Original Article

Comparison of the Developmental Potential and Clinical Results of *In Vivo* Matured Oocytes Cryopreserved with Different Vitrification Media

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Abstract

Background: Oocyte vitrification is widely used throughout the world, but its clinical efficacy is inconsistent and depends on the vitrification media. This study compared the developmental potential and clinical results of *in vivo* matured oocytes cryopreserved with different vitrification media.

Methods: This retrospective study involved vitrified-warmed oocytes at one *in vitro* fertilization laboratory. Vitrification media kits comprised the MC kit (ethylene glycol [EG] plus 1,2-propanediol [PROH]), the KT kit (EG plus dimethyl sulphoxide [DMSO]), and the Modified kit (EG plus DMSO and PROH kit). Rates of oocyte survival and subsequent developmental potential were recorded and analyzed. The *t*-test and the Chi-square test were used to evaluate each method's efficacy.

Results: Oocyte survival rate was significantly higher for the Modified kit (92.0%) than for the MC kit (88.2%) ($P < 0.05$) and the KT kit (77.3%) ($P < 0.001$). The rate of high-quality embryo development in the Modified kit group (35.8%) was significantly higher than in the MC kit group (29.0%) and the KT kit group (28.3%) ($P < 0.001$). No significant differences were observed in the clinical pregnancy and implantation rates among the MC, KT, and Modified kit groups (37.2% vs. 30.2% vs. 39.6%; 21.9% vs. 18.8% vs. 27.4%, respectively) ($P > 0.05$). The high-quality embryo rate per warmed oocyte was significantly higher (23.4%) in the Modified kit group than in the other groups ($P < 0.001$). The embryo utilization and live birth rates per warmed oocyte were the highest in the Modified kit group, but not significantly ($P > 0.05$).

Conclusions: Modified vitrification media are efficient for oocyte vitrification and, with further verification, may be able to replace commercially available media in future clinical applications.

Key words: Fertilization; Live Birth; Oocytes; Pregnancy; Vitrification Media

Introduction

In the most recent three decades since 1986, oocyte cryopreservation, an essential female fertility preservation method, has gained importance as a useful adjunct to routine *in vitro* fertilization (IVF) in various clinical scenarios. The recent removal of the "experimental" label for oocyte cryopreservation by the American Society for Reproductive Medicine Practice Guidelines Committee has opened a new era for this technology.[1] Oocyte cryopreservation, especially using vitrification, has proven to be an efficient technique, resulting in pregnancy outcomes similar to those of IVF with fresh oocytes.[2]

In spite of the promising results for oocytes cryopreservation mentioned above, multimolar concentrations of cryoprotectants employed in the vitrification process may cause damage

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from excessive osmotic pressure changes and resultant cell volume excursions.[3-5] The combination of ethylene glycol (EG) plus dimethyl sulphoxide (DMSO) and EG plus 1,2-propanediol (PROH) are both effective in oocyte vitrification during clinical applications.[6-9] However, in spite of successful results of previous studies, oocyte survival instability often interfered with the media's clinical efficacy. We believe that the long shelf life and transit time could affect the stability of these media. Thus, we devised a more efficient method for oocyte vitrification, preparing a media with modified composition. We prepared a combination media using three penetrating cryoprotectants and named it the Modified kit. This combination media improved when more than two penetrating cryoprotectants were used. After successful experiments in 1-day-old oocytes in which fertilization failed, and maturation was delayed (data not published), we tested this combination media in the clinical application of vitrification for fresh oocytes. In this paper, we retrospectively analyzed the effects of three kinds of vitrification media on vitrified-warmed oocytes. We hope that these data will supply the needed information for developing more effective oocyte vitrification methods.

Methods

Patients and oocyte collection

The oocytes vitrified in this study were extracted from patients at our center. The reasons for oocyte vitrification were fertility preservation or failure to obtain semen on the day of oocyte retrieval.

