Cryopreservation of oocytes after vitrification

A. Tripodi, E. Sturlese, F. Cremonesi

[1]*Faculty of Medicine and Surgery, Institute of Gynecology and Obstetrics, University of Messina*
[2]*Faculty of Veterinary Medicine, Institute of Gynecology and Obstetrics, Division of Physiopathology of Reproduction, University of Milan (Italy)*

Summary

Recently the majority of studies and research in the field of physiopathology of reproduction have been oriented towards cryopreservation of female eggs. This could resolve the ethical problems connected with the preservation of the embryo and with the gestation of the surplus oocytes collected after cycles of ovarian hyperstimulation.

Notwithstanding that extraordinary results have been obtained in the field of cryopreservation of embryos and spermatozoa, the freezing of oocytes involves unfortunately many difficulties in relation, above all, to deep morphostructural and functional modifications which take place due to the low temperatures and to the toxic action of cryoprotective agents.

The aim of this work was to test and then verify whether an efficient method of preserving the female egg cells by freezing in order to be able to subsequently use them for in vitro fertilization.

The conclusions of this study, even if not highly encouraging, have allowed us to better evaluate the damage caused by low temperatures and at the same time should stimulate us and other researchers in this field to find new methods which will allow us to reach higher aspirations.

Key words: Cryopreservation; Oocytes; Vitrification.

Introduction

All the laboratory procedures used for in vitro fertilization in the field of human medicine have come from studies of the embryos and oocytes of experimental animals such as the mouse and rabbit and zootechnical animals like cows and sheep.

In the same way, methods for the cryopreservation of human oocytes and embryos at the preimplant stage have also come about from laboratory experiments with animals. The capacity to cryopreserve embryos at the preimplant stage has become of primary importance in the application of the clinical protocols to increase the potential of in vitro pregnancy [1, 2].

The possibility of freezing human embryos is particularly relevant when considering the fact that ovarian hyperstimulation frequently produces an excessive number of oocytes and consequently of embryos with respect to the number that can be placed in the Fallopian tubes and uterus.

The presumed risk of multiple pregnancies and the possibility of causing selective intrauterine abortions has limited the number of oocytes and pronucleated eggs that can be transferred to the Fallopian tubes during the transfer procedure of some gametes and zygotes and has reduced the number of embryos at the division stage that can be transfered to the uterus after in vitro fertilization.

Many artificial reproduction programs try to maximize the number of embryos produced following a single cycle of ovarian stimulation and to freeze the resulting excess embryos of that treatment.

Presently human embryos are frozen within the first and second day of fertilization. This development period is considered to be the most practicable method. However in these first stages, and especially in the pronuclear stage, an adequate estimate of developmental potential is not always possible and moreover one has to also keep into account the growing capacity of the human embryo after freezing.

Consequently it would be preferable to prolong the stage of development of the excess embryos up to the stage of morula before initiating the freezing procedure. In this way only the embryos that have shown a certain potential would be used for such a procedure [3].

However the ethical and legal problems associated with the attempts to define when life really begins and the rights and the legal state of the embryo have led to the belief that cryopreservation of oocytes could be an alternative approach for the management of excess gametes usually collected after controlled ovarian hyperstimulation [4].

At present the literature reports few cases of births resulting from in vitro fertilization of frozen oocytes of which the majority are in mice, rabbits and in humans [5, 6].

Siebzehnruebl *et al.* (1989) reported a case of birth deriving from the transfer to the uterus of seven out of 14 mouse oocytes that were fertilized and arrived at the first stage of division, obtained from in vitro fertilization of frozen MII (metaphase II) oocytes with the addition of 1.5 mol DMSO (dimethysulphoxide), according to the procedure of slow freezing [7]. Even if these results can be encouraging for those who apply cryopreservation to oocytes, the injurious effects of cryoprotectors on freezing and thawing to the structure of the cytoplasm and the integrity of the meiotic spindle must be kept into account. Cryoprotector agents are necessary to freeze tissue and cells but the molar concentrations required can cause damage due to osmotic effects and chemical toxi-

Revised manuscript accepted for publication March 30, 2001

city. A brief exposure of mouse oocytes at 1.5 mol of DMSO promotes the depolarization of the fusiform microtubes and cytoplasmatic anomalies while the exposure of rabbit oocytes at 1.5 mol of glycopropeline produces, in addition to negative effects to the tubular structure, also depolarization of the cortical band of the threadlike actin in mature oocytes [8].

