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# Exposure time to cryoprotectants influences oocyte survival rate

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Oocyte cryopreservation may have significant potential for assisted reproductive technology. The success depends on morphological and biophysical factors. The main biophysical factor affecting human oocyte survival is the intracellular ice formation which generally pierces the membranes causing cell lysis. Intracellular ice formation can be affected by the presence of cryoprotectants in the freezing solutions. Thus, the aim of this study was to examine the effect of the exposure time to cryoprotectants on human oocytes before freezing. Oocytes were cryopreserved in 1,2 propanediol added to sucrose, using a slow-freezing-rapid-thawing method. Exposure time longer than 5 min to cryoprotectant solutions, before lowering of the temperature, significantly increased the oocyte survival rate. These data are also supported by morphological parameters, e.g. ooplasm size and perivitelline space. Adequate oocyte dehydration should be obtained before lowering of the temperature and this can be achieved by sufficient exposure to cryoprotectants. That could further contribute to avoiding of formation of cytoplasmic ice crystals, which are the main factor influencing oocyte survival rate during cryopreservation.

**Key words:** oocyte, cryopreservation.

## INTRODUCTION

In humans, the main application of oocyte cryopreservation technique is fertility restoration in women at risk of premature menopause, which should depend on several causes: recurrent or severe ovarian diseases such as cysts, benign tumors and endometriomas; ovary removal to treat endometriosis or genital cancer; and chemotherapy or radiotherapy to treat cancer or other systemic diseases (Amorim *et al*., 2003). For women with certain types of chromosomal anomalies, such as Turner's syndrome (Aubard *et al*., 2000), which can also cause premature ovarian failure, oocyte cryopreservation might be a good option for fertility treatment. It has been reported that follicles may be found in ovaries from adolescent girls with Turner's syndrome (Hreinsson *et al*., 2002). This finding is very important as it indicates that infertility in these patients can be avoided by rescuing ovarian fragments for cryopreservation procedures (Amorim *et al*., 2003). The oocyte cryopreservation technique could also be used in healthy women who choose to delay childbearing until later in life. It further facilitates oocyte donation, as the procedure of harvesting of the ovarian fragment is very simple and does not require hormonal stimulation, which can cause side effects to the donor (Van den Hurk *et al*., 2000). In addition, oocyte cryopreservation is a successful alternative for storing the excess of oocytes during the ART therapies, thus avoiding ethical, moral and religious dilemmas. How-

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ever, in spite of several successes being reported (Porcu *et al*., 1997; Kuleshova *et al*., 1999; Porcu *et al*., 2000; Van der Elst, 2003), there are still technical problems associated with oocyte freezing.

It should also be mentioned, that nowadays an important problem limiting oocyte cryopreservation procedure is the survival rate after thawing. Studies using metaphase II oocytes showed that the oocyte survival rate after cryopreservation could be affected by morphological and biophysical factors.

The main biophysical factor affecting human oocyte survival is the intracellular ice formation that generally pierces the membranes causing cell lysis. Since the human oocyte is a large cell containing a large quantity of water, it requires a long time for adequate dehydration (osmotically balanced by the cryoprotectant solution) before lowering of the temperature, and thus it is more difficult to avoid ice crystal formation. Intracellular ice formation can be affected by the presence of cryoprotectants in the freezing solutions, and by the freezing and thawing rate (Shaw, 1993).

The cryoprotectants generally used in oocyte freezing protocols are 1,2 propanediol (PROH, membrane-permeating cryoprotectant) and sucrose (membrane non-permeating cryoprotectant). Their protective action is very complex and attributable to a number of properties, the most important of which is the beginning of the dehydration process. In particular, sucrose does not enter the cells, but exerts its beneficial effects by causing cellular dehydration through changes in osmotic pressure (Friedler *et al*., 1988). The increase of the extracellular solute concentration generates an osmotic gradient across the cell membrane, which draws water out of the cell, causing the cell to dehydrate before the freezing procedure.

Furthermore, it is extremely important to establish the optimal exposure time of the oocyte to the cryoprotectant solutions. This has to be long enough to permit sufficient dehydration of the cell, but not so long as to damage the cell since it alters the intracellular pH as well as the developmental potential as seen in mouse zygotes (Damien *et al*., 1990).

