New methods for cooling and storing oocytes and embryos in a clean environment of −196 °C

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Abstract  It is well documented that oocyte vitrification using open systems provides better results than closed systems. However, its use is limited owing to risks of contamination posed by direct exposure to liquid nitrogen and cross-contamination when stored in liquid nitrogen tanks. A device that produces clean liquid air (CLAir) having similar a temperature as liquid nitrogen and a sterile storage canister device (Esther) that keeps samples sealed in their own compartment while in regular liquid nitrogen tanks were developed. The following experiments were performed: temperature measurements, bioburden tests, vitrification and storage experiments with mice embryos and human oocytes. Results showed similar cooling rates for liquid nitrogen and liquid air. Bioburden tests of CLAir and Esther showed no contamination, while massive contamination was found in “commercial” liquid nitrogen and storage canisters. Mice blastocysts had a survival rate of over 90%, with 80% hatching rate after vitrification in CLAir and 1 week storage in Esther, similar to the fresh (control) results. Human oocytes vitrified in CLAir and in liquid nitrogen for three consecutive vitrification/warming cycles showed 100% survival, seen as re-expansion in both groups. These new systems represent a breakthrough for safe vitrification using open systems and a safe storage process generally.

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KEYWORDS: closed systems, contamination, liquid nitrogen, open systems, storage, vitrification

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Introduction

Cryopreservation of biological samples by direct exposure to liquid nitrogen and their storage in standard liquid nitrogen tanks and canisters is problematic owing to the potential risk of contamination by viruses, bacteria, fungi and spores, which survive in liquid nitrogen and pose a real threat (Arav and Natan, 2013; Bielanski, 2012; Bielanski and Vajta, 2009; Vajta et al., 2015). The risk of contamination might be due to contaminants already residing in the liquid nitrogen when supplied to the laboratory or, alternatively, might be due to cross-contamination between samples (Grout and Morris, 2009; Vajta et al., 2015).

This risk of microbial infection becomes a serious threat when the biological samples are intended to be transplanted back to patients, as done for IVF procedures (Vajta et al., 2015), for ovarian cortex transplantation (Herraiz et al., 2014) and for future stem cell and germ cell therapies (Kurita et al., 2015; Tournaye et al., 2014). Therefore, the availability of safe cryopreservation protocols is very important and highly necessary for preventing the risks of contamination of biological samples during cooling and storage.

Vajta et al. (2015) described a series of procedures, normally carried out during cryopreservation, which highlight the potential sources and risks for contamination (see Table 1).

At present, heat-sealing is considered the best and safest method for the hermetical closure of storage carriers. However, as demonstrated in many publications, the viability results after vitrification in closed systems is lower than that compared with vitrification in open systems (Paffoni et al., 2011; Saragusty and Arav, 2011; Vajta et al., 2015). In fact, more live births have been reported using open systems (Chang et al., 2013; Cobo et al., 2015) than closed systems (Papatheodorou et al., 2013; Stoop et al., 2012). In addition, other sealing methods such as cotton plugs, beads (commonly used for spermatozoa), screw caps of cryovials or protective caps of several vitrification devices, do not provide an appropriate protection and allow the penetration of liquid nitrogen (Vajta et al., 2015).

To overcome these potential risks of contamination during vitrification and cross-contamination during storage, two devices were developed. The first (called CLAir; FertileSafe, Nes-Ziona, Israel), produces clean liquid air, which has the same temperature and properties as liquid nitrogen. The second (called Esther; FertileSafe, Nes-Ziona, Israel), is an insulating canister device enabling the sterile, compartmentalized storage of biological samples in standard liquid nitrogen tanks while preserving the liquid nitrogen temperature. This study reports experiments from the usage of these two devices for the vitrification and storage of mice embryos as well as for human oocytes donated to research. In addition, bioburden test results for these devices and temperature measurements are also provided.

Materials and methods

CLAir description

CLAir (FertileSafe Ltd) (Figure 1) is a bench-top device for the production of sterile liquid air made of two stainless steel containers, one inside the other, having a gap between them that is filled with commercial liquid nitrogen. The liquid air is produced inside the cooled, inner stainless steel container, which collects filtered room air (equipped with a 0.22 μm filter) and

Table 1 Procedures performed in assisted reproductive treatment laboratories that carry a risk of contamination.