Johnson & Parcking (1987) report that the exposure of mouse oocytes at the MII stage of DMSO for one hour is associated with the loss or the presence of spindle residue. In this stage DMSO was united directly to 1.5 mol or incremented with 0.25 mol with ten minutes of exposure for each concentration [9]. Both protocols showed that the loss of spindles is associated with a dispersion of chromosomes from the equatorial plane. After removal of DMSO the restoration of some oocytes has been observed while in a major part no spindle is any longer observed or the recomposition of the spindle is abnormal or incomplete. However subsequent studies by Vincent show that the freezing of mature oocytes without a cryoprotector favors the early release of cortical granules and a premature reaction of the zone [10].

Structural cytoplastic alterations have been described in mouse oocytes that had been cryoprotected and subsequently thawed according to the two methods presently used for the embryo which are:

– Slow freezing where the temperature is lower than 1°C/min up to –7°C and after having performed the seeding, it is again lowered to 0.5°C/min up to –35°C and then finally the paillettes are transferred into liquid nitrogen.

– Vitrification consists of very fast freezing leaving the paillettes in a nitrogen vapor for 5 mins before immersing them directly into liquid nitrogen [11].

In addition to the cytoplastic lysis there can also be structural and/or cytogenetic alterations such as small interuptions in the pellucid zone and of the cytoplasmatic membrane, extensive disorganization of the cytoplasm, early exocytosis of the cortical granules and depolarization of the microtubular axis with the resulting dissemination of chromosomes and formation of micronuclei [12, 13]. It has also been shown that before the exposure to a temperature below 0, progressive freezing of mature oocytes destroys the microtubular organization of the spindles.

Van der Elst (1988) showed that about 90% of mouse oocytes at the metaphase II level, exposed to 1.5 mol of DMSO at room temperature, showed spindle anomalies [14]. It also seems that frozen oocytes undergo a hardening of the pellucid zone which is similar to the physical chemical changes (zone reaction) caused by the exocytosis and solubilization of cortical granules (cortical reaction) after the contact between oolemma and fertilized spermatozoa. Both reactions are primary mechanisms that prevent the fertilization by one or more spermatozoa. The hardening of the pellucid zone is shown by the increased resistance to chemotreptisin [15]. Although the degree of the disorganization of the spindle and the dispersion of chromosomes observed in mouse oocytes at the MII stage frozen to 4°C are variable the displacement of chromosomes from the axis has the capacity to create cytogenetic anomalies that can compromise normal development even if the recomposition of the spindle at 37°C takes place and the oocytes can be fertilized [16].

These results show that both cooling before freezing and exposure to cryoprotectors such as DMSO can have significant effects on the stability of the microtubular systems of mature mouse oocytes. Cryomicroscopic studies of mature human and mouse oocytes have shown the following physical and physiochemical changes that can cause lethal damage during cryopreservation:

1) Deformation of oocytes can be caused as a direct consequence of the initial contact with the ice front and consequent formation of solutions through which they exit the cytoplasm after thawing.

2) The generation of intracellular gas bubbles after thawing can damage the cytoplasmatic structure and lead to cell death due to lysis.

It has been reported that the haploids which are formed following the destruction of the meiotic spindle occur in a very high percentage in mouse oocytes at the thawed MII stage and fertilized in vitro [17]. The recurrence of haploid oocytes and the seeming increment of risk of polyploidy observed in an animal model such as the mouse are of significant interest for the application of the cryopreservation to oocytes. Therefore, physiochemical modifications of the pellucid zone which occur normally during fertilization can be a dangerous consequence of the exposure to cryoprotectants and to cooling.

