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Developmental consequences of mouse cryotop-vitrified oocyte and embryo using low concentrated cryoprotectants

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Abstract

Background: The risk of multiple pregnancies, often present in programs of In Vitro Fertilization (IVF), is an important force for embryo cryopreservation. On the other hand, ethical restriction and assurance of potential fertility following chemo/radio therapy has led scientists to focus on female gamete preservation.

Objective: Optimizing vitrification protocol by using less concentrated cryoprotectants (CPAs) in order to decrease CPAs toxicity.

Materials and Methods: Mouse Metaphase-II (M-II) oocytes and four cell-stage embryos were collected. Oocytes Survival, Fertilization and Developmental Rates (SRs, FRs, DRs) were recorded after cryotop-vitrification/warming. As well as comparing fresh oocytes and embryos, the data obtained from experimental groups (exp.) applying 1.25, 1.0, 0.75 molar (M) CPAs were analyzed in comparison to those of adopting 1.5 M CPAs [largely-used concentration of Ethylen Glycol (EG) and Dimethyl-sulphoxide (DMSO)].

Results: The data of oocytes exposed to 1.25 M concentrated CPAs were in consistency with those exposed to 1.5 M and control group in terms of SR, FR and DR. As less concentration was applied, the more decreased SRs, FRs and DRs were obtained from other experimental groups. The results of embryos which were exposed to 1.25 M and 1.0 M were close to those vitrified with 1.5 M and fresh embryos. The results of 0.75 M concentrated CPAs solutions were significantly lower than those of control, 1.5 M and 1.0 M treated groups.

Conclusion: CPAs limited reduction to 1.25 M and 1.0 M instead of using 1.5 M, for oocyte and embryo cryotop-vitrification procedure may be a slight adjustment.

Key words: *Cryotop, Cryoprotectant, Embryo, Mouse, Oocyte, Vitrification.*

Introduction

Avoiding the risk of multiple pregnancies, scientists have led to not transfer all embryos produced in-vitro and do cryopreserve the surplus embryos as efficiently as possible (1). On the other hand, embryo cryopreservation has been restricted or even forbidden in some countries (2). One solution to these problems could be the cryopreservation of female gametes. In another aspect, the condition of iatrogenic sterility after chemo/radiotherapy in neoplastic pathologies would be avoided by the preservation of oocytes. In addition, the utilization of frozen oocytes could be included in a program of oocyte donation (3).

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Through ultra-rapid freezing (vitrification), liquid is so rapidly cooled that it forms into a glassy, vitrified solid state from the liquid phase at low temperature, not by ice crystallization, but by extreme elevation in viscosity during cooling (4). Basically, vitrification approach eliminates totally one source of chilling injury, ice crystal formation, and avoids zona and blastomere fracture (5); however, it exposes cells to a considerably elevated toxic and osmotic effect (6). Recently, Kuwayama has gone into great details of terms and conditions, to summarize arguments supporting or disapproving the use of vitrification, and to outline its role among Assisted Reproductive Technologies (ARTs) (7). Not only Kuwayama, but also other researchers have held firmly to the belief that vitrification does not necessarily require high CPA concentrations, because even pure water can be vitrified if the cooling rate is high enough (-10^{7}°C/s) and, on the other hand, with concentrated CPA solutions, vitrification can also be achieved with a moderate or even slow cooling rate (8, 9).

A mixture of CPAs can decrease individual specific toxicity. The most common used mixture is EG, DMSO, and sucrose (10). To optimize results, besides an appropriate selection of CPAs, these agents should be used at as low a concentration as possible, while still achieving vitrification (9). By dramatically increasing the cooling rate, the CPA concentration can be reduced, preserving the cells at non-toxic concentrations of CPA (9). Minimizing the volume of the vitrification solution, containing oocytes or embryos, not only offers the obvious benefit of increasing both cooling and warming rates, but also decreases the chance of ice crystal nucleation/formation in the small samples. The higher cooling rate, the lower concentrated CPAs solutions can be used (11).

The latest approach to minimum volume vitrification is the cryotop device (Kitazato Supply Co., Fujinomiya, Japan). The producers make the point that advantage of this method is that an extremely fast cooling rate is achieved (23,000°C/min) and chilling injury is avoided. The extremely small volume, also helps achieving a faster warming rate (42,000°C/min), thereby avoiding ice crystal formation during warming (7).

