; Almodin et al., 2010; Rienzi et al., 2010; Ubaldi et al., 2010). The current study obtained a pregnancy rate per transfer of 35.5% (11/31) and seven live births. No significant statistical differences were observed between fresh versus sibling vitrified–warmed oocytes. Other authors have previously demonstrated that vitrified human oocytes may have the same potential to fertilize and develop as their fresh counterparts, both in case of young donors (Cobo et al., 2008, 2010b) and in case of infertile patients (Almodin et al., 2010; Rienzi et al., 2010). In particular, performing a strict prospective randomized trial, Rienzi et al. (2010) clearly demonstrated the non-inferiority of Cryotop vitrified oocytes versus sibling fresh oocytes in infertile patients. The current study fully confirms the observation of Rienzi et al. and, in addition, reports for the first time, as far as is known, a straightforward method to eliminate the potential risk of cross-infection without modifying the principles of the Cryotop protocol, a wish expressed by Rienzi et al. in their study. Taking all these studies together, it is clear that UV LN_2 sterilization combined with hermetical cryostorage does not adversely affect the developmental competence of vitrified oocytes. This procedure allows the use of all kinds of carriers for human oocyte vitrification, avoiding as it does any risk of contamination and thus improving the safety of oocyte cryobanking programmes. Furthermore, by demonstrating for the first time the high efficiency of hermetical storage of vitrified human oocytes, this study may also encourage manufacturers in the design and production of specific hermetical containers for cryostorage of each type of carrier.

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