<table>
<thead>
<tr>
<th>Procedures performed in assisted reproductive treatment laboratories that carry a risk of contamination.</th>
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<tbody>
<tr>
<td>Semen collection is not a sterile procedure.</td>
</tr>
<tr>
<td>Oocytes are contaminated with blood during collection.</td>
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<tr>
<td>Many containers are inappropriately sealed or closed by non-hermetical methods.</td>
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<tr>
<td>The outer surface of straws and vials is not sterile.</td>
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<tr>
<td>Storage tools (canisters, holders) are not sterile.</td>
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<tr>
<td>Openings of Dewars mix air with N₂ vapour and might cause contamination.</td>
</tr>
<tr>
<td>Factory-produced liquid nitrogen is not transported under aseptic conditions, and accordingly, cannot be regarded as sterile, even if during production the infective agents are largely destroyed.</td>
</tr>
<tr>
<td>Biological samples (sperm cells, oocytes, etc.) cannot be safely decontaminated.</td>
</tr>
<tr>
<td>In most IVF laboratories Dewars are not decontaminated regularly. Accordingly, tanks and the liquid nitrogen in the tanks should always be regarded as contaminated.</td>
</tr>
<tr>
<td>Cutters for opening straws*</td>
</tr>
</tbody>
</table>

Adapted from Vajta et al., 2015.

*This procedure was not included in Vajta et al., 2015.

Figure 1 CLAir®.
liquefies it. The CLAir device produces 250 ml of clean liquid air every 10 min when operated according to the instruction manual. The liquid air is collected into a specially designed sterile Styrofoam cup, which can be used for cryopreservation with open carrier systems. Liquid air has the same temperature as liquid nitrogen (−195.7°C).

Esther description

Esther is a device intended for storing biological samples in standard liquid nitrogen tanks avoiding direct contact with the surrounding liquid nitrogen. The device is made of stainless steel and it resembles a standard nitrogen canister having a top with an opening connected with a 0.22 μm syringe filter situated above the level of liquid nitrogen (see Figure 2). After the biological samples are loaded in Esther, the device is hermetically closed and placed inside the standard liquid nitrogen tank thus providing a sterile storage compartment. When there is a need to take out a sample or add another one, Esther is simply lifted up to the opening of the liquid nitrogen tank, the sealed ring is removed and the lid is opened. The Esther canister is then hermetically resealed by placing a new ring and repositioned back in the liquid nitrogen tank.

Temperature measurements

Experiments to measure cooling rates of different volumes (from 2.5 ml up to 50 ml) of cryogenic fluids (liquid air produced by CLAir as compared with standard liquid nitrogen) were carried out using a T-type thermometer connected to a data logger (Amemo 2290–4, MRC Ltd. Holon, Israel). The temperature of the clean liquid air and of the liquid nitrogen were measured immediately after being poured into Styrofoam cups. A thin thermocouple was connected to the tip of the Cryoleaf (Origio) carrier and was plunged into the cryogenic fluids at each volume. These temperature measurements were repeated three times for each volume (Figure 4).

Additionally, temperature measurements of 50 ml clean liquid air using a T-type thermocouple connected to a data logger (Amemo 2290–4, MRC Ltd, Holon, Israel) were carried out for more than 15 consecutive minutes, recording temperature readings every 10 s.

Experiments with biological samples stored in Esther and temperature curve measurements obtained daily for 1 week using a thermometer connected to a data logger (Amemo 2290–4, MRC Ltd) were also carried out.