Moreover, in addition to the chromosomally abnormal zygotes that develop from the fertilized oocytes after exposure to cryoprotectors, teratogic effects on fetal development have been observed. The age of the oocyte measured in hours from the restart of meiosis may be one of the factors associated with a generation of chromosome anomalies. The aging of the oocytes, both in vivo and in vitro, is often associated with a deterioration of the integrity of the spindle and the consequent dispersion of the chromosomes [18]. In addition, since it cannot be that all the oocytes at the MII stage in vivo simultaneously undergo meiosis the age of the oocyte obtained at the time of recovery can vary significantly and consequently it is possible to observe a set of mature and immature oocytes.

A prolonged preincubation before cryopreservation can for some oocytes lead to the generation of anomalies which cannot then be classified as a consequence of the cooling process. The same applies to oocytes obtained from stimulated ovaries. In fact, Van Blerkom has estimated that approximately 10-15% of human oocytes at metaphase II stage obtained from hyperstimulated ovaries can have cytoplasmatic abberations associated with a reduced fertilizability of the oocyte or with an ability of the zygote to progressively develop after fertilization.

This can be due to:

1) Massive accumulation in the tubes of smooth surface endoplasmic reticulum (SER);

2) Intracellular degeneration or necrosis;

3) Aggregation of SER vesicles and mitrochondria;

4) Depletion of organelles from portions of cortical cytoplasm;
5) Cytoplasmic vacuoles;
6) Accumulation of perivitellin fluid in endocyte vacuoles;
7) Premature or partial oxidation of cortical granules.

These cytoplasmic anomalies not only interfere with the possibility of fertilization and consequent embryonal development but also with freezing and defreezing [19]. The freezing of the fully developed meiotically immature oocyte (GV stage) can be an alternative approach to cryopreservation of the female gamete.

A potential advantage of cyropreservation at the GV stage is the fact that the freezing is done before the chromosomal condensation and as a consequence there is no haploid that could derive from the destruction of the spindle at MII due to cooling [20]. Moreover, because of the complete growth the oocytes at the GV stage can be obtained from small follicles that are not subject to high levels of circulating gonatropins.

A second advantage of freezing of human oocytes at the GV stage is that the frequency of haploids after the spontaneous restart of meiosis in vitro is somewhat low. Another benefit of cyropreservation at the GV stage is the possibility of monitoring the progress of nuclear maturation, the chromosomic maturation, and to estimate the normality of the cytoplasmic maturation by optical microscope. Presently attempts at obtaining vital oocytes after freezing at the GV stage have had limited success. A significant advantage of the method of vitrification for the cyropreservation of oocytes is due to the fact that the cooling takes place so quickly that the intracellular fluid passes directly from the liquid to the solid stage. Moreover, being a very rapid procedure also the exposure time to cryoprotectors, which act negatively on the cytoskeletal elements, the meiotic axis, cortical granules, and on the pellucid zone is reduced. Consequently, the cellular damage that takes place in the normal procedure of slow freezing due to the formation of numerous little intra- and extracellular crystals does not occur. However the very rapid cooling does not allow osmotic equilibrium to be reached which is sufficient to fully dehydrate the cell causing the destruction of the meiotic spindle and chromosome dispersion.

Materials and Methods

Throughout the study an incubator, Haereus model 3066, kept at 39°C, 5% with CO_2 and 95% humidified air, was used.

Collection of ovaries

The ovaries, the object of the experiment, were collected from cows raised in farms without leucoma, tuberculosis, and brucellosis, butchered at the public meathouse of Milan. The ovaries were preserved in a thermus of phophate buffered saline by Dulbecco in addition to 10.1% polyvinyl alcohol taking care to open and close it quickly only when the ovaries were introduced. The ovaries were then immediately transported to the laboratory where the time, the temperature at arrival and the quality of the ovaries were noted. Time and temperature are very important for the quality of the oocytes; the optimal temperature of preservation of the ovaries should be 25°C. Before aspiration the ovaries were washed two times in PBS-PVA and then preserved in a sterile container with PBS-PVA during the withdrawal.

