Studies on Cryopreservation of Human Oocytes using Verification Technique

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ABSTRACT--- ***This study aims to extend the applications of oocytes cryopreservation into the clinical practice of ART. Using the vitrification method applied on 676 human oocytes divided into four groups based on their maturation stage, presence of corona cells and the time to start cryopreservation. Group (A) includes 177 MII oocytes, fully denuded and vitrified 3-5 hours after pick up, (B) includes 194 GV/MI oocytes fully denuded and vitrified 3-5 hours after pick up, (C) contains 103 oocytes vitrified 24 hours from retrieval at the MII stage and fully denuded and (D) contains 202 oocytes vitrified with the corona cells at the MII stage 3-5 hours after pick up. Survivability is determined one hour after thawing then the survived oocytes undergo ICSI procedure. Group (A), after thawing 161 oocytes (91%) survived, 111 (69%) got fertilized, and only 38 (34%) developed into blastocysts. Group (B), 167 oocytes (86%) survived then 60 (36%) matured to MII, and 40 (67 %) got fertilized, then 11 embryos (28%) developed into blastocyst. Group C, 67 oocytes survived (65%), 44 got fertilized (66%) and 8 embryos (18%) reached blastocyst stage. Group D, 188 oocytes survived (93%), 126 got fertilized (67%) and 39 embryos developed into blastocyst (31%). The vitrification method of oocytes is very successful in preserving viability and fertilization .The presence or removal of the corona cells before vitrification didn't affect the results. Delaying vitrification 24 hours significantly lowered them. Vitrifying GV oocytes resulted in a reasonable survival rate; however the fertilization rate was very low.***

Keywords--- Oocytes cryopreservation, vitrification, IVF, ICSI.

1.INTRODUCTION

Cryogenic preservation of the reproductive tissues (gametes and embryos) has become of greater importance nowadays in the programs of the *in vitro* fertilization (IVF) all over the world and it might play a significant role over the next few years [1]. The aimed oocytes cryopreservation or freezing may have the potential to be an important adjunct to assisted reproductive technologies (ART) in humans and animals as well [2]. The results from the recent studies suggest that the survival of human oocytes after cryopreservation can be affected by many factors including the oocytes stage of maturation, their quality and other biophysical factors resulting from the freezing technique used [3] .The incorporation of oocyte cryopreservation into the clinical practice of assisted reproduction programs was the aim of many practitioners in order to extend its applications to other cases [4].

Many reasons that bring the human oocytes cryopreservation into the interest, those reasons are widely known and can be summarized including: diseases, treatments, legal, ethical, social, and practical problems may also require oocyte cryopreservation [5].

For many cases cryopreservation offers the opportunity for preserving women oocytes who are at risk of losing ovarian function as many women experience certain conditions that may result in diminishing or even losing fertility due to age as in premature menopause and other circumstances as in oncology treatment, pelvic disease, surgery, or clinical treatment involving radiotherapy or chemotherapy [6] . Also, women who are in their late reproductive or menopausal years, who have hereditary diseases, abnormal oocytes, or who have had a history of difficulty with oocyte retrieval for in vitro fertilization (IVF) treatment may also benefit from oocyte banking [7] .

Despite approximately 20 years of effort, the results in oocytes cryopreservation remain highly variable, and human oocyte cryopreservation is still considered as an experimental procedure. However, both methods have resulted in a successful cryopreservation of human oocytes and embryos, but the slow freezing technique has given much doubted efficiency and consistency. The relatively poor performance of slow-cooling has been highlighted in recently published reports. Therefore, during the past few years, opinion articles as well studies initiated to underline that vitrification might be a better alternative for cryopreserving human zygotes and embryos rather than the slow-rate method. Vitrification methods have been applied to cryopreserve human oocytes to determine if improvements can be made, some of these

investigations have demonstrated remarkable success, but, to date, too few reports on vitrification have been published to reach definitive conclusions [8,9] .

The present study is based on comparing the effect of vitrification and the biophysical factors associated with the freezing technique used on the oocytes at different stages of nuclear maturation, different time to start vitrification after their pick up and the degree of oocytes denuding. The rates of survival, fertilization and blastomeric formation are determining the achievement of efficiency along with observing several criteria including the maintenance of a stable vitreous state during cooling and warming, using the minimum amount of permeating cryoprotectant, and to reduce the potential for osmotic damage to the cell.