Patients underwent conventional IVF or intracytoplasmic sperm injection (ICSI) cycles and offered the option of oocyte preservation on the oocyte retrieval day when >20 oocytes were retrieved. Based on the number of oocytes collected, 15–20 were to be inseminated in fresh cycles. The reason for choosing patients from whom >20 oocytes were retrieved was based on the data of the patients who visited our hospital. In our hospital, the embryo cryopreservation rate per cycle was >70%. Usually, 10–15 oocytes would result in baby delivery for most patients without other complex etiology, so, we chose enrolling patients from whom >20 oocytes were retrieved. All these patients were fully informed of the advantages and disadvantages of oocyte cryopreservation. Oocyte cryopreservation would reduce the number of embryo frozen and avoid ethic and legal controversy companied by embryo cryopreservation in the future. For the patients got delivery success in fresh oocytes cycle, these cryopreserved oocytes would facilitate oocyte donation. Another reason for oocyte vitrification was failure to obtain semen in a timely manner. All participants had a normal karyotype and were treated with controlled ovarian hyperstimulation after gonadotropin-releasing hormone agonist downregulation or antagonist protocols. An 8000–10,000 U dose of human chorionic gonadotropin (hCG) was given when at least two follicles in the two ovaries reached a diameter of 20 mm. Oocyte retrieval was performed by transvaginal ultrasound–guided follicle aspiration 35–37 h later.

Informed consent was obtained from all patients whose oocytes were vitrified, and the number of oocytes to be vitrified was specified by the patients in the consent forms. All these oocytes were cryopreserved for the patients. The oocytes used in this study were warmed during the period January 2012 to December 2013, prior to which they had been vitrified and cryopreserved in liquid nitrogen at any time during 2008–2013. Patients who failed to achieve pregnancy in both, fresh cycle and frozen embryo transfer cycles and had supernumerary oocytes cryopreserved were provided the option to transfer embryos derived from the vitrified-warmed oocytes. This study was approved by the ethics committee at our hospital.

Oocyte preparation and vitrification kit

After being cultivated for 3–4 h in a 37°C, 6% CO_2 incubator, the oocytes for vitrification were put into hyaluronidase media (SAGE BioPharma, NJ, USA) for denudation of cumulus cells and corona radiata. Only mature oocytes were vitrified.

The vitrification kit used in this study included two commercially available kits – the so-called MC kit and KT kit – and one Modified kit prepared in our lab. The penetrating cryoprotectants in the MC kit were EG and PROH (MediCult Vitrification Cooling, Copenhagen, Denmark). To obtain more efficient clinical results, the Kitazato KT kit (Kitazato Biopharma Co., Ltd., Shizuoka, Japan), which includes EG and DMSO, was then used. The Modified kit was made up of three kinds of penetrating cryoprotectants: EG (Sigma-Aldrich, St. Louis, MO, 102466, USA), DMSO (Sigma-Aldrich, St. Louis, MO, D2650, USA), and PROH (Sigma-Aldrich, St. Louis, MO, 544324-068, USA). All these vitrification kits were prepared with equilibrium solution (ES) and vitrification solution (VS). As for the MC kit and KT kit, ES included 7.5% EG + 7.5% PROH (DMSO), and the VS constituted of 15% EG + 15% PROH (DMSO) + 0.5 mol/L sucrose, per the instructions. The Modified kit was prepared with M-199 (Gibco Invitrogen Corp., Grand Island, NY, USA) as the basal media. A 20% serum plasma substitute (SPS) (SAGE, Trumbull, CT, USA) was also added. The ES for the Modified kit comprised 7.5% EG + 3.75% DMSO + 3.75% PROH, and the VS comprised 15% EG + 7.5% DMSO + 7.5% PROH + 0.5 mol/L sucrose in a M-199 medium with 20% SPS. The commercial kits were used before the expiration date on the package. The Modified kit was generally prepared in 10 days.

Oocyte vitrification and warming

Oocyte vitrification was performed at the room temperature. Regardless of whether they were washed in basal media, the oocytes were equilibrated in ES for 5–10 min until they recovered their shape. They were then placed into the VS for 1-min. Finally, the vitrified oocytes were placed on a CryoLoop (Hampton Research, Laguna Niguel, CA, USA) and immediately immersed in liquid nitrogen. No more than four oocytes were loaded onto each CryoLoop.

Oocyte warming was performed at room temperature, except for the first step. The CryoLoop with the vitrified oocytes was taken out of the liquid nitrogen and immediately placed in

1.0 mol/L sucrose in a M-199 + 20% SPS solution at 37°C for 1.5–2.0 min. Next, the oocytes were placed in 0.5 mol/L sucrose in an M-199 + 20% SPS solution for 3 min at the room temperature, after which they were transferred into another M-199 solution with 0.25 mol/L sucrose for 3 min. Finally, they were washed in M-199 + 20% SPS for 5–10 min while the stage was warmed slowly. After warming, the surviving oocytes were cultured for 2 h in G-IVF (Vitrolife, Göteborg, Sweden) in an incubator with 37°C, 6% CO_2 before being inseminated by ICSI.