Procedure for the collection of oocytes

The oocytes were collected by means of aspiration from the follicles with dimensions between 2-7 mm using a 10 ml syringe with a 18 G needle. The withdrawn liquid was placed in a sterile container in which PBS-PVA had previously been introduced, up to the time the ovaries were withdrawn. After having left the liquid sediment for a few minutes the floating particles were eliminated and the precipitation was poured on Petri tiles and diluted with PBS-PVA until a not too dense solution was obtained so as to allow easy identification of oocytes. By using a stereoscopic microscope x 16 the oocytes which had compact cumulo cells displaced on many layers were detected and collected.

Maturation of oocytes

Those oocytes that were thought to be of good quality – with homogeneous cytoplasm and cumulo cells – were transferred to Petri tiles (30 mm) containing 2 ml of culture medium (M199+FCS) and put for 22 hours to mature in an incubator at a temperature of 39°C and at 5% of CO_2.

Vitrification procedure of oocytes

After the period of incubation the oocytes were collected with a pipette and placed in a 1 ml Eppendorf test tube. This was shaken for 3 mins by the vortex which by its retrotranslatory movements favors the accumulation of oocytes by distancing the granulosa cells. Some of the accumulated oocytes were placed, in groups of 5 or 10, for 5 mins in a solution of Vitrification Solution (VS) of 25% in PBS+ 15% of FCS at 20°C and then transferred into a solution of VS at 65% in PBS+ 15% of FCS for about 30 secs taking care during the transfer to collect the least amout of solution possible; others were immersed in a solution of 2.0 mol, 4.0 mol and 6.0 mol for 1 min and 30 secs. After these two steps the oocytes were transferred to the previously prepared paillettes according to the following method:

1) The cylinder of cotton polyvinyl alcohol was pushed 4 cm inside the paillette.
2) A fraction of 1 cm of VS was introduced in the paillette at 3 cm from its free margin. The oocytes were placed inside this fraction.
3) The paillettes containing the oocytes were placed in nitrogen vapor at a distance of 5 cm from the level of the nitrogen itself and left for 5 min before being immersed.

Procedure for thawing oocytes

The paillettes to be thawed were left at room temperature for 10 secs and then immersed in water at 20°C for another 10 secs. Subsequently the paillettes were opened and the contents of each one were poured and slowly mixed in 1 ml of PBS+ 15% of FCS containing 1 mol of saccharose at room temperature for 5 mins. The oocytes were subsequently transferred to PBS+ 15% of FCS at room temperature for 10 mins and after to PBS+ 15% of FCS at 37°C for 10 mins before being washed in fertilization medium.

In vitro fertilization with frozen sperm

Fertilization tiles, which were the same kind as those used for the maturation of the oocytes, were prepared using micro drops of 46 µl of heparin tyroide lactate in Petri tiles 35 x 10 mm.

Every tile contained 12 micro drops. The tiles were incubated for at least two hours in an incubator before use.

The preparation of the sperm and its capacitation was done an hour and a half before the fertilization.

One or two paillettes of sperm, according to the protocol, were thawed by immersing for one minute at 37°C in a double sauce pan, and the contents were collected in 12 x 55 mm Rohren test-tubes. We proceeded to take the sperm with a 1 ml syringe with a 27 G needle and then deposited and divided them into equal parts in four previously prepared test-tubes containing sperm modified tyroide lactate medium (Sperm TL) used for the separation of spermatozoa on the basis of their mobility by the Swim-up technique. The four test-tubes were kept in the incubator for one hour during which care was taken not to move them. The contents of the four test-tubes were collected into a single test-tube and transferred to a centrifuge.

The solution was centrifuged for 10 mins at 120 rpm (350 g). At the end of the centrifugation the material floating on the surface was withdrawn with a Pasteur pipette. Since many of the mobile spermatozoa were not centrifuged at the first washing, it was necessary to do another washing with 3-4 ml of TL-HEPES which was centrifuged at the same speed for 10 mins.

The percentage of mobility of spermatozoa was estimated after every washing. The material on the surface was again aspirated leaving at the bottom of the test-tube 0.2-3 ml of residual material containing spermatozoa; these were resuspended in 0.5 ml of medium for each paillette and diluted at 25 x 10^6 spermatozoa/ml. The test-tubes, in which heparin (100 µl or 10 µl) was added, were incubated for 15 mins; from these 2 µl were taken and added to fertilization drops in order to obtain a sperm concentration cocoltura of 1 x 10^6 spermatozoa/ml.