2.MATERIALS AND METHODS

The oocytes were obtained from young and healthy women (age 25-35). All couples participating in the study signed an approval consent form prior to treatment. The females underwent ovarian stimulation using a daily dose of 200 IU of recombinant FSH (Follistim; Organon, Roseland, NJ, USA). Gonadotrophin releasing hormone (GnRH) antagonist (ganirelix acetate; Organon) was administered, starting on day 6, for LH surge prevention. Recombinant human chorionic gonadotrophin (HCG; Ovidrel; Serono, Rockland, MA, USA) was administered to trigger nuclear maturation of oocytes when two or more follicles reached 18 mm.

The oocytes retrieved were divided into four groups according to their nuclear maturity, time to start vitrification and the presence of corona cells as follows (table 1):

Group A (fig. 1): Include the oocytes in the metaphase II that were denuded by brief exposure to hyaluronidase. The corona cells were completely removed by pipetting through micropipettes. For this group of oocytes freezing was routinely initiated within 3-5 hours after retrieval.

Group B (fig. 2): Include the oocytes in the metaphase I or the germinal vesicle stage that were denuded by brief exposure to hyaluronidase. The corona cells were completely removed by pipetting through micropipettes. For this group of oocytes freezing was routinely initiated within 3-5 hours after retrieval.

Group C (fig. 3): Include the oocytes in the metaphase II that were denuded by brief exposure to hyaluronidase. Then corona cells were completely removed by pipetting through micropipettes. This group of oocytes that was incubated for 24 hour in culture media then freezing was routinely initiated after 24 hours from retrieval.

Group D (fig. 4): Include the oocytes in the metaphase II that were denuded by brief exposure to hyaluronidase to remove the cumulus cells only while the corona cells were not removed, then freezing was routinely initiated within 3-5 hours after retrieval.

The oocytes were vitrified by the minimum volume cooling (MVC) method described by Kuwayama *et al.* [10]. Then the polypropylene strip carrying the oocytes was submerged in liquid nitrogen and was ready for storage. Then the vitrified oocytes were subjected to the thawing process and after the completion of their recovery prior to manipulation, morphological evaluation of the oocytes, cell membrane and perivitelline space is made under zoom stereomicroscope. Morphologically normal oocytes are selected for ICSI as described by Nagy *et al.* [11]. Fertilization was noticed on day 1 while embryonic development was assessed on day 3.

3.RESULTS

The morphologic survival of the thawed oocytes included in group (A) was 91% (177/161), group (B) was 86% (167/194), group (C) was 73% (76/103) and group (D) was 93% (188/202). The highest proportion of morphologically normal oocytes were seen in group (D) which is nearly the same in group (A) and slightly higher than group (B) while the lowest were in group (C). Non-significant ($P > 0.05$) difference was seen between the oocytes recovered in Group (A) and those recovered in Group (C), while groups (A) and (D) are showing a significant variation ($P \leq 0.5$).
The survived oocytes from group (B) were in GV stage of maturation. These oocytes were cultured for IVM and only 60 oocytes completed their maturation into fully mature MII oocytes with the percentage 36% of the survived oocytes and 31% to the starting number of the vitrified oocytes, the proportion of oocytes attaining MII along with the survived oocytes form the other groups were then underwent ICSI procedure.

Fertilization is detected by observing the pronuclei (PN) which is a pair of nuclei undergoing fusion in karyogamy, each nucleus contains the haploid number of chromosomes and it is the nucleus of an ovum or a spermatozoon after fertilization but before fusion of the chromosomes to form the nucleus of the zygote Fertilization of the injected oocytes included in group (A) was 69% (111/161), group (B) was 67% (40/60), group (C) was 66% (44/67) and group (D) was 67% (126/188). A significantly higher proportion of fertilized oocytes were seen in group (A) while the fertilization in group (C) was the least. A significant ($P \leq 0.05$) difference was seen between the percentages of fertilization in the four groups.
Comparing the number of the fertilized oocytes to the total number of the vitrified oocyte the results were: 63% for group

(A), 21% for group (B), 43% for group (C) and 64% for group (D). A significantly higher proportion of fertilized oocytes were seen in group (D) which is nearly the same in group (A) while the fertilization percent in group (B) was the least.

