Oocyte vitrification/storage/handling/transportation/warming, effect on survival and clinical results in donation programmes

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Summary

Nowadays human gamete and embryo cryopreservation is integral to Assisted Reproductive (AR) techniques in optimizing treatment management and results. Cryopreserved cells can be stored for years and transported between authorized AR centres. Particularly in the case of heterologous insemination techniques, the use of frozen gametes facilitates donor/recipient matching and allows time for donor testing. Cryopreserved oocytes, spermatozoa and embryos can be shipped between specialized donation and cryopreservation centres (gamete cryobanks) and AR centres carrying out treatment on recipient patients. However, appropriate procedures need to be in place to minimize physical shocks and contamination during cryostorage, handling and shipping. The aim of the meeting “Shipping Gametes and Embryos” held in Milan on February 4th, 2017 was to address these critical aspects, including regulation and legal requirements, and to propose some troubleshooting procedures in the case of emergencies. A specific example of the possible negative impact of handling and transportation on survival of oocytes shipped between Spain and Italy was reported during the meeting: an inappropriate method of shipping reduced by 20% the vitrified oocytes’ survival at warming. The influence of different transportation methods and, in addition, the impact of the number of surviving oocytes used are described in detail through the analysis of original data presented for the first time at this meeting and reported in this article. The general suggestion emerging from this study is to take into account the possible negative impact of handling and different transportation methods on survival at warming and to assign an adequate number of oocytes per treatment, in order to inject a minimum of five oocytes.

KEY WORDS: gamete shipping, transportation, shipping cryo-injury, oocyte donation programme, vitrification.

Introduction

Human reproductive cells, particularly oocytes, are very sensitive to cryoinjury. Oocytes and embryos are frozen mainly by vitrification protocols and the frozen specimen are no longer protected by a layer of ice as used to be the case with “slow freezing”, the cryopreservation technique generally used until the last decade. For this reason, vitrified cells are highly sensitive to temperature shocks and the suggestion is that the temperature during handling, shipping and storage should never rise above safety threshold.
of -180°C. In fact, it has been demonstrated that vitrified oocyte survival rate can be lowered by 20% with less than optimal handling (1, 2).

Thus, handling and transportation between cryobanks and user clinics is critically important: complex shipping procedures require skilled staff not only for the biological procedures involved but also for preparation of documents to accompany biological specimens, procedures for shipment tracking and for Quality Assurance and notification of import/export to relevant national authorities.

**Survival rate of transported oocytes**

During the “Shipping Gametes and Embryos” meeting held in Milan on February 4th, 2017 our original data on survival rates of donor oocytes after transportation were presented for the first time. In this study we observed that survival was significantly affected by the temperature inside the dry shipper and by the type of courier used.

**Material and methods**

This was a trans-national multi-center, descriptive, retrospective study, performed from March 2015 to December 2016. The study was performed on 1,535 oocytes warmed in Italy, at GynePro Medical Center, Bologna. Oocyte were frozen by vitrification in 2 Cryobanks in Spain (Bank n°1 and Bank n°2) and 1 IVF clinic in Italy (GynePro Medical Center, Bologna). The oocytes from Spain were transported to Italy in three ways: by plane as hold baggage, carried by an operator as cabin baggage, or by road courier. Outcome measure was oocyte survival rate, observed at 2 hours from warming.

**Vitrification procedure**

Cryotop (Kitazato BioPharma Co, Japan) oocyte vitrification was performed at room temperature in a solution comprising 15% dimethylsulphoxide (DMSO) and 15% ethylene glycol (EG) and 0.5 mol/L sucrose. After a gradual initial equilibration of 15 minutes in a solution comprising 7.5% DMSO and 7.5% EG (3). For the ultra-rapid cooling, the Cryotops containing 1-3 oocytes were plunged into UV-sterilized LN₂ and closed with their plastic caps.

**Oocyte handling for transportation and warming**

The dry-shippers for oocyte transportation were equipped with temperature probes and data logger to monitor the temperature during the whole journey. The temperature graphics were downloaded on arrival of the dry-shippers at final destination. The handling between Spanish cryobanks and the Italian destination centre (GynePro Medical Center) followed these steps (Figure 1):

**Spain**

1. Oocytes were moved from the styrofoam box used for vitrification to the freezing centres’ cryo-tank for temporary storage;
2. Oocytes were moved from the cryo-tank to a dry-shipper. The dry-shipper was completely filled with liquid nitrogen only in the case of road courier shipment;
3. Shipment by A) plane as hold baggage or B) transportation by plane by an operator as cabin baggage or C) by road courier Italy;
4. The dry-shipper was opened and re-filled with liquid nitrogen;
5. Oocytes were moved from the dry-shipper to a styrofoam box to enable the identification of the cryocarriers;
6. Oocytes were moved from the styrofoam box to a cryo-tank for temporary storage before warming;
7. Oocytes were moved from the cryo-tank to the styrofoam box used for the warming procedure.