Oxygen percentage measurements of clean liquid air

To evaluate the percentage of oxygen in the liquid air sample, an oxygen measuring system was devised, consisting of a 200 ml Styrofoam cup (Dart, Michigan, USA) covered by another smaller one (100 ml), upside down, and connected to an oxygen meter (PO2–250, MRC, Holon, Israel) (see Figure 3). The oxygen present in the air volume of the cup had to be removed, since it could have affected the measurements (false positive readings), by pouring 5 ml of liquid nitrogen into the cup and letting complete evaporation occur. After documenting zero percentage in the oxygen reading, 1.5 ml of liquid air was poured into the oxygen measuring system. The oxygen percentage was recorded at the beginning (when oxygen level was zero) until the liquid air sample had completely evaporated and the oxygen level reached its peak.
representing the oxygen concentration of the liquid air. This process of filling the cup with liquid nitrogen and then pouring 1.5 ml samples of liquid air and measuring the oxygen was repeated three times.

**Mice embryo experiments**

Five CBA male mice were bred with 10 BL C57 female mice (Harlan Laboratories, Rehovot, Israel). The mice were kept on a 12-h photoperiod schedule with unlimited water and food supply (Israeli animal ethics authorization no. IL-15–04–119 approved on 15 April 2015). Six to eight weeks after receiving F1 offspring, each female mouse received an intraperitoneal injection of 5 IU pregnant mare’s serum gonadotropin (PMSG) (Sigma, St Louis, USA). After 48 h each female received an intraperitoneal injection of 5 IU human chorionic gonadotrophin (HCG) (Sigma, St Louis, USA). Male CBA mice proven to be fertile were then put together with the superovulated females. The next morning the female mice were checked for copulatory plugs. If the plugs existed, the females were killed 24 h later (approximately 36 h post-coitus) and embryos at the 2-cell stage were retrieved from the oviducts. The oviducts were dissected in Quinn’s Advantage cleavage media (SAGE, Origio, Malov, Denmark) and the recovered embryos were transferred to 50 μl drops of Quinn’s Advantage cleavage media overlaid with mineral oil, cultured at 37°C under 5% CO2 and atmospheric oxygen in an incubator (Thermo Fischer Scientific, Waltham, MA USA) and monitored until they reached blastocyst stage.

**Vitrification, storage and rewarming**

Mice embryos were vitrified using an open carrier Cryoleaf (Origio, Malov, Denmark) and vitrification kit (Origio). One group of embryos (group A) were vitrified in clean liquid air and the other (group B) in liquid nitrogen. In brief, the embryos on the Cryoleaf were cooled by plunging the carrier into a sterile Styrofoam cup filled with 50 ml clean liquid air produced by CLAir (FertilSafe) (group A) or by plunging the Cryoleaf into liquid nitrogen (group B). Of note, the Styrofoam cups (with either liquid air or liquid nitrogen) are fitted with a special sterile holder immersed in them, which holds the Cryoleaf carriers when they are plunged. While still in the holder, the Cryoleaf carriers were inserted into sterile plastic goblets and then placed in a sealed, sterile storage canister device (Esther). After 1 week the Cryoleafs were warmed rapidly in 37°C warming solution (Origio) and washed in the same warming solution (Origio). The embryos were then kept in culture for two additional days and evaluated for hatching.

**Human oocyte experiments**

This study utilized existing cryopreserved oocytes that had been obtained from consenting patients and available for research studies (IRB-approved on 18 November 2013, approval number 15/13). The oocytes were first warmed, then re-vitrified using either clean liquid air (produced by CLAir, FertilSafe) or standard commercial liquid nitrogen and re-warmed.

The oocyte survival rate was evaluated by their morphological appearance 2 to 3 h after warming. Oocyte insemination could not be done, since this type of research is not permitted, but by using the same oocytes for three consecutive cycles of cooling, warming and dilution, a robust indicator for oocyte survival was provided. A stereo and an inverted microscope was used to assess the appearance of the zona pellucida, the perivitelline space, the plasma membrane (oolemma), the cytoplasm (ooplasm) and the intactness of the polar body. The oocytes were vitrified using Cryotop, an open system, (Kitazato BioPharma Co., Shizuoka, Japan) with Kitazato medium according to the Kitazato protocol (Cobo et al., 2013).

