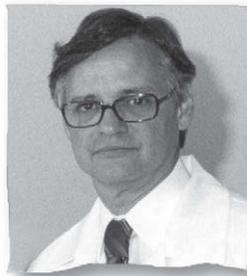


Article

Comparison of embryo quality between sibling embryos originating from frozen or fresh oocytes



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Abstract

Human embryo cryopreservation techniques allow storage of surplus embryos created during assisted reproduction procedures; however, the existence of these same surplus embryos has sparked further debate. What can be their fate once they are no longer desired by their parents, or if the parents are deceased? Thus, the level of interest in the cryopreservation of oocytes has increased, as has the necessity for further scientific study. This study had the objective of comparing embryo quality from 16 women who underwent intracytoplasmic sperm injection, where approximately half of the retrieved oocytes per cycle were inseminated fresh after collection, and the remainder cryopreserved for subsequent fertilization. Normal fertilization rates were not significantly different between the two oocyte-treatment groups. There was no significant difference in the frequency of good quality embryos (morphology grades I and II) on the second day of laboratory evaluation between embryos derived from the two oocyte-treatment groups. It is interesting to note that embryo transfer from fresh oocytes produced no pregnancies, which shows that even embryos derived from frozen oocytes that are fragmented or have a slower cleavage rate are viable embryos, capable of producing healthy babies.

Keywords: cleavage, embryo quality, freezing, human, ICSI, oocytes

Introduction

After almost three decades since the first human birth resulting from IVF, several developments have taken place. IVF was created originally to treat women presenting severe tubal factors, but was then prescribed for other forms of infertility, thus becoming increasingly popular.

The IVF domain has also brought about many issues that called for profound reflections, among which is the debate about the fate of surplus embryos that result from those technologies. Embryo cryopreservation techniques have improved, allowing storage of viable surplus embryos for an indefinite period of time. On the other hand, the disposition of these same surplus frozen embryos has sparked further debate. What is to be done with these embryos? What is their fate once they are no longer required by their parents, or even if the parents

are deceased? Thus, interest in oocyte cryopreservation grew and, by 1986, Chen produced the first birth with this technique (Chen, 1986). The subsequent improvement of this craft heated the controversies that surrounded the freezing of embryos and created new designations, such as the preservation of the reproductive capacity for young women with premature loss of ovarian function, and of those who require oncological treatment, or even for those women who simply wish to postpone child bearing to a later phase of reproductive life when the chances of natural conception would be greatly reduced due to the deleterious effects of ageing on gonadal function.

Oocyte freezing techniques are challenged by the physical characteristics of oocytes, e.g., they are large, highly specialized cells that are dynamic in their development up to metaphase II. Despite isolated reports of gestation with frozen oocytes (Chen, 1986; Van Uem *et al.*, 1987), the global rates of success

in surviving thawing and in pregnancy first reported were very low, discouraging cryopreservation of oocytes as a clinical routine (Gook and Edgar, 1999; Coticchio *et al.*, 2005). Ovarian freezing has also been proposed as a measure to preserve female fertility, but this application does not correspond precisely with that of oocyte storage (Hovatta, 2005). Attempts to improve our understanding of oocyte cryobiology have been in many cases empirical, while only limited experience has been gained by adopting models able to predict the intensity of various forms of stress imposed on oocytes during freezing (Fuller and Paynter, 2004; Paynter, 2005). Isachenko *et al.* (2004) suggested the possibility of combining ovarian tissue cryopreservation with in-vitro maturation (IVM) of germinal vesicle (GV) oocytes (retrieved during ovarian tissue preparation) and later cryopreservation at metaphase I/II. Recently, Porcu *et al.* (2000) strengthened these studies by reporting the birth of 13 children with no chromosome alterations using frozen oocytes. Today, with over 150 registered births and the absence of chromosome abnormalities, it seems that the method is potentially safe and has growing applicability worldwide.

The aim of this paper is to assess the reproductive performance of women who have undergone intracytoplasmic sperm injection (ICSI) using frozen oocytes by comparing the fertilization, cleavage rates, and embryo quality between sibling oocytes submitted to ICSI after thawing and those that were fertilized about 4 h after follicle aspiration. This is the first study to compare fertilization, cleavage rate and embryo quality between frozen-thawed and fresh oocytes from the same cohort.

Materials and methods

Study design and patients

This was an observational retrospective cohort study. The study population consisted of patients who sought medical assistance for treating conjugal infertility. They were submitted to IVF with the ICSI technique. The data were collected from the medical records at the Fertilitat (Reproductive Medicine Centre) clinic between January 2001 and July 2004 with administrative authorization. All the patients signed a term of consent that was informed before the fertilization treatment granting use of their data for scientific purposes, with the procedure being approved by the Ethical Research Committee at Pontifícia Universidade Católica-RS (PUCRS). No other procedure, except those required for treating infertility, was carried out.

The sample comprised 16 women who underwent ICSI and did not wish to freeze the supranumerary embryos. All 16 patients had an average of 22 oocytes per cycle; therefore the possibility of freezing oocytes was offered in order to avoid freezing embryos. Consequently, based on the patients' consent, some oocytes were inseminated while fresh, and some were frozen, for future use if necessary. The mean maternal age was 32.9 ± 3.5 years. The inclusion criteria were: women less than 40 years of age, with no apparent infertility cause, or with defined infertility cause; men with oligospermia regardless of age, sperm collected by masturbation, percutaneous epididymal sperm aspiration (PESA), testicular sperm aspiration (TESA) or testicular sperm extraction (TESE). The sperm quality from the 16 patients was as follows: six oligospermia, one necrospermia, three asthenospermia and six normal concentration.