Blastocysts formation is detected on day five of injection. The blastocysts formation of the fertilized oocytes included in group (A) was 34% (38/111), group (B) was 28% (11/40), group (C) was 18% (8/44) and group (D) was 31% (39/126). The highest proportion of morphologically normal oocytes were seen in group (A) which is near the result in group (D) and higher than group (B) while the lowest were in group (C). A significant ($P \leq 0.05$) difference was seen between the percentages of blastocysts formation in the four groups. Comparing the number of the blastocysts formed to the total number of the vitrified oocyte the results were: 22% for group (A), 6% for group (B), 8% for group (C) and 22% for group (D). A significantly the higher proportion of blastocysts formed was seen in groups (A) and (D) while the blastocysts formation percent in group (B) was the least.

The comparison between the final results collected of the four groups including number of oocytes vitrified, survived, number of normal MII oocytes (underwent ICSI), fertilized and blastocysts formed are showed in table (2) and demonstrated by the histogram in (fig. 5).

4. FIGURES AND TABLES

	Nuclear Maturation	Start Vitrification	Corona Cells
Group A	MII	3-5 hours	Completely removed
Group B	GV / MI	3-5 hours	Completely removed
Group C	MII	24 hours	Completely removed
Group D	MII	3-5 hours	Not removed

Table 1: Showing the oocytes selection protocol dividing the oocytes retrieved into four groups according to their nuclear maturity, time to start vitrification and the presence of corona cells.

Group	Number of oocytes vitrified	Number of oocytes survived	Number of normal MII oocytes	Number of oocytes fertilized	Number of blastocysts Day 5
Group A	177	161(91%)	161(91%)	111(69%)	38(34%)
Group B	194	167(86%)	60(31%)	40(67%)	11(28%)
Group C	103	67(65%)	67(65%)	44(66%)	8(18%)
Group D	202	188(93%)	188(93%)	126(67%)	39(31%)

Table 2: Showing a comparison between the final results collected of the four groups including the number of oocytes vitrified, survived, underwent ICSI (number of normal MII oocytes) fertilized and blastocysts formed.

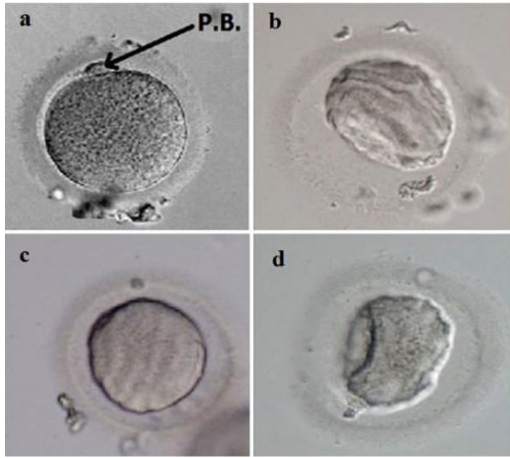

Fig. 1: A photomicrograph showing mature MII oocyte from group (A) ongoing vitrification procedure:
a) Oocyte in the mature MII stage and the polar body (P.B.) is evident (completely denuded).
b) Shrinking of the oocyte should occur within 90 sec. after merging in the equilibration solution.
c) Normal oocytes should regain their original appearance after equilibration within 9 min.
d) Extreme shrinkage of the oocyte when placed in the vitrification sol.