**Bioburden test**

A bioburden test was carried out by an independent company (Hy Labs, Rehovot, Israel) comparing samples of “commercial” liquid nitrogen taken from the conventional liquid nitrogen tank and samples of clean liquid air produced by CLAir. Briefly, 50 ml of clean liquid air produced by CLAir and 50 ml of liquid nitrogen taken from the conventional storage tank were poured into specially designed sterile Styrofoam cups and were allowed to evaporate. Then 10 ml of alpha modified-MEM medium (Sigma, St Louis, USA) was poured into the cups, collected in a sterile manner and sent to Hy-Labs for the bioburden test (Hy-Labs, Rehovot, Israel). Bioburden tests were also performed for the Esther canister and for the regular canister placed in a commercial liquid nitrogen storage tank (MVExc 35/12, Chart Industries, Garfield Heights, OH, USA) for 1 week. The canisters were then taken out of the tank and 10 ml of alpha modified-MEM medium (Sigma, St Louis, USA) was poured into each canister, collected in a sterile manner and sent to Hy Labs for the bioburden test (Hy Labs, Rehovot, Israel).

It should be stated that the above bioburden tests were not validated for negative results, i.e. the sterilization process. Usually when performing bioburden tests on commercial batches of products that are supposed to be sterile, a sample will be infected on purpose with a defined amount of bacteria; thus, when the samples undergo the sterilization process and are then evaluated by bioburden tests the results will be negative (unless the sterilization process was done correctly).

**Statistical analysis**

At least three cooling rate measurements for each sample’s volume were performed. Means were calculated and differences between treatments were examined by t-tests using the general linear model procedure of JMP (SAS Institute, 1994, Cary, NC). Significance was P < 0.05 unless otherwise stated. Different letters represent statistically different samples. Results are reported as mean ± standard error (SE).

**Results**

Cooling rates between liquefied air and liquid nitrogen were above 20,000°C/min for volumes of 50, 25 and 10 ml. At a
volume of 5 ml, liquid air had a lower cooling rate than liquid nitrogen, and at a volume of 2.5 ml, liquid nitrogen had a slower cooling rate than liquid air, but both were below 20,000 °C/min (statistically different only at the 2.5 ml volume; \( P < 0.05 \)) (Figure 4). Since the thermocouple introduces heat into the system, there is variability in the cooling rates between each measurement. Nevertheless, at or above the 10 ml volume samples all measured cooling rates were above 20,000 °C/min, which stands as a threshold for most successful vitrification protocols.

The oxygen percentage measured in clean liquid air was 20%, 20% and 21%, respectively, in the three measurements performed. In addition, the temperature measurements that were carried out for more than 15 consecutive minutes showed that liquid air temperature was stable over time and in the range of \(-194.4°C \) to \(-193.3°C \) (see Figure 5a). The temperature of liquid nitrogen was also measured and was \(-195.4°C \). Temperature readings for 1 week in the Esther device showed stable \(-196°C \) measurements throughout the week (Figure 5b). These observations demonstrate that samples stored in the Esther device (a closed dry canister) do not need to be in direct contact with liquid nitrogen for storage at liquid nitrogen temperature.

Bioburden tests showed no contamination in liquid air as opposed to commercial liquid nitrogen (Table 2) and no contamination in Esther compared with the regular canister (Table 2).

Survival of mice blastocysts after vitrification using liquid air (CLAir), storage for 1 week in an insulated canister (Esther) and rewarming was assessed by the percentage of re-expansion. The survival rate was 98% (117/120) and the hatching rate was 80% (96/120). The survival of the embryos vitrified in liquid nitrogen was 100% (30/30) and hatching rate was 90% (27/30). The hatching rates of both groups was similar to the fresh control group 80% (24/30) (kept only in culture and not vitrified at all).

A total of 25 metaphase II oocytes were used for the preliminary human experiments, of which 22 were vitrified with liquid air by CLAir and three with standard liquid nitrogen. Three consecutive vitrification/rewarming cycles were done for both groups, using a volume of 50 ml clean liquid air (CLAir). The survival rate was assessed by the degree of re-expansion after warming (no degeneration). In both groups the rewarming results showed 100% survival rate (22/22 for liquid air and 3/3 for liquid nitrogen), demonstrating no differences between the two cryogenic media and furthermore demonstrating that relatively low volumes of either liquid air or liquid nitrogen (50 ml) are sufficient for the vitrification process.