The fertilization tiles were incubated for six hours. After 22 hours of incubation, the oocytes were ready to be fertilized. A sample of oocytes was colored each time with aceto-orcein at 1% to evaluate the degree of maturation reached.

The rest of the oocytes were washed two times with TL HEPES and divided among the different fertilization drops so that each contained ten.

Culturing of the embryos

The oocytes were taken from the fertilization tiles after six hours of gamete culture and then were washed with fertilization medium and with another washing in M199+FCS.

Subsequently the embryos were placed on the tiles used for the maturation of the oocytes and then incubated. After 20 hours of the cocoltura (14 hours after the beginning of the incubation of the fertilized oocytes) we proceeded with the coloring of a sample of oocytes with 1% aceto-orcein in order to determine the fertilization quota.

Composition of the used media

Dulbecco's Phosphate Buffered Saline with 0.1% of polyvinyl alcohol (PBS-PVA):

– 1 package of D-PBS powder and 1 package of calcium choride (GIBCO Lab, Grand Island, NY; No. 450-1500);

– 8 ml antibiotics-antimicotics (GIBCO Lab, Cat. No. 600-5240);

– 1 g polyvinyl alcohol diluted in 1,000 ml of highly purified water in a Milli-Q system.

Medium 199 containing 25 mmol of HEPES with 7.5% of fetal calf serum (M199+FCS):

– 50 ml medium 199 (GIBCO Lab, Grand Island, NY; no. 380-2340);

– 4 ml of fetal calf serum (FCS).

Vitrification solution:

– 60 mol; 4.0 mol, 2.0 mol of dimethysulphoxide (DMSO);

– PBS+15% FCS solution.

Thawing solution (VS):

– PBS+15% FCS solution;

– 1.0 saccharose.

Results and Conclusions

It has already been noted in the literature how it is more difficult to freeze a single cell, extraordinarily large like the oocyte, which has peculiar characteristics, rather than a unit made up of more cells such as the embryo. One also has to consider the fact that the permeability of the oocyte membrane is different in various species and that little is known about the biochemical composition of its bilayer.

According to the experimental protocol a group of oocytes was exposed to a final concentration of 6.0 mol of DMSO obtained by having the oocytes pass through scalar concentrations from 2.0-4.0 and 6.0 mol of DMSO for periods of 1 min and 30 secs with 2.0 mol, 30 secs with 4.0 mol and 1 min with 6.0 mol.

We used high concentrations of DMSO only as a cryoprotector, considering that vitrification requires a high percentage of cryoprotector and based on the work published by Hamano and Kuwayama in which they obtained a percentage of division of 64.5% using as a medium a mixture consisting of 2.0 mol of DMSO and of 3.0 mol of propylene glycose [21].

Thus, with the same method of vitrification, we froze a group of oocytes treated with 6.0 mol of DMSO, another with 4.0 mol of DMSO, and the last one with 2.0 mol of DMSO – concentrations which are very low for such

Table 1. — *Percentage of oocyte division treated with DMSO without undergoing vitrification and the control group. Percentage of blastocytes is in reference to divided oocytes.*

	2m-4m-6m*		6 mol		6 mol		2 mol	
	Treated group	Control group	Treated group	Control group	Treated group	Control group	Treated group	Control group
Treated oocytes	70	–	70	–	70	–	194	–
Fertilized oocytes	70	70	70	70	70	70	194	194
Divided ooctyes	0	49	0	57	0	56	89	153
Blastocytes	0	33	0	36	0	37	13	100
% of blastocytes	0%	67%	0%	63%	0%	66%	15%	65%

* m = mol.