To further improve the performance of egg freezing, technical variations are proposed. In spite of the vast efforts invested, advances were rather slow (12). Reasons for this may include low permeability of the oocytes membrane to CPAs and susceptibility to cooling and toxic effects of CPAs that affect various aspects of the oocytes' physiology (13). To embryo freezing, the main problems are the lack of consistency as well as differences in SRs and DRs after warming between species, developmental stages and the systems of embryo production (14).

To avoid a degree of uncertainty surrounding the outcome of the IVF procedure and to achieve success to overcome infertility, using the most qualified gametes plays the central role in ART program (1). Cryopreservation protocols efficiency is evaluated by the point that how much they are able to preserve the quality of the freezed-gametes or embryos (15).

Regarding efficiency, assessing current vitrification protocols is not an exception. Obviously decreasing CPAs concentration and therefore the toxicity would be a step toward achieving the more efficient cryopreservation technique. In the present study, investigating developmental consequences of cryotop-vitrified mouse M-II oocytes and four cell-stage embryos using low concentrated CPAs made a case for us to re-examine the vitrification protocol and improve the technique.

Materials and methods

This was an experimental study. Animals were cared according to the university guide for the care and use of laboratory animals. All chemicals were purchased from Sigma unless otherwise stated.

Experimental design

M-II mouse oocytes and four cell stage-embryos were allotted randomly to one of the followings; one control and five experimental groups. The oocytes of exp. 1 to 5 were subjected to Partial Zona Dissection/ Intra Cytoplasmic Sperm Injection (PZD/ICSI) procedures and in vitro culture after vitrification/warming, using 0.75 M Equilibration Solution (ES) and 1.5 M Vitrification Solution (VS), 0.625 M ES and 1.25 M VS, 0.5 M ES and 1.0 M VS, 0.75 M ES and 0.75 M VS and 0.375 M ES and 0.75 M VS, in order. Similarly vitrified embryos, assigned to exp. 1 to 5, were cultured in vitro after warming. Non-vitrified fresh oocytes and embryos were considered as control group.

Experiments in each series were repeated at least seven times.

Reagents and media

The vitrification kit (Kitazato Biopharma, Mitojima, Japan) included: Base Medium (BM)

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that was HEPES-buffered TCM 199 medium, ES and VS were Serum Substitute Supplement (SSS) enriched-BM and CPAs, EG (E9129) and DMSO (D2650), without or with sugar (sucrose) (S1888) respectively, Thawing Solution (TS), Diluents Solution (DS) and Washing Solution (WS) contained SSS enriched-BM and different concentration of sucrose. The medium for oocyte manipulation and embryo culture was Hypermedium, as Eroglu *et al* indicated (16). Before use, drops of the Hypermedium were overlaid with embryo-tested mineral oil (M5310) and equilibrated overnight under a humidified atmosphere of 6% CO2 in air at 37°C.

Embryo and egg collection

M-II oocytes were obtained from 8 to 10 weeks old C57BL/6J mice; Pasteur Institute animal house, Iran. Superovulation was induced intraperitoneally as the same as that described previously (17).

To collect M-II oocytes and four cell stage-embryo, the oviducts were excised from euthanized mice 13–14 h or 56-57 h after hCG injection, respectively. Oocyte-cumulus masses were released from the ampulla by a needle incision and embryos were collected by flushing the oviducts.

To remove cumulus cells, the oocyte-cumulus masses were exposed to 120 IU/ml bovine testis hyaluronidase (H3506) at ambient temperature for 3–4 min. After oocytes/embryos collection, they were washed in HEPES-buffered Hypermedium twice and then transferred to the Hypermedium for recovery before experimentation. For each experiment, oocytes/embryos were isolated from three or more female mice, pooled, and then randomly distributed among the experimental groups.

Vitrification / warming

Mouse M-II oocytes and four cell stage-embryos were vitrified/ warmed by the minimum volume cooling method using cryotop as exactly as mentioned by Kyono *et al* (18). There were five or six oocytes/embryos on each cryotop. The oocytes/embryos with a poor grade, irregular contours, dark coloration, or fragmented unequal blastomeres were excluded from cryopreservation. The appropriate time for ES to permeate inside of the oocytes and embryos was measured 7-8 or 5 min. respectively, for perfect recovery of cell size and shape.

Cryostorage was done in Liquid Nitrogen (LN2) for 5 days. Following warming re-expanded oocytes/embryos were considered to have survived and transferred to the Hypermedium for recovery before experimentation.