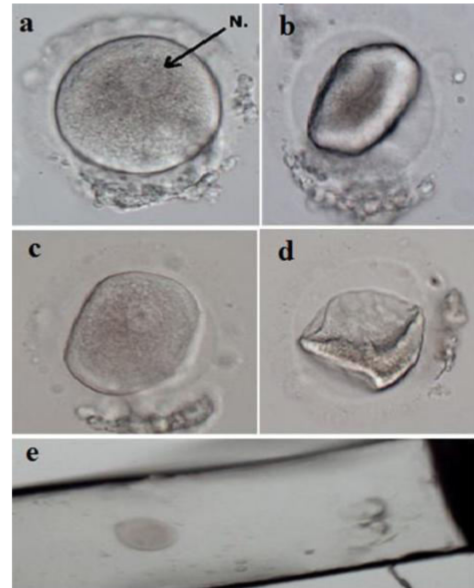

Fig. 2: A microscopic micrograph showing immature GV oocytes from group (B) ongoing vitrification procedure:
a) Oocyte in the germinal vesicle stage and the nucleus is evident (completely denuded).
b) Shrinking of the oocyte should occur within 90 sec. after merging in the equilibration solution.
c) Normal oocytes should regain their original appearance after equilibration within 9 min.
d) Extreme shrinkage of the oocyte when placed in the vitrification solution
e) Oocytes placed on the strip of the Cryotop near a black mark.

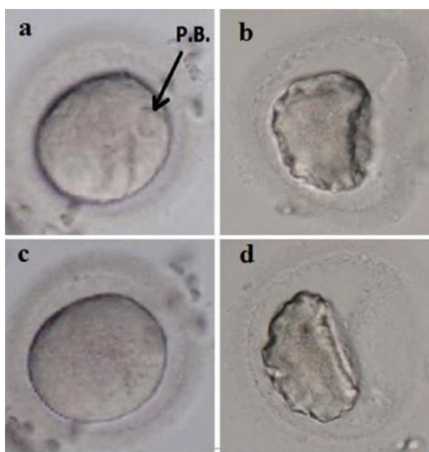

Fig. 3: A photomicrograph showing mature MII oocytes from group (C) ongoing vitrification procedure which was delayed for 24 hours:
a) Oocyte in the MII stage and surrounded with corona cells cultured.
b) Shrinking of the oocyte should occur within 90 sec. after merging in the equilibration solution.
c) Normal oocytes should regain their original appearance after equilibration within 9 min.
d) Extreme shrinkage of the oocyte when placed in the vitrification solution.

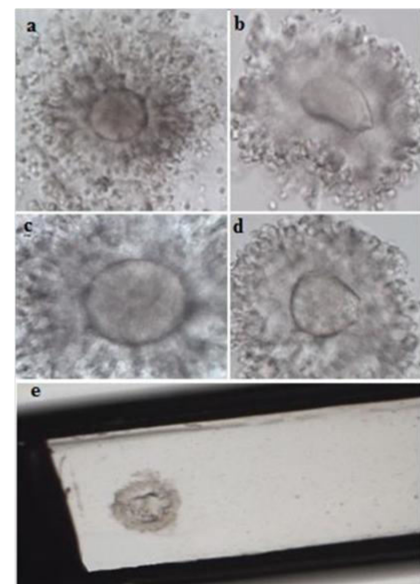

Fig. 4: A photomicrograph showing mature MII oocytes with corona cells from group (D) ongoing vitrification procedure:

a) Oocyte in the MII stage and surrounded with corona cells cultured.
b) Shrinking of the oocyte should occur within 90 sec. after merging in the equilibration solution.
c) Normal oocytes should regain their original appearance after equilibration within 9 min.
d) Extreme shrinkage of the oocyte when placed in the vitrification solution.
e) Oocytes placed on the strip of the Cryotop near a black mark.

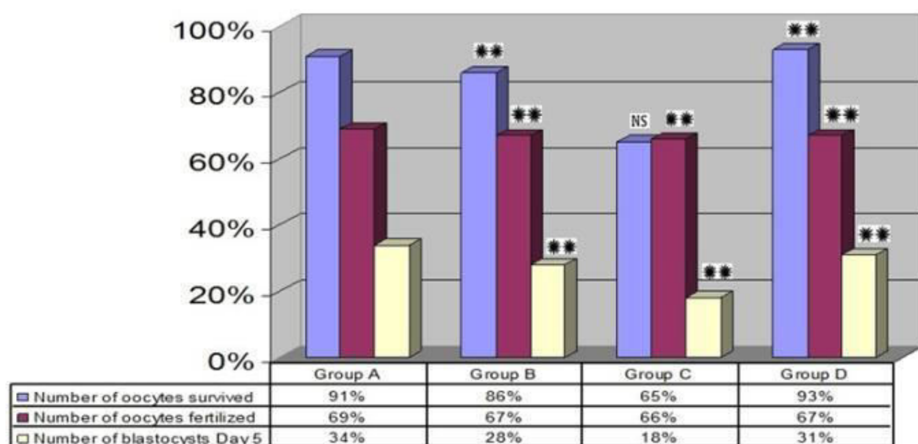

Fig. 5: histogram showing the total percentages of oocytes survival, fertilization and blastocysts formation in the four groups. (**) significant variation, (NS) non-significant.