Oocyte fertilization and embryo culture

The surviving oocytes with intact zona pellucida and plasma membranes were given ICSI procedure for fertilization. A fertilization check was performed 16–20 h later, and the 2 pronuclei (2PN) zygotes were followed for another 64–72 h in G-1 (Vitrolife, Göteborg, Sweden) for cleavage. According to Puissant's standard for embryo grades, seven- to ten-cell embryos with <30% fragmentation were deemed high-quality embryos. On day 3, 2 to 3 high-quality embryos were selected for embryo transfer, and others were cultured for another 3 days for blastocyst vitrification. According to Gardner's standard,[10] only blastocysts evaluated with grade 4BC and higher quality was cryopreserved. Motile sperm used for ICSI was obtained from each patient's husband via masturbation, percutaneous epididymal sperm aspiration (PESA), or testicular sperm aspiration (TESA), on the day of oocyte warming. Several patients had to accept donor sperm because no sperm was available from the husband. All semen was prepared by discontinuous density gradient centrifugation.[11]

Uterine preparation and clinical outcomes

Endometrial preparation included 17-β-estradiol (Schering, Germany) dosed at 4–6 mg/d for 14–16 days until a trilaminar endometrium >8 mm in thickness was identified. Progesterone 40 mg/d was injected, and oocytes were warmed on the next day. Embryo transfer was performed on the 4th day of progesterone injection, which was continued up to 12 weeks of pregnancy.[12] No more than three embryos were transferred per cycle.

Pregnancy was confirmed by a rise in serum hCG concentrations 14 days after embryo transfer. Clinical pregnancy was determined by ultrasonography, which demonstrated a gestational sac at 7 weeks. Miscarriage was classified as either "early" (before 12 weeks of gestation) or "late" (after 12 weeks of gestation). Implantation rate was defined as the number of gestational sacs per transferred embryo. The live birth rate per embryo transferred and per warmed oocyte were both calculated.

Statistical analyses

Age, oocyte survival rate, 2PN rate, high-quality embryo rate, number of embryos transferred, number of embryos cryopreserved, clinical pregnancy rate, implantation rate, early miscarriage rate, and live birth rate were recorded and compared. To evaluate the utilization of vitrified-warmed oocytes more precisely, embryo utilization rate per warmed oocyte was also calculated and compared. Statistical analyses were performed using the *t*-test and the Chi-square test. IBM SPSS software version 16.0 (IBM SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Values were considered statistically significant when $P < 0.05$.

Results

The vitrified-warmed oocyte cycles in the MC kit group, the KT kit group, and the Modified kit group separately totaled 46, 58, and 56 from 2012 to 2013. There were three, four, and three separately canceled embryo transfer cycles owing to poor embryo quality in the three groups, respectively. One total embryo cryopreservation cycle was performed in the KT kit group for other reasons. There were no significant differences in age, endometrial thickness, semen quality, and oocyte cryopreservation storage time in the three groups. Although the three cryopreservation kits were not applied at the exact same time, there was no change in the selection criteria for oocyte cryopreservation and thawing. The culture medium used, laboratory environment and operating technology remained constant during this study.

The oocyte survival rate in the Modified kit group was higher than in the other two groups (92.0% vs. 88.2% for the MC kit, χ^2=4.537, $P < 0.05$; 92.0% vs. 77.3% for the KT kit, χ^2=80.286, $P < 0.001$). The high-quality embryo rate in the Modified kit group was significantly higher than in the other groups (35.8% vs. 29.0% for the MC kit and 28.3% for the KT kit) (χ^2=27.072, $P < 0.001$). There was no significant difference in clinical pregnancy rates and implantation rates in the three groups (37.2% vs. 30.2% vs. 39.6%; 21.9% vs. 18.8% vs. 27.4%, respectively) ($P > 0.05$). However, the number of embryos transferred in the Modified kit group was lower than in the MC kit group, and the difference was significant (1.89 ± 0.59 vs. 2.28 ± 0.83, t = 2.751, $P < 0.001$) [Table 1].