Fertilization and development of cryopreserved oocytes

12-14 weeks old C57BL/6J male mice were used to obtain sperm. Sperm preparation was performed as previously described (19). Mouse oocytes PZD/ ICSI were performed after 120 minutes incubation period (20) following the method of Balaban *et al* (21) with brief modifications. The zona pellucida of cryosurvived oocytes (assessed by translucent appearance of cytoplasm, integrity of the plasma membrane and the zona pellucida, the size of the perivitelline space and extruded polar body) was dissected on 10–15% of its circumference with a fine glass needle, far from the polar body area at 37°C. Morphologically normal, motile spermatozoa were randomly selected for ICSI. Separated sperm heads were injected into the PZD-oocytes at 17°C on a pre-cooled inverted microscope. Injected oocytes were left to rest for 20 min. at 17°C followed by 15 min. at room temperature (RT). All oocytes after ICSI were washed four times in Hypermedium 0.4% Bovine Serum Albumin (BSA) (w/v) (A3311). Fertilized oocytes were cultured in a 60 mm tissue culture dish in 20 µl of Hypermedium 0.4% BSA (w/v) under mineral oil. The presumptive zygotes and developed to two cell-stage embryos were scored 6 and 24 h after insemination, respectively.

Culture of cryopreserved embryos

Cryosurvived embryos (assessed by morphologically normal blastomeres with apparent zona pellucida integrity) were cultured in a 60 mm tissue culture dish (Falcon; BD 35-3037) in 20 µl of Hypermedium 0.4% BSA (w/v) under mineral oil with incubation at 37°C in a 6% CO2 atmosphere. Within a few hours, the embryos were assessed for their morphological appearance. Then the survival of embryos was assessed by their ability to develop to 8-cell, morulla, young blastocyst and hatching/hatched-stage blastocyst after 48, 72, 96, 100-120 h in culture medium, respectively.

Statistical analysis

Mean percentage differences in rates of survival and development between treatment groups were tested for significance with one-way ANOVA and LSD (least significant difference) as post hoc test. The level of significance was set at less than 0.05.

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Results

Studies on oocytes

Oocytes in exp. 1 (larger-used concentration of EG and DMSO) showed a statistically non-significant different SR with exp. 2 ($p<0.05$) and significant higher SR with the other exp. ($p<0.001$). Comparing the results of exp. 3, 4 and 5 revealed statistically significant decreased SR in proportion to using less CPAs concentrations ($p<0.001$).

In other words, the less CPAs concentrated solutions were used, the less SRs were obtained. When we compared FRs, the control and exp. 1 and 2 exhibited no differences ($p<0.05$), although FRs in exp. 1 and 2 were lower than fresh oocytes. The FR in exp. 3 was significantly lower than those recorded for fresh and exp. 1 and 2 oocytes ($p<0.001$). None of cryosurvived oocytes in exp. 4 were fertilized. The DRs to two cell-stage embryo were not different between control and exp. 1 and 2 oocytes ($p<0.05$). DR to two cell-stage embryo in exp. 3 was significantly lower than control and exp. 1 and 2 ($p<0.001$). Table I details our observations after using different freezing solutions in order to vitrify mouse mature oocytes.

Studies on embryos

Embryos in exp. 1 (larger-used concentration of EG and DMSO) showed a statistically non-significant different SRs with exp. 2 and 3 ($p<0.05$) and significant higher SR with exp. 4 and 5 ($p<0.001$). Cryosurvived embryos in exp. 4 showed significant lower SR than exp. 1, 2 and 3 ($p<0.001$). There were significant differences between embryos SR in exp. 5 and the rest of exp. ($p<0.001$).

There were no statistically differences between DR to 8 cell-stage embryo of exp. 1 and 2 compared to control group ($p<0.05$), whereas DRs of exp. 1 and 2 were lower than control embryos. DR to 8 cell-stage embryo in exp. 3 was significantly lower compared to control ($p<0.001$) and exp. 1 ($p<0.001$) and 2 ($p<0.05$). The description was the same for exp. 4 ($p<0.001$). None of cryosurvived embryos in exp. 5 were developed further. The rates of embryos developed to morulla were lower but not-statistically different between control and exp. 1, 2 and 3 ($p<0.05$). DR to morulla in exp. 4 was significantly lower than that recorded for fresh and exp. 1 ($p<0.05$), whereas, there were no significant difference compared to exp. 2 and 3.