**Warming procedure**

The Cryotops were opened into the UV-sterilized LN₂ to minimize any potential microbial contamination (4-7) and immediately transferred from the goblet to the warming solution without having contact with the LN₂ in which the goblet is plunged. Each Cryotop was submerged in 4 mL of warming solution containing 1 M sucrose at 37°C. Then, the oocytes were incubated at room temperature for 3 minutes first in 0.5 M and finally washed for 6 minutes in ba-
Warmed oocytes were considered to have survived in absence of negative characteristics: dark or contracted ooplasm, vacuolization, cytoplasmic leakage, abnormal perivitelline space, cracked zona pellucida.

**Endometrial preparation and embryo transfer**

Preparation of endometrium for the embryo transfer (ET) was performed as described elsewhere (8). Embryo transfer was carried out after two (day 2) or three days (day 3) from oocyte warming and ICSI. Clinical pregnancy was defined as the presence of a gestational sac with or without Fetal Heart Beat (FHB) at ultrasound examination, two weeks after positive hCG testing.

**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 Lilliefors 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 $\chi^2$-method with Yates correction if needed. Difference was considered significant when a P-value was <0.05.

**Results**

Survival rate was 59.6% (34/57) when the temperature was at -180°C, 65.5% (288/439) at between -180°C and -190°C, 81.1% (619/763) at lower than -191°C (P<0.001). In the same way, survival was 65.4% (104/159) when the specimen was transported by plane, as hold baggage 57.1% (89/156) when carried by an operator as cabin baggage and 78.8% (705/895) when transported by road courier (P<0.001). Perhaps surprisingly, survival rate was not observed to be affected by donor age: 73.9% (536/725) at age 20-25, 75.7% (316/416) at age 26-30, 74.7% (74/99) at age 31-35 (P=N.S.; Tables 1-3; Figures 2, 3).

When we analyzed the survival rates we noticed that these were significantly higher (P=0.038)
for oocytes donated, retrieved, vitrified and stored in our centre compared with oocytes vitrified and shipped by Bank n°1: 87.5% (21/24), versus 64.7% (317/490) despite the slightly higher mean age of the donors in the first group (28.0±2.5 vs 26.0±0.4, P=N.S.). No significant differences were noticed between the other groups (Table 4, Figure 2).
In 227 thawing cycles performed with 1,432 donated oocytes, the mean number of vitrified oocyte per treatment was 6.2±0.1 and the mean number of surviving and injected oocytes was 4.7±0.2. We noticed a better trend in cumulative clinical pregnancy rate when injecting more than five oocytes (41.0%-48/117) rather than when using fewer than 4 (29.1%-23/79). Interestingly, oocyte survival rate was significantly higher in the batch from which more than five oocytes were injected (89.4%-741/829 vs 61.0%-368/603; P<0.00; Figure 4).

**Discussion**

Cryopreserved reproductive cells can remain stored for years (9, 10) and transported between authorized AR centres. It has been demonstrated that frozen spermatozoa, oocytes, and embryos perform as well as their fresh counterparts (4, 11, 12); this is the main reason why frozen gametes are widely used for human heterologous insemination techniques. Human oocytes are particularly sensitive to cryoinjury; thus, appropriate procedures are needed to minimize physical shocks and contamination during their handling, transportation and cryostorage.

In this multi-centre descriptive study of oocyte transported from Spain to Italy, for the first time, we demonstrate that shipping temperature and type of transportation can significantly affect oocyte survival at warming. We observed that the safest means of transportation is by road courier, where the dry shipper is kept completely filled with liquid nitrogen; this maintains the temperature below -196°C for the whole journey. Despite the fact the shipping temperature...
remains below the safe threshold of -180°C in both the transportation as hold baggage and as cabin baggage, these two transport methods seem to negatively affect the survival rate (Tables 1, 2), which can decrease by up to 20% compared with oocytes always kept submerged in liquid nitrogen (LN₂). This confirms previous observations made by McDonald et al. (1). This cryo-survival decline is probably due to the extreme sensitivity of vitrified oocytes, which are no longer protected by a layer of ice as used to be the case with “slow freezing”, the cryopreservation technique generally used until the first years of this century. Oocytes are probably injured during vapor-phase transportation and the handling necessary to move them from departure cryo-bank, to dry-shipper, and finally to destination cryo-bank (Figure 1). During transfer between cryo-vessels, moving from the liquid to vapor phase of nitrogen and back, temperature and humidity can potentially change in uncontrolled ways which may harm the oocytes. In clinical practice, a low survival rate can have a knock-on effect on the number of oocytes available for in vitro fertilization. Thus, the final number of surviving oocyte is a key factor that potentially influences the final clinical outcome: in our study we observed the highest pregnancy rates in egg-donation treatments where more than five oocytes were injected.

In the light of this study, in order to maximise the clinical results of a trans-national egg-donor programme, it seems important to consider the possible negative impact of handling and transportation methods on cryo-survival and to assign an adequate number of oocytes per treatment, preferably injecting a minimum of five oocytes.

References