**Discussion**

Cryopreservation of oocytes and embryos are essential procedures for fertility treatments. Most protocols for oocyte
vitrification require direct exposure to liquid nitrogen for the cryopreservation procedures. This method, called open system vitrification, has been demonstrated to be superior (Vajta et al., 2015) to closed system vitrification. Open systems have higher chances for successful vitrification (Saragusty and Arav, 2011), since they employ low volumes (0.035 μl) (Arav, 2016) and allow very high cooling rates (>20,000°C/min) (Larman and Vanderzwalmen, 2016). This high cooling rate is important.

Figure 5  (A) Temperature measurements of clean liquid air (50 ml) poured into a specially designed Styrofoam cup. Measurements were recorded every 10 s consecutively for 18 min and 50 s. (B) One-week temperature measurements recorded within the Esther® storage device.
for reducing chilling injury of oocytes and embryos and, as shown in Figure 4, is achieved with as little as 10 ml of liquid air. The cooling rate is in the order of ~20,000°C/min (Figure 4) when using small volumes, what is referred to as the minimal drop size (MDS) technique, with carriers such as Cryotop and Cryoleaf that are plunged into 10 ml of liquid air or liquid nitrogen. However, when plunged into volumes less than 10 ml, there is a decrease in the cooling rate, and in fact with 5 ml of liquid air the cooling rate is 16,333.3°C/min. Therefore, exposing samples to low volumes results in lower cooling rates and as reported for the rapid-I method for vitrification the cooling rate is about 1200°C/min (Larman and Gardner, 2011). This low cooling rate, probably due to the small amount of liquid air that exists in the straw (less than 10 μl), is in contrast to the higher cooling rate achieved when using 1000 times larger volumes of liquid air.

Studies of closed systems have been published by isolated research groups, resulting in only 13 births and 20 ongoing pregnancies in total (Papatheodorou et al., 2013; Stoop et al., 2012), while thousands of births have been documented using vitrification with open systems (Chang et al., 2013; Cobo et al., 2013, 2015; Nagy, 2016; Vajta et al., 2015). However, when working with open carriers the risk of potential contamination must be taken into account (Bielanski, 2012; Bielanski and Vajta, 2009; Vajta et al., 2015). The two new devices reported in this study, CLAir for the production of clean liquid air and Esther for the insulated sterile storage, eliminate this potential risk. The results of this study demonstrated that using liquid air with two of the most used open systems, i.e. Cryoleaf and Cryotop (Cobo et al., 2013) for mice embryos and human oocyte vitrification, respectively, was as effective as liquid nitrogen, but without the risk of contamination. It also showed that clean liquid air has the same cryogenetic properties as liquid nitrogen. The oxygen percentage results and temperature measurements of clean liquid air indicate that liquefied air preserves the same ratio between nitrogen and oxygen that is presented in the air. By definition, liquid air contains 78% nitrogen, 21% oxygen and 1% argon. The boiling point of liquid air is the same as the lowest of its constituent gases, which is nitrogen; thus air remains liquid below (~195.79°C).

The results of the present study confirm and show that liquid air and liquid nitrogen have the same temperature. Furthermore, this temperature is constant for more than 15 min, providing sufficient time for the embryologists to perform the precooling handling. If this time is not sufficient, then of course more clean liquid air can be poured into the Styrofoam cup.

Storing biological samples in the Esther canister immersed in regular liquid nitrogen tanks is another step to prevent potential contamination (in this case cross-contamination of samples within liquid nitrogen tanks). Storage in a sterile closed compartment can also be used for quarantine of samples without the need of additional separate liquid nitrogen tanks.

In summary, this preliminary study describes two novel and simple methods that can be easily adopted in every assisted reproduction treatment laboratory to eliminate the potential risks of contamination and cross-contamination during cryopreservation and storage of oocytes, embryos or other biological samples.

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References


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Declaration: A Arav is the founder of FertileSafe Ltd., and both A Arav and Y Natan are employees of FertileSafe Ltd. PE Levi-Setti is medical advisor of FertileSafe Ltd., and P Patrizio is co-founder and medical advisor of FertileSafe Ltd. The study was funded by FertileSafe Ltd.

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