Table 2. — *Percentage of oocyte division that underwent vitrification and the control group. Percentage of blastocytes is in reference to divided oocytes.*

	2m-4m-6m*		6 mol		6 mol		2 mol	
	Treated group	Control group	Treated group	Control group	Treated group	Control group	Treated group	Control group
Treated oocytes	300	–	110	–	142	–	272	–
Fertilized oocytes	280	300	82	110	135	142	264	272
Divided ooctyes	0	240	0	85	0	113	14	206
% of divisions	0%	80%	0%	77%	0%	80%	5%	76%
Blastocytes	0	150	0	57	0	73	0	132
% of blastocytes	0%	63%	0%	67%	0%	65%	15%	64%

* m = mol.

freezing technique – to observe the different percentages of division and to test the possible toxicity of this cryoprotector.

To have more reliable results we fertilized another group of oocytes treated with the same concentrations of DMSO without however using the vitrification procedure in order to establish if the possible lack of division was due to the cryoprotector or to the vitrification.

In each experiment the oocytes obtained by withdrawing the ovaries were divided into two groups, one of which was submitted to the procedures required by the experimentation while the other was used as a control group and fertilized to test the effective capacity of the development of the oocytes and of the used sperm.

The oocytes contained in the paillettes were preserved in liquid nitrogen during the entire experiment period (1 wk - 2 mos) and then frozen and fertilized all at the same-time with the sperm coming from the same container to avoid vitrification that could have compromised the reliability of the experiment. It should be noted that a modest group of frozen oocytes were lost during the thawing due to explosion of the paillettes due to an excessive quantity of residual air inside them caused by the particular packaging system.

As reported in Tables 1 and 2, for both the oocytes treated but not frozen and those that were not vitrified concentrations of DMSO of 4.0 and 6.0 mol and of 2.0 mol-4.0-mol-6.0 mol scalars showed a zero percentage of division while those treated with a concentration of 2.0 mol of DMSO showed a 46% cleavage, and the those frozen with the same concentration showed 5%. These results could suggest that DMSO is toxic at high concentrations but that at the same time low concentrations are insufficient to guarantee efficacious protection during the process of vitrification.

Moreover, observing that the oocytes that were treated but not vitrified had a percentage of division of 46% vs 5% of those vitrified, it should be noted that these negative results can not be ascribed only to the toxicity of DMSO but must also be ascribed to the vitrification that intervenes and thus compromises the development of the oocyte.

The results have shown that differently from what has been observed from mouse and hamster oocytes vitrified with 6.0 mol of DMSO, bovine oocytes cannot be preserved with the described technique. Particularly it has also been shown that simple exposure to concentrations higher than 2.0 mol of DMSO is lethal for bovine oocytes. This is probably due to toxic phenomena that act on the cellular proteins at high concentrations of DMSO. Only the exposure to concentrations of 2.0 mol resulted to be compatible with a good percentage of fertilization; but such concentration cannot be proposed for cyropreservation by vitrification since it does not allow an optimal cellular dehydration to be reached.

Also, dehydration done at scalar exposures of DMSO did not give percentages of development after in vitro fertilization while in other researches this method of the addition of DMSO has been shown to be the best system of dehydration of the oocyte before the vitrification

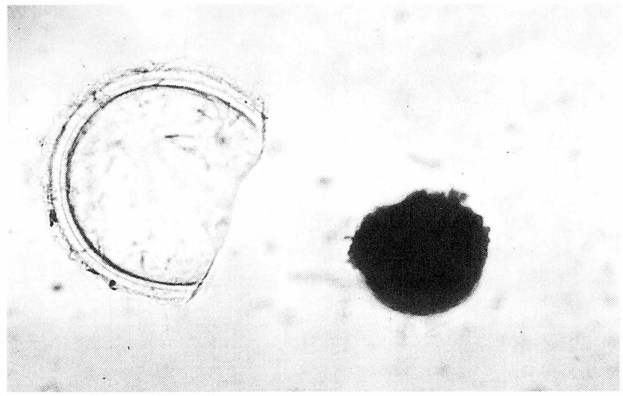

Figure 1. — Oocytes after vitrification in 6 mol of DMSO (cytoplasm accumuation, complete breakage and detachment of the pellucid zone).