There was a higher early miscarriage rate in the Modified kit group, but no significant difference compared with the other groups ($P > 0.05$). Fortunately, no later miscarriages occurred in the three groups. Nineteen healthy babies were delivered from 14 women in the MC kit group, 20 babies from 15 women in the KT kit group and 22 babies from 17 women in the Modified kit group. All these babies were healthy except for two, which were injured during the birthing process.

Regarding oocyte utilization, the high-quality embryo rate per warmed oocyte in the Modified kit group was higher than in the other groups (χ^2=16.487, $P < 0.001$). The embryo utilization rate per warmed oocyte and the clinical pregnancy rate per warmed cycle were also higher in the Modified kit group than in the other two groups, but without significant difference ($P > 0.05$). The live birth rates per warmed oocyte in the three groups were 3.5%, 3.2%, and 4.1% for the MC kit group, KT kit group, and Modified kit group, respectively ($P > 0.05$) [Table 1].

Table 1: Laboratory and clinical results using vitrified-warmed oocytes with different vitrification media

Variables	MC kit	KT kit	Modified kit
Cycles	46	58	56
Patient age (years), mean ± SD	29.7 ± 4.2	30.5 ± 4.7	30.1 ± 4.8
Number of oocytes warmed	549	628	539
Oocyte survival rate, % (*n*/*N*)	88.2 (484/549)	71.3 (448/628)	92.0 (496/539)*,†
2PN rate, % (*n*/*N*)	62.6 (303/484)	72.5 (325/448)	71.0 (352/496)
High-quality embryo rate, % (*n*/*N*)	29.0 (88/303)	28.3 (92/325)	35.8 (126/352)†
High-quality embryo rate per armed oocyte, % (*n*/*N*)	16.0 (88/549)	14.6 (92/628)	23.4 (126/539)†
Number of cycles performed embryo transfer	43	53	53
Number of embryos transferred	105	117	106
Number of embryos transferred per cycle, mean ± SD	2.28 ± 0.83	2.02 ± 0.93	1.89 ± 0.59†
Number of embryos cryopreserved	19	21	35
Embryo utilization per warmed oocyte‡	22.6 (105 + 19/549)	22.0 (117 + 21/628)	26.2 (106 + 35/539)
Implantation rate, % (*n*/*N*)	21.9 (23/105)	18.8 (22/117)	27.4 (29/106)
Clinical pregnancy rate per transfer cycle, % (*n*/*N*)	37.2 (16/43)	30.2 (16/53)	39.6 (21/53)
Early miscarriage rate per pregnant cycle, % (*n*/*N*)	12.5 (2/16)	6.3 (1/16)	19.0 (4/21)
Live birth rate per embryo transferred, % (*n*/*N*)	18.1 (19/105)	17.1 (20/117)	20.8 (22/106)
Live birth rate per oocyte warmed, % (*n*/*N*)	3.5 (19/549)	3.2 (20/628)	4.1 (22/539)

*P<0.05, oocyte survival rate in the MC kit group compared to that in the Modified kit group; †P<0.001, oocyte survival rate in KT kit group versus that in the modified group; high-quality embryo rate in KT kit group and MC kit group versus the modified group; number of embryos transferred per cycle in the Modified kit group versus the MC kit group. ‡: The value is expressed as No. of embryos transferred + No. of embryos cryopreserved / No. of oocytes warmed. 2PN: 2 pronuclei. SD: standard deviation.

Discussion

The aim of oocyte cryopreservation is to efficiently preserve female fertility and to obtain optimal clinical results using fewer oocytes. This retrospective study showed that using a modified vitrification media, vitrified-warmed oocytes had a higher survival rate and resulted in more high-quality embryos. The modified vitrification media might bring about the higher oocytes vitrification efficiency based on the number of embryo transfers.