The rates of embryos developed to young blastocyst-stage in all the vitrified groups were lower than those of control, however the difference between control and exp. 1 and 2 were not statistically significant ($p<0.05$). DR to young blastocyst in exp. 3 and 4, were significantly lower compared to control ($p<0.001$) and exp. 1 ($p<0.001$) and 2.

The rates of embryos developed to hatching/hatched blastocyst-stage in all the vitrified groups were lower than those of control; however the difference between control and exp. 1, 2 and 3 were not statistically significant ($p<0.05$). DR to hatching/hatched blastocyst in exp. 4 was significantly lower compared to control and vitrified groups ($p<0.001$). Table II summarizes DRs of fresh versus frozen embryos.

Table I. M-II oocytes survival, fertilization and developmental rates after using different concentrations of the CPAs.

Groups	No. of oocyte vitrified	No. of oocyte recovered	No. of oocyte morphologically survived after warming	No. of oocyte inseminated	No. of oocyte fertilized	No. of zygote developed to 2 cell
Control	—	—	—	138	122 (88.9±5.2)	115 (94.4±3.9)
Exp. 1	106	103	97 (94.3±5.4)	97	84 (85.6±11.11)	76 (90.3±7.2)
Exp. 2	145	141	127 (90.3±7.3)	127	110 (87.3±8.51)	97 (88.6±8.1)
Exp. 3	140	137	98 (72.4±10.9)*	98	37 (38.1±6.42)*	21 (58.5±17.4)*
Exp. 4	157	151	33 (21.5±4.0)*	33	0*	0*
Exp. 5	187	186	0*	0	0*	0*

Data presented in parentheses as mean ± standard division.
Control group includes fresh oocytes.
Exp.1 includes oocytes vitrified by 1.5 M VS following equilibration by 7.5 M ES.
Exp.2 includes oocytes vitrified by 1.25 M VS following equilibration by 6.25 M ES.
Exp.3 includes oocytes vitrified by 1.0 M VS following equilibration by 5 M ES.
Exp.4 includes oocytes vitrified by 7.5 M VS following equilibration by 7.5 M ES.
Exp.5 includes oocytes vitrified by 7.5 M VS following equilibration by 3.75 M ES.
* $p<0.001$

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Table II. Four cell-stage embryo developmental rates after using different concentrations of the CPAs.

Groups	No. of embryo vitrified	No. of embryo recovered	No. of embryo morphologically survived after warming	No. of embryo cultured	No. of embryo developed to 8 cell	No. of embryo developed to morula	No. of embryo developed to young blastocyst	No. of embryo developed to hatching/hatched blastocyst
Control	—	—	—	141	135 (95.9±3.6)	128 (95.0±5.6)	126 (98.4±2.9)	113 (89.9±5.33)
Exp. 1	142	141	134 (95.1±4.7)	134	125 (93.3±3.8)	118 (94.4±4.0)	115 (97.6±4.7)	95 (82.4±5.7)
Exp. 2	147	145	135 (93.3±6.1)	135	125 (92.5±4.1)	116 (92.9±5.3)	112 (96.7±4.8)	91 (81.4±8.0)
Exp. 3	150	148	136 (91.9±4.8)	136	117 (86.2±7.5)*	108 (93.4±5.9)	91 (86.1±9.7)*	74 (78.2±7.9)
Exp. 4	242	239	124 (52.1±5.8)*	124	85 (68.6±10.0)*	74 (87.9±12.7)*	31 (42.3±8.2)*	10 (31.9±30.53)*
Exp. 5	338	333	50 (15.0±3.6)*	50	0*	0*	0*	0*

Data presented in parentheses as mean ± standard division.
Control group includes fresh 4 cell-stage embryos.
Exp.1 includes 4 cell-stage embryos vitrified by 1.5 M VS following equilibration by 7.5 M ES.
Exp.2 includes 4 cell-stage embryos vitrified by 1.25 M VS following equilibration by 6.25 M ES.
Exp.3 includes 4 cell-stage embryos vitrified by 1.0 M VS following equilibration by 5 M ES.
Exp.4 includes 4 cell-stage embryos vitrified by 7.5 M VS following equilibration by 7.5 M ES.
Exp.5 includes 4 cell-stage embryos vitrified by 7.5 M VS following equilibration by 3.75 M ES.