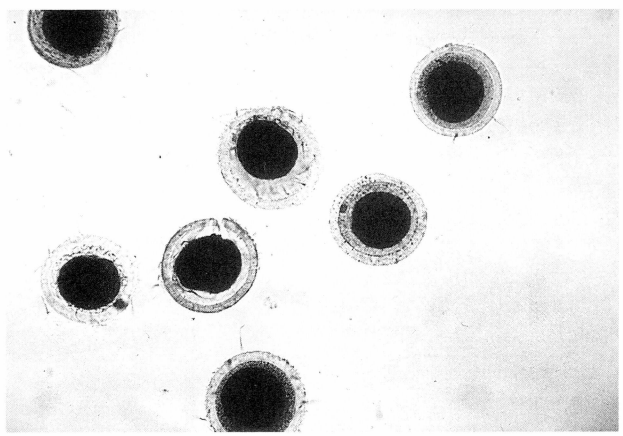

Figure 2. — Oocytes after vitrification in 4 mol of DMSO and fertilization (breakage in the pellucid zone and accumulation of cytoplasm).

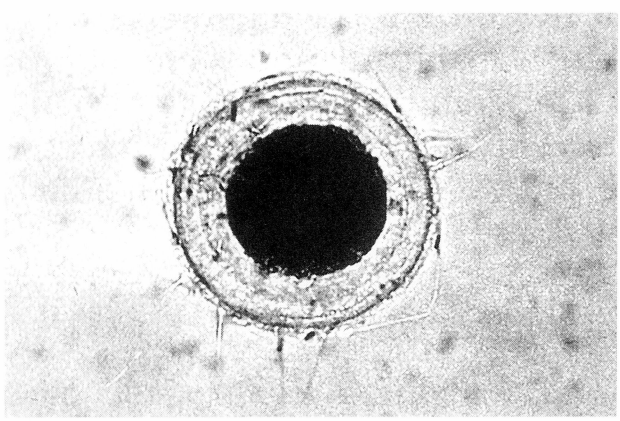

Figure 3. — Oocytes treated with 2 mol of DMSO.

period. In fact the partial dehydration of the cytoplasm is a fundamental prerequisite to avoid the formation of intracellular ice and to guarantee the survival of the oocytes and of the embryos during the freezing period. Particularly, the balancing of the oocytes, first in a solution containing low concentrations of cryoprotector fol-

lowed by a brief exposure to the vitrification solution to reach maximum dehydration and to concentrate the endocellular substance, is the most used technique to obtain a cytoplasm able to resist vitrification.

In the case of bovine oocytes not even the gradual addition of DMSO or exposure for 1 min and 30 secs at a maximum concentration of DMSO gave positive results. After thawing, many of the oocytes showed variable morphologic alterations, from a simple little rupture in the pellucid zone up to fragmentation and loss of cytoplasm (Figures 1 & 2). Evidently the quick changes in temperature, which are characteristics of the technique of vitrification in oocytes perfectly prepared by an adequate concentration of cryoprotector, result in causing remarkable morphologic alterations.

Instead in the case of the oocytes which look normal after thawing we can consider the pellucid zone which increases the resistance of the same zone to proteolytic digestion by the spermatozoa (Figure 3). In this case such alterations evidently are of such high degree to compromise the fertilization of all the frozen oocytes. This failure of fertilization is according to us much more explainable when one considers that the mechanisms that rule the interaction between spermatozoa and oocytes are based on molecular membrane receptors that can easily be altered by vitrification.

In conclusion even if the data are preliminary we can confirm that DMSO is inadequate, at least in our experimental conditions, for use as a single cryoprotector for the vitrification of oocytes.

References

[1] Mandelbaum J., Junca A. M., Plachot M., Alnot M. O., Salat-Baroux J., Alvarez S. *et al.*: "Cryopreservation of human embryos and oocytes". *Human. Reprod.*, 1988, *3* (1), 117.

[2] Quigley M. M.: "The new frontier of reproductive age". *JAMA*, 1992, *268* (10), 1320.

[3] Osborn J. C., Moor R. M.: "An assessment of the factor causing embryonic loss after fertilization in vitro". *J. Reprod. Fertil.*, Suppl., 1988, *36*, 59.

[4] Ethics Committee of The American Fertility Society: "Ethical consideration of the new reproductive technologies". *Fertil. Steril.*, 1990, *53* (6 Suppl. 2), 1S.