Since the birth of the first baby through IVF using oocyte vitrification in 1999,[13] EG has been commonly utilized in the vitrification process in conjunction with either DMSO[7,8] or PROH.[6,14] As a cryoprotectant with low molecular weight and toxicity, EG is an important component of oocyte vitrification kits. We found that the combinations of EG plus DMSO and EG plus PROH were both feasible for cryopreservation in the clinic, although it was not easy to determine which combination was superior. To achieve more effective oocyte vitrification, we included DMSO and PROH in our modified oocyte vitrification media, which contained a constant EG concentration, as is customarily used. As expected, a higher oocyte survival rate was achieved with the Modified kit compared with the other commercially available kits. The oocyte survival rate for the Modified kit was also higher than had been reported by Siano *et al*. (86.7%)[15] and a meta-analysis (88%).[16] The 2PN rate was 71% for the Modified kit group, similar to the 73% rate for nondonor oocytes reported in an equivalent meta-analysis.[16] The high-quality embryo rate in the Modified kit group was significantly higher (35.8%) than in the other groups, although it was lower than had been previously reported (48.1%).[17] This difference might have resulted from using a different embryo evaluation system. The implantation rate of the Modified kit group was 27.4%, which was similar to the 25% reported by Siano *et al*.[15] The laboratory and clinical data for the Modified kit group were very encouraging compared with the groups that used the two commercial kits at our center. When compared with other reported oocyte vitrification data, ours were equivalent if not superior. As previously reported, Papatheodorou *et al*. achieved an oocyte survival rate of 91.0%, but the clinical pregnancy rate was only 33% and the implantation rate was only 10.1%,[18] not comparable with the results from the Modified kit in this study (39.6% and 27.4%, respectively). Although the 19% miscarriage rate of the Modified kit group was higher than in the other groups, it was still comparable with rates from the previous report (20%).[16]

When discussing oocyte vitrification efficiency, the quality of the oocytes and semen should be mentioned. In this study, the reasons for undertaking oocyte vitrification and warming were similar in the three groups, so we can conclude that the results might have related closely to oocyte quality. One important indicator of the vitrification method's efficacy is the oocyte survival rate, which in the Modified kit group was comparable with the high survival rates reported by Cobo *et al*. (92.5%) and Figueira *et al*. (94.8%); however, it should be noted that these studies used donor oocyte cycles.[2,19] The clinical pregnancy rate of 40% in the study of Figueira *et al*. and the 50.2% rate in the study of Cobo *et al*. for donor oocyte cycles were comparable with the rates we obtained with vitrified oocytes in commercial media, that is, the KT kit and the MC kit (data not published). Unquestionably, the oocytes used in this study, which came from women with a history of failed pregnancies in fresh IVF cycles, were

not comparable in quality with donor oocytes. However, we did not obtain sufficient data on the results of donor oocyte cycles that used the Modified kit media, so these data were not reported in this paper. Sperm quality was another important factor in clinical efficacy. In a case-control study that strictly limited patient age and body mass index, the clinical pregnancy rate for initial IVF cycles using sperm that was not obtained through PESA or TESA was 53.9%, which was comparable with results obtained using fresh oocytes cycles.[20] In addition, keeping oocytes in liquid nitrogen for 15 min is not comparable with storing them long-term. Song *et al*. reported a clinical pregnancy rate of 53.3% when vitrified-warmed oocytes were inseminated with donor sperm,[21] a higher rate than the 39.6% achieved in this study. Thus, semen quality might explain the difference in success rates.

Based on the above discussion, we concluded that the modified vitrification media used in our study was superior to the commercial vitrification media and comparable with previously reported data. Our results can be explained in two ways. One is that our vitrification media combined a lower concentration of penetrating cryoprotectants with increased variety of cryoprotectants, thereby retaining optimal permeability. Lower cryoprotectant concentration meant lower cytotoxicity to the oocytes. The other explanation for our results is that less time in transit and shorter storage times meant less denaturation of the protein or serum substitute added to the media. All those factors helped boost the efficiency of the vitrification process.

Regarding the reasons for oocyte vitrification, failed pregnancy during fresh IVF cycles for any reason meant poor clinical results from these vitrified-warmed oocytes. When not enough cycles were included, the data analysis for oocytes vitrified for different reasons was not available. Data analysis that included such detailed classification would have been more conclusive. Even so, cumulative pregnancy rates are able to testify to the quality of the warmed oocytes that were used. We did not include these data, however, because an insufficient number of vitrified embryos from vitrified-warmed oocytes were transferred. If more robust clinical trials are conducted in the future, the resultant data can be used to verify the efficiency of the Modified kit. Until then, the safety of oocyte vitrification using this modified media should be considered. This newly designed modified vitrification media led to efficient oocyte vitrification, and in future, might take the place of commercially available vitrification media because of its lower cost and ease of preparation.

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Conflicts of interest

There are no conflicts of interest.

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