The aim of this study was to examine the optimal exposure time to cryoprotectants for oocyte survival.

## MATERIALS AND METHODS

### *Source of oocytes*

Immature oocytes were obtained from consenting patients from our IVF-ICSI program, after ovarian hormonal stimulation only when adequate number of normal MII oocytes was retrieved.

### *Oocyte freezing/thawing program*

The cryopreservation protocol consisted of a slow-freezing-rapid-thawing method. All the oocytes were transferred to Petri dishes containing Dulbecco's phosphate buffered saline (PBS) supplemented with 25 mg $ml^{-1}$ human serum albumin (Vitrolife). One or two oocytes were placed in the equilibration solution containing 1.5 M PROH, and maintained for 10 min at room temperature before transfer to the loading solution with 1.5 M PROH supplemented with 0.1 M sucrose (Vitrolife). The oocytes were loaded in plastic straws and transferred into an automated biological vertical freezer.

The initial chamber temperature was 20°C. Then the temperature was slowly reduced to -7°C at a rate of 2°C $min^{-1}$. Ice nucleation was induced manually at -7°C. After a hold time of 10 min at -7°C, the straws were cooled slowly to -30°C at a rate of 0.3°C $min^{-1}$ and then rapidly to -150°C at a rate of 50°C $min^{-1}$. After that, the straws were transferred into liquid nitrogen tanks and stored until thawing.

For thawing, the straws were air-warmed for 30 sec and then immersed in a 30°C water bath for 40 sec until all traces of ice had disappeared. The cryoprotectant was removed at room temperature by stepwise dilution of PROH in the thawing solutions. The contents of the melted straws were expelled in a 1 mol $l^{-1}$ PROH-sucrose solution (0.1 mol $l^{-1}$) and the oocytes were equilibrated for 5 min. The oocytes were transferred to a 0.5 mol $l^{-1}$ PROH-sucrose solution (0.1 mol $l^{-1}$) and then to a sucrose solution (0.1 mol $l^{-1}$), before the final dilution in a PBS solution added to human serum albumin. Finally, the oocytes were cultured in an IVF medium at 37°C in an atmosphere of 5% $CO_2$ in air.

The oocytes were checked for survival and classified as survived if the zona pellucida and plasma membrane were intact, the perivitelline space was clear and normal in size, there was no evidence of cytoplasmic leakage or oocyte shrinkage and there was virtually no space between the zona pellucida and the cytoplasm.

### *Oocyte survival rate*

#### *A. Influence of exposure time to 1,2 propanediol*

In a group of 85 oocytes, the effective exposure time to 1,2 propanediol solution was calculated from the

time the oocytes were placed into the Petri dish with the equilibration solution to the time that they were placed into the Petri dish with the loading solution. Oocytes after *in vitro* culture were randomly assigned to three experimental groups: (i) oocytes remained for 5 min to the equilibration solution, (ii) oocytes remained from 5.5 to 10 min, and (iii) oocytes remained from 10.5 to 15 min.

*B. Influence of exposure time to 1,2 propanediol added with 0.1 M sucrose*

In a group of 50 oocytes, the effective exposure time to the loading solution was retrospectively calculated from the time the oocytes were placed into the Petri dish to the time that they were loaded into the straws. This calculation was performed considering that a time of 30 sec was necessary between the loading of one straw and the next: this is the time required to load the oocytes into the straws and to put them into the biological freezer, before starting the cooling program. The oocytes were divided into two groups; the first one remained in the loading solution up to 5 min and the second one up to 10 min.

*In vitro culture*

Only intact oocytes with a refringent cytoplasm and presenting no signs of atresia or degeneration were used. Oocytes were cultured in 4-well dishes, in a maturation medium G-1 (Vitrolife) covered with oil. Serial observations under the inverted microscope were performed for the determination of any maturation processes.

*Statistical analysis*

Statistical analyses between groups 1, 2 and 3 were carried out using the R × C test of independence. Differences were considered significant when $p \leq 0.05$.

## RESULTS

*A. Influence of exposure time to the equilibration solution 1,2 propanediol*

Results of the survival rates in the three different groups are summarized in Table 1. Exposure time to the equilibration buffer (consisting of 1.5 mol $l^{-1}$ 1,2 propanediol) for more than 5 min, before lowering of the temperature, significantly increased the oocyte survival rate ($p < 0.05$) (Table 1).