Discussion

Although there have been numerous studies on vitrification of mouse oocytes and embryos, the majority of them have used at least 1.5 M concentrated CPAs as freezing solution. The purpose of the experiment described herein was to examine the possibility of applying lower CPAs concentrations and obtaining the similar results to those using higher concentrations. As it is, the toxicity of the CPAs used in oocytes/ embryos vitrification approach will be diminished. In designing the experiment, we drew upon earlier finding published by Tucker *et al.* The actual cooling rate during vitrification, and therefore the efficiency, may still vary extremely depending on the device used (4). Regarding the capability of the new tool, cryotop, to allow for an even smaller volume of vitrification medium (<0.1µl) to be used and therefore yield quicker cooling and warming rate (23,000˚C/min and 42,000˚C/min) (7), it appears logical to assume that it is an adjustment to use CPA agents at lower concentration, while maintaining the necessary concentration to achieve vitrification. The biophysical detail of CPAs and the mechanisms of freezing/warming rates are beyond the scope of this paper. Briefly, it is noted that CPAs are organic solutes that simply by increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature; but to be biologically acceptable: 1) The compound must be highly soluble in water and remain so at low temperatures in order to produce a profound depression of the freezing temperature; 2) It must be able to penetrate into the cells; and 3) It must have a low toxicity so that it can be used in the high concentrations that are required to produce these effects (22). Novel approaches have been tested to reduce the toxicity of various solutions that are used to vitrify oocytes/embryos. One of the candidate CPA agents was EG, which was very effective and less toxic for mouse oocytes and embryos vitrification (23). Kartberg *et al* realized that vitrification with DMSO protects embryo membrane integrity better than solutions without DMSO (24). Mixtures of CPAs may have some advantages over solutions containing only one solute (10). The incorporation of DMSO into an EG containing medium has at least two advantages: firstly, vitrification is facilitated because of the greater glass-forming characteristics of DMSO and, secondly, the permeability of each CPA is enhanced in the presence of the other (10). For these reasons, we were more attracted by current mixed vitrification solution. Applying the cryotop method and usual CPAs concentration (1.5 M) to human oocyte, Cobo *et al* obtained excellent 96.9 % SR after vitrification (25). Kuwayama *et al* (26) and Katayama *et al* (27) have reported a 91% and 94% SR, 81% and 90% Cleavage rates (CRs), respectively. Morato *et al* have scored 94.5% SR and 46.1% CR (9). 100% morphologically survived and 93% CRs of human pronuclear stage vitrified embryo are by far the highest published results so far (7). Above mentioned were the teams