5.DISCUSSION

Our study evaluated oocytes cryopreservation, using the vitrification method applied on 676 oocytes divided into four groups based on their maturation stage, presence of corona cells and the delay in oocytes cryopreservation.

The efficient oocyte cryopreservation may be used to avoid embryo cryopreservation with its associated moral and ethical concerns .One of the goals in oocyte cryopreservation is using the minimum number of oocytes to achieve a successful implantation [12,13,8].

The results calculated for group (A) that includes normal MII oocytes, completely denuded and vitrified 3 hours after retrieval, the survival rate is 93% with 69% fertilization rate, such results are along with many other reports published [5,14,15].

It was very remarkable by our crew that many of the oocytes after thawing show a zona pellucida hardening with the plasma membrane. However, zona hardening may not be of great problem after adoption of ICSI technology.

In our study we have a survival rate for immature oocytes about 86% of 194 oocytes included in group (B) and a fertilization rate 67% of 60 oocytes that complete their maturation in vitro. Those results among previously published reports didn't show a considerable variation; in the study carried by Dr Ching-Chien Chang et al [12] on vitrification of immature oocytes.

In studying the efficiency of vitrification for the oocytes that spent 24 hours in vitro those included in group C and are described as aged oocytes survival rate was 65% out of 96 oocytes and fertilization rate was 66% which can be considered as a reasonable result. Such result gives a hint that the fertilization power of the oocytes delayed in cryopreservation, even for 24 hours, is not lost.

In the group (D) 202 oocytes were cryopreserved directly with their corona cells around them with a survival rate of 93%, and a fertilization rate of 67% and 31% of the oocytes reach the blastocyst stage, The results show that oocyte survival, fertilization and cleavage rate were not significantly different in this group when compared with the results of group (A) that contains MII oocytes.

It was reported that oocytes depend on the surrounding cells through gap junctions to provide nutrients and regulatory signals [12] . The success achieved in vitrifying the oocytes surrounded by corona cells by our study ensures that the cryoprotecants used can pass through these cells and completely vitrify the cytoplasm.

The results we got in the study for the oocytes survival, fertilization and cleavage rates are very successful proving that using vitrification method in the cryopreservation of the oocytes is a very reliable way in preserving the viability of the oocytes and so preserve the fertility power in such cases when oocyte preservation is needed, it provides valuable information about the efficiency of oocyte cryopreservation if it is used routinely as an adjunct to a human IVF programs.

Cryopreservation of immature oocytes could provide a partial solution for damage inflicted to cytoskeleton, as these oocytes do not contain polymerized tubules at this stage. However, oocytes at the GV stage are extremely sensitive to chilling temperatures, with the damage occurring at the cytoplasmic membrane level.

Our research is involved in examining fundamental cryobiological properties of human oocytes by vitrification solutions as a step towards optimizing vitrification methods in applications of oocyte cryopreservation. Managing the potential damage from the solution used to achieve vitrification and preventing ice formation during cooling and warming are the main goals of studying such protocols. The results of our analysis suggest that successful vitrification

can be achieved, as the resulting theoretically optimized method is similar to one shown to improve vitrification with human oocytes. The data collected in our study support the use of oocytes vitrification technique in the routine work for the ART labs as suitable adjunct or a standing by to the IVF programs. Vitrification is a reliable way in preserving viability and fertility power of the oocytes in such cases when oocyte preserving is needed. Applications of oocyte freezing include using it as an alternative to embryo freezing, improving the efficiency of IVF, oocyte preservation for patients with ovarian hyperstimulation syndrome, and preventing fertility loss through surgery. Future work should be directed at comparing this method with other methods currently in practice and more research into the fundamental cryobiological properties of human oocytes is warranted.

6. ACKNOWLEDGMENTS

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