These data are also supported by morphological changes, such as the ooplasm size and the perivitelline space. By a single observation performed under an inverted microscope to assess the oocyte morphological changes, it was found that for a period shorter than 5 min, the oocytes did not restore to their initial size (Fig. 1A) and these were the oocytes that showed lower survival rates (Fig. 1B). The restoration was achieved from 5.5 to 10 min exposure time to the equilibration solution (Fig. 1C). Exposure time longer than 10 min did not show any other morphological changes (Fig. 1D).

TABLE 1. Human oocyte survival: influence of exposure time to 1.5 mol $l^{-1}$ 1,2 propanediol concentration (a *vs* b and c: $p < 0.05$)

| Exposure time (min) | Frozen-thawed oocytes (n) | Number survived (%) |
|---|---|---|
| 0.5-5 | 25 | 4 (16)[a] |
| 5.5-10 | 32 | 15 (46.8)[b] |
| 10.5-15 | 18 | 8 (44.4)[c] |

TABLE 2. Human oocyte survival: influence of exposure time to 1.5 mol $l^{-1}$ 1,2 propanediol supplemented with 0.1 M sucrose (a *vs* b: $p < 0.05$)

| Exposure time (min) | Frozen-thawed oocytes (n) | Number survived (%) |
|---|---|---|
| 0.5-5 | 25 | 4 (16)[a] |
| >10 | 25 | 11 (44)[b] |