[5] Whittingham D. G.: "Fertilization in vitro and development to term of unfertilized mouse oocytes previously stored at –196 degrees C". *J. Reprod. Fertil.*, 1977, *49* (1), 89.

[6] Al-Hasani S., Kirsch J., Diedrich K., Blanke S., van der Ven H.: "Successful embryo transfer of cryopreserved and in-vitro fertilized rabbit oocytes". *Hum. Reprod.*, 1989, *4* (1), 77.

[7] Siebzehnruebl E. R., Todorow S., van Uem J., Koch R., Wildt L.: "Cryopreservation of human and rabbit oocytes and one-cell embryos: a comparison of DMSO and propanediol". *Hum. Reprod.*, 1989, *4* (3), 312.

[8] Shaw J. M., Trounson A. O.: "Parthenogenetic activation of unfertilized mouse oocytes by exposure to 1,2-propanediol is influenced by temperature, oocytes age, and cumulus removal". *Gamete Res.*, 1989, *24* (3), 269.

[9] Johnson M. H., Pickering S. J.: "The effect of dimethylsulphoxide on the microtubular system of the mouse oocyte". *Development*, 1987, *100* (2), 313.

[10] Vincent C., Pickering S. J., Johnson M. H.: "The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present". *J. Reprod. Fertil.*, 1990, *89* (1), 253.

[11] Rall W. F., Wood M. J.: "High in vitro and in vivo survival of day 3 mouse embryos vitrified or frozen in a non-toxic solution of glycerol and albumin". *J. Reprod. Fertil.*, 1994, *101* (3), 681.

[12] Sathananthan A. H., Trounson A. O., Freeman L.: "Morphology and fertilizability of frozen human oocytes". *Gamete Res.*, 1987, *16* (4), 343.

[13] Tripodi A., Pullè C., Cremonesi F., Stacchezzini S.: "Cryopreservation of female gametes after the second meiotic division". *Minerva Ginecologica*, 1997, *49* (7-8), 305.

[14] Van der Elst J., Camus M., Van der Abbeel E., Maes R., Devroey P., Van Steirteghem A. C.: "Prospective randomized study on the cryopreservation of human embryos with dimethylsulfoxide or 1,2-propanediol protocols". *Fertil. Steril.*, 1995, *63* (1), 92.

[15] Van Blerkom J., Davis P. W.: "Cytogenetic, cellular, and developmental consequences of cryopreservation of immature and mature mouse and human oocytes". *Microsc. Res. Tech.*, 1994, *27* (2), 165.

[16] Ashwood-Smith M. J., Morris J. W., Fowler R., Appleton T. C., Ashorn I.: "Physical factors are involved in the destruction of embryos and oocytes during freezing and thawing procedures". *Hum. Reprod.*, 1988, *3* (6), 795.

[17] Eichenlaub-Ritter U.: "Genetics of oocytes ageing". *Maturitas*, 1998, *30* (2), 143.

[18] Van Blerkom J., Bell H., Weipz D.: "Cellular and developmental biological aspects of bovine meiotic maturation, fertilization, and preimplantation embryogenesis in vitro". *J. Electron Microsc. Tech.*, 1990, *16* (4), 298.

[19] Wood M. J., Barros C., Candy C. J., Carroll J., Melendz J., Whittingham D. G.: "High rates of survival and fertilization of mouse and hamster oocytes after vitrification in dimethylsulphoxide". *Biol. Reprod.*, 1993, *49* (3), 489.

[20] Goud A., Goud P., Qian C., Van der Elst J., Van Maele G., Dhont M.: "Cryopreservation of human matured MII oocytes: influence of cryopreservation media on the survival, fertilization, and early cleavage division". *Fertil. Steril.*, 2000, *74* (3), 487.

[21] Kuwayama M., Hemenos S. I., Nagei T.: "Vitrification of bovine blastocysts obtained by in vitro culture of oocytes matured and fertilized in vitro". *J. Reprod. Fertil.*, 1992, *96* (1), 187.

Address reprint requests to:
A. TRIPODI, M.D.
Via Carcere Nuovo, 16
89133 Reggio Calabria (Italy)