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with dramatic improvement in their oocyte cryopreservation method. In the current study the SR, FR, and CR of the oocytes and developmental consequences of the embryos which were subjected to 1.5 M and 1.25 M of CPAs (exp.1 and 2) were near to those findings. This seems to support the claim that using 1.25 M DMSO+EG and 0.5 M sucrose for vitrification medium and cryotop as cryocontainer, we are able to obtain the findings comparable with largely-used higher concentration (1.5 M). Needless to say that to demonstrate the proof-of-principle that mature mouse oocyte and four cell-stage embryo cryopreserved using reduced concentration of CPAs can develop to term, more researches should be conducted. The proposed protocol application to preserve other more cryosensitive oocytes of mammalian species, stage-dependant sensitive embryos to damage during vitrification and the embryos developed in vitro, needs to be tested. According to the results of embryos treated by 1.0 M CPAs (exp.3) and two early studies showing that mouse embryos can be frozen using lower concentrations of CPAs (i.e., 1.0 M DMSO and 1.2 M EG) with good success rates (17), further studies are stimulated. The statistics of SRs and DRs to morulla and hatched blastocyst do not show any significant trends between exp. 3 and control, exp. 1 and 2. In contrast, development to 8-cell as resuming normal development after warming, in addition development to blastocyst stage as preparing to produce inner cell mass were impaired. Adopted low concentration of CPAs might be insufficient to enter into and protect vital cell organelles such as mitochondria, endoplasmic reticulum (28, 29) and embryo mitotic apparatus (30). Intracellular ice formation can be affected by the presence of the CPAs in the freezing solutions, and by the freezing and thawing rate (31). To touch on important issue such as temperature reduction rate, it is noted that at rates of cooling slower than the optimum, cell death is due to long periods of exposure to hypertonic conditions. At rates of cooling faster than the optimum, cell death is associated with intracellular ice formation, which is inevitably lethal (22). The actual value of the optimum rate is determined by a number of biophysical factors: 1) Cell volume and surface area, 2) Permeability to water, 3) Arrhenius activation energy (temperature-dependent energy required for the rate of chemical reactions) and 4) Type and concentration of CPA additives (22). The last item establishes a connection between the concentration of the CPAs used and the cooling/warming rate. Achieving optimum rate for oocyte (because of small surface-to-volume ratio) and embryo (because of large surface area and low water permeability) is a matter of numerous researches (32). Based on the SR, FR and CR of oocytes exposed to 1.0 and 0.75 M of CPAs (exp.3-5) there are no supporting evidence of restoring freezing oocytes to normal physiology and functioning while applying these lower CPAs concentrations. Although the precise nature of the damage caused by cryopreservation remains to be exactly determined (17), findings of several studies suggest that the major obstacles in successful oocyte cryopreserving are the characteristics of the oolema (33), the presence of cortical granules, spindle system at the metaphase of meiosis II (34) and zona pellucida hardening (35). In addition, the oocytes must be fertilized by sperm at the appropriate time (23). As documented earlier, a major limiting factor that may be responsible for the success of a cryopreservation methodology is the preservation of the integrity of the spindle apparatus. The cryovulnerability of this structure has been pointed out by several investigators, as has its plasticity, which allows repolymerization (36). We should point out equilibration issue which is often critical in the case of oocyte cryopreserving. Because the oocyte is a large cell containing a large quantity of water, it requires a time to reach adequate dehydration (osmotically balanced by the CPA solution) before lowering the temperature and thus it is more difficult to avoid ice crystal formation (23). The pretreatment (or equilibration) time before cooling might affect the viability and developmental ability of oocytes (23). Comparing the SRs of oocytes/embryos pretreated to 0.75 M (exp. 4) with those exposed to 0.375 M CPAs (exp. 5) lays emphasis on the vital effect of equilibration in terms of CPAs concentration, exposure temperature and duration. Any intervention causing even temporary change in the equilibrium of the physiological state could potentially be toxic to cells. These include ICSI, which was introduced as a unique technique to overcome cryo-induced zona hardening and sequential IVF failure (37). Because of mechanical stress and small amounts of Polyvinylpyrrolidone (PVP) into oocytes during procedure, it has fallen into harmful category (17). These can be the reason of why none of the cryosurvived oocytes in exp. 4 were fertilized. Moreover, our statistics supported the view that the embryo cryopreservation using 0.75 M concentrated CPAs (exp. 4) was not perfectly successful.

In addition to the immediate causes of the cryodamage explained above, we shall make

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reference to the work of Tucker *et al* who have provided evidence to offer that although the cell nucleus has the ability to reassemble morphologically following cryopreservation, the future development of the embryo could be suboptimal (15). Except for the results published by Schroeder *et al* (38) who were able to successfully cryopreserve mouse oocytes using a slow-cooling protocol and 1.0 M DMSO, this appears to be the first time that a 1.0 M and 0.75 M concentrated CPAs + 0.5 M sucrose have been evaluated for cryotop-vitrified mouse M-II oocytes (exp. 3 and 4). Experience led us to the expectation that in the course of cryopreservation with 1.0 M concentrated CPAs for oocyte and 0.75 M concentrated CPAs for embryo freezing, the oocytes'/embryos' viability will be reduced, although not necessarily to an extent that makes them incapable of becoming fertilized or developed further. It is suggested that the current reduced concentrated solutions i) to combine with other additives [such as CSK stabilizers (39), ice blocking polymers (40) and high concentration of the sugars (41)] into, or ii) to deplete some of the supplements from freezing solutions [such as sodium (42, 43) and calcium ions (44)], in such circumstances with iii) different equilibration temperature, duration and the concentration of the CPAs (23), to be assessed. To put the finishing touches to the suggestions, we draw your attention to a large body of experimental evidences indicated a major positive impacts of cumulus cells on vitality of oocytes (39, 45-47). It would be effective to study the efficiency of the new protocol on cryopreserving cumulus oocyte complex; furthermore, because of the mechanical stress of PZD/ICSI procedures, monitoring oocyte parthenogenetic activity is a safer alternative to scoring oocyte functioning after warming (23). These, in turn, may lead to some improvements in cryopreservation unfertilized oocytes/embryos procedures. The data from this study made us to draw the conclusion that vitrification by cryotop technology using minimal volume approach increases both cooling and warming rates, so CPAs limited reduction to 1.25 M and 1.0 M instead of using 1.5 M, for oocyte and embryo cryotop-vitrification procedure may be a slight adjustment.

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