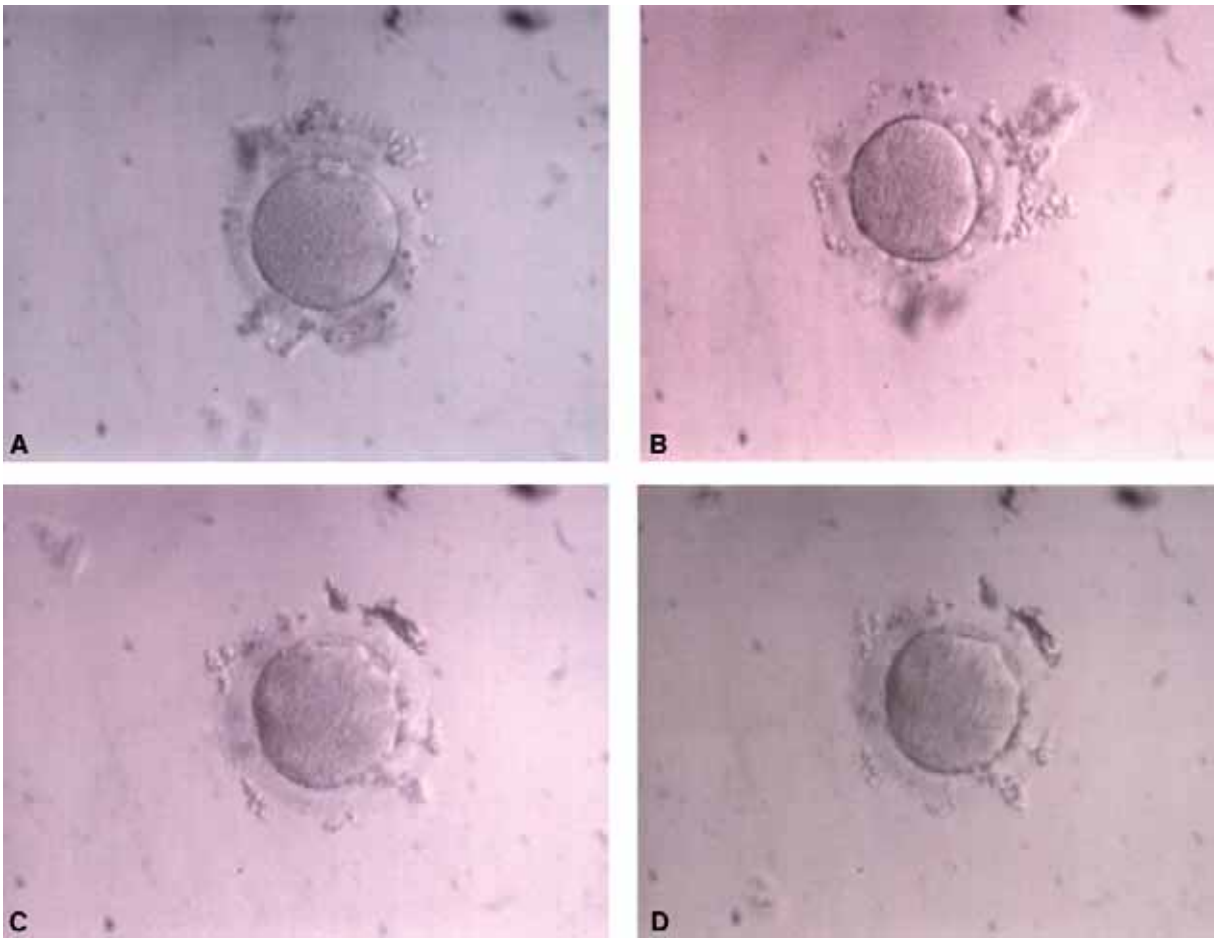


FIG. 1. Morphological changes occurring in an oocyte with the cumulus oophorus exposed to 1.5 mol $l^{-1}$ 1,2 propanediol (equilibration solution) at room temperature. (A) The human oocyte at the moment placed in the equilibration solution; (B), (C), (D) The same oocyte after 5 min, 10 min, and 15 min exposure to the cryoprotectant solution.

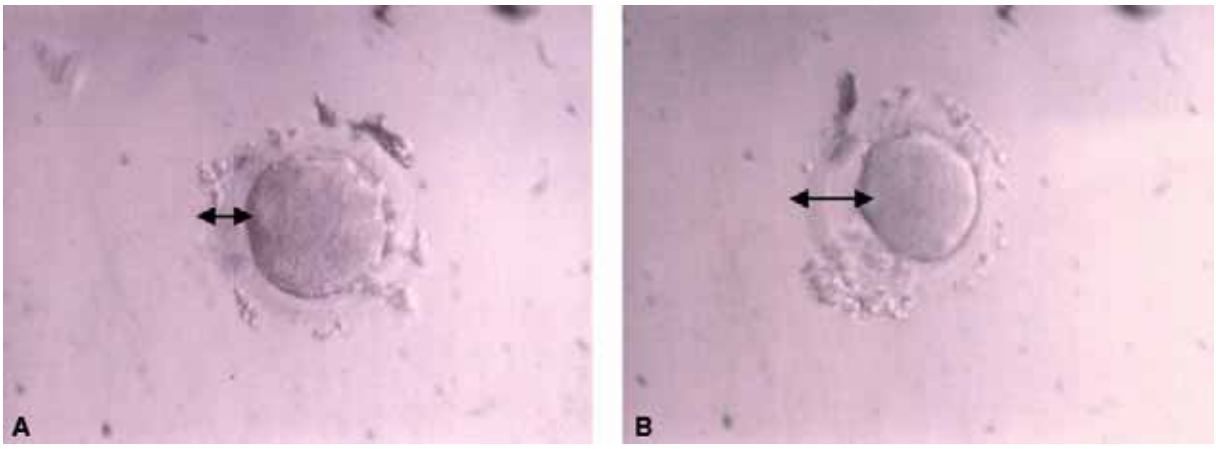


FIG. 2. Morphological changes occurring in an oocyte with the cumulus oophorus exposed to 1.5 mol $l^{-1}$ 1,2 propanediol supplemented with 0.1 mol $l^{-1}$ sucrose (loading solution) at room temperature. (A) The human oocyte at the moment placed in the loading solution. The oocyte shows an ooplasm with regular dimensions and a regular perivitelline space; (B) The same oocyte after 10 min of exposure showing a shrunken ooplasm and an increased perivitelline space.

*B. Influence of exposure time to the loading solution consisted of 1,2 propanediol added to 0.1 M sucrose*

By a single observation performed under an inverted microscope to assess the oocyte morphological changes it was found that at the time when the oocyte had just been put into the loading solution, the ooplasm showed a regular size and a regular perivitelline space that did not change when the exposure time was less than 5 min (Fig. 2A). The same oocyte after an exposure time longer than 10 min in 1,2 propanediol supplemented with 0.1 M sucrose (Fig. 2B) showed a shrunken ooplasm and a plasmalemma with an increased perivitelline space as a consequence of contact with the high molarity solution. This reduction, reached after 10 min of exposure, was estimated as 17% of the diameter. The oocyte did not exhibit any other changes in its morphology after 10 min of exposure.

A gradual enhancement of the oocyte survival rate was observed in relation to the increase of the exposure time to the loading solution and a significantly higher survival rate was found when the exposure time to the solution was lower than 10 min (Table 2).

## DISCUSSION

Human embryo cryopreservation provides an effective technique in the treatment of infertility. However, oocyte cryopreservation is another method offering a chance to young women, at risk of reducing or losing ovarian function because of chemotherapy or professional career development, to extend their fertility. Successful pregnancies achieved through oocyte cryopreservation have been reported at mature (Porcu *et al*., 1997; Young *et al*., 1998; Hreinsson *et al*., 2002; Boldt *et al*., 2003; Chen *et al*., 2003) and immature stages (Tucker *et al*., 1998). However, both mature and immature oocyte cryopreservations have drawbacks of giving lower survival rates when compared with the contemporary techniques of embryo cryopreservation (Goud *et al*., 2000).

In this study, one factor influencing human oocyte survival rate before cryostorage was investigated, namely the effects of different exposure times to the freezing solutions.

The presence of cryoprotectants (both permeating and non-permeating) in the freezing solution should minimize cell damage during the freezing and thawing processes. For oocyte cryopreservation procedures, cryoprotectant concentrations were usually 1.5 mol $l^{-1}$, many times higher than any other components in the medium. While cryoprotectants readily cross cell membranes, water usually crosses them even more readily (Shaw, 1993).

The results presented here showed that exposure time to the equilibration buffer (consisted of 1.5 mol $l^{-1}$ 1,2 propanediol) longer than 5 min, before lowering of the temperature, significantly increased the oocyte survival rate. At the time when the oocytes were placed into the solution with the propanediol buffer they lost their intracellular water and a reduction of their size was started. After a period of time, which in this study was calculated to be longer than 5 min, the cryoprotectant entered the cell and the size recovery began. Presumably, exposure time shorter than 5 min is not sufficient for the passage of the cryoprotectant through the cell membranes and for the recovery of the cell volume after the dehydration process. The data obtained from the morphological changes confirmed this hypothesis.

Concerning the exposure time to the loading solution, a time longer than 10 min seems to be optimal. In particular, sucrose does not enter the cell, but exerts its beneficial effects by causing cellular dehydration through changes in the osmotic pressure (Fabbri *et al*., 2001). In this study, the effects of different exposure times to the loading solution on oocyte freezing were investigated. We suggest that the increase of the exposure time to the loading buffer generates an osmotic gradient across the cell membrane, which draws water out of the cell, causing the cell to dehydrate sufficiently before and during the freezing procedure. Moreover, the intracellular ice formation is the main reason for the oocyte's death after the cryopreservation procedures. Based on these morphological parameters, it is observed that no changes occur after exposure shorter than 5 min in the loading buffer (consisted of 1.5 mol $l^{-1}$ 1,2 propanediol added to 0.1 mol $l^{-1}$ sucrose). When the time was longer, the same oocyte showed a shrunken ooplasm and plasmalemma with an increased perivitelline space. A significantly higher survival rate was observed when the exposure time to the sucrose solution was between 10.5 and 15 min. Moreover, Fabbri *et al*. (2001) showed that shorter times in 0.1 and 0.2 mol $l^{-1}$ sucrose reflect insufficient oocyte dehydration and that the 15 min time in 0.2 mol $l^{-1}$ is approaching the extent of dehydration.

This appears to be the first time that a similar evaluation was performed, and it is suggested that a

satisfactory oocyte dehydration should be obtained before lowering of the temperature. This could further result in avoiding of the formation of cytoplasmic ice crystals which are the main factor influencing oocyte survival rate during the cryopreservation procedure. In addition, based on the results of this study, it appears possible to achieve a higher survival rate of cryopreserved human oocytes that could lead to successful fertilization and embryo development.

These encouraging results indicate that an increased exposure time enhances oocyte survival and suggest that insufficient dehydration results in low survival rates. Further studies are required to verify whether the longer exposure time could improve the oocyte survival rate after thawing.

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