From the 16 women participating in this study, 362 oocytes were collected, of which 135 followed the ICSI procedure, 158 were frozen, and 69 were either immature or degenerated. All the frozen oocytes were thawed in under two years, which resulted in 99 (62.7%) oocytes viable for undergoing ICSI. The comparative analysis of the laboratory evaluation of the embryos that were inseminated either fresh or after thawing was carried out on the second day of culture following fertilization.

ICSI procedure

The patients underwent ovarian stimulation with human menopausal gonadotrophin (HMG, Menogon, Ferring, São Paulo, Brasil) after hypophysis desensitization with leuprolide acetate (Lupron, Abbott, São Paulo, Brasil) according to the clinic's protocols. Follicular development was controlled through transvaginal echography and human chorionic gonadotrophin (HCG, Profasi 10000, Serono, São Paulo, Brasil) was administered when at least two follicles reached diameters of 18 mm. Follicular aspiration was carried out 36 h after HCG administration, transvaginally under echographic view. The aspirated follicular fluid was then immediately sent to the assisted reproduction laboratory for oocyte screening. Oocytes at the metaphase II (MII) stage were inseminated 4 hrs following follicular aspiration.

The semen was collected through masturbation and prepared by Percoll gradient (Vitrolife, Mölndalsvägen, Göteborg, Denmark) separation. Some patients underwent PESA, TESA or TESE due to obstructive or unobstructive azoospermia. ICSI was performed 4 h after oocyte collection (Palermo *et al.*, 1992). The fertilized oocytes were cultivated in an HTF (human tubal fluid, Irvine Scientific, Santa Ana, CA, USA) culture supplemented with 15% SSS (serum substitute solution, Irvine Scientific) and placed at 37°C in an atmosphere of 5% CO₂ in air. Embryo cleavage was observed 24 and 48 h after fertilization.

Freezing and thawing of oocytes

The oocytes, after removal of cumulus cells, were observed for maturation stage. Some of the oocytes in MII were inseminated by ICSI and the rest were placed in a four-well dish (Nunclon, Roskilde, Denmark) for further cryopreservation. The cryopreservation protocol consisted of a slow freezing-rapid thawing method. After 4 h of incubation, all oocytes were transferred to a four-well dish) containing human tubal fluid-HEPES (HTF-HEPES, Irvine Scientific), supplemented with 10% SSS at room temperature. The oocytes were held for 15 min in a choline-based medium with 1.5 M PrOH (Stachecki *et al.*, 2002) followed by an additional 15 min in a choline-based medium with 1.5 M PrOH plus 0.2 M sucrose. The oocytes were loaded in plastic straws (IMV International, Minneapolis, MN, USA) and transferred into an automated Cryo Bath freeze control (Cryologic CL 8000, Victoria, Australia).

The initial chamber temperature was 20°C. The temperature was slowly reduced to -7°C at a rate of -2°C/min. Ice nucleation was induced manually at -7°C. After a hold time of 10 min at -7°C, the straws were cooled slowly to -33°C at a rate of 0.3°C/min and then rapidly to -120°C. After a hold time of approximately 10-15 min to allow stabilization of the final temperature, the straws were transferred into liquid nitrogen tanks and stored until thawing.

To thaw the oocytes, straws were air-warmed for 60 s (Stachecki *et al.*, 2002). The cryoprotectant was removed, at room temperature, by stepwise dilution of PrOH in the thawing solutions as follows: the contents of the warmed straws were expelled into 1.0 M of PrOH with 0.2 M of sucrose, and equilibrated for 5 min. Then the oocytes were transferred to 0.5 M PrOH with 0.2 M of sucrose for an additional 5 min, then transferred to 0.2 M sucrose, 0.1 M sucrose and then finally out of sucrose, respectively for 5 min each. Finally the oocytes were cultured in HTF medium at 37°C in an atmosphere of 5% CO₂ in air until ICSI according to protocol. Oocyte survival following warming was evaluated and those cells that were degenerate were considered non-viable.

Oocyte survival and fertilization rate

Those oocytes in which cells were degenerated after thawing were considered non-viable. The criterion used to define normal fertilization was the presence of two pronuclei and a view of the second polar corpuscular 18 h after oocyte insemination. Those oocytes that presented one, three or more pronuclei were considered to be abnormally fertilized. These oocytes were considered non-viable and were discarded.

Embryo quality

Embryo quality was assessed according to the following three variables: degree of cytoplasmic fragmentation, blastomere symmetry, and cleavage rate.

The morphological grade assigned to the embryos was based on criteria from the Latin American Assisted Reproduction Network and modified by the Fertilitat clinic team (Table 1). The classification is based on blastomere symmetry parameters and percentage cytoplasmic fragmentation (RED criteria, Red Latino Americana, 1998). The embryos were graded as I, II, III, and IV, with the best embryos being considered those of grades I and II. Data for the comparison of sibling embryo quality were restricted to the second day of embryo culture, since their transfer was carried out on that day.

The development of sibling embryos was also evaluated with regard to cleavage rate, as measured by comparison of actual number of cell divisions against the expected number of cell divisions on the second day of culture. The presence of three to five blastomeres was considered to be the most appropriate (expected) measure of cellular division for the second day of evaluation.

Endometrial preparation

All cycles where embryos from frozen oocytes were transferred followed the same protocol, starting at the luteal phase with gonadotrophin-releasing hormone analogue (GnRH_a, Lupron; Abbott, São Paulo, Brasil) prior to the transfer and maintained until starting with progesterone. When the hypophysis blockage was reached, confirmed by an oestradiol concentration below 40 pg/ml, then 17- β -oestradiol (Estrofen 2 mg; Medley, São Paulo, Brasil) was administered. The first day of the cycle was the start of oestradiol administration at 2 mg/day, followed by 4 mg/day, reaching a maximum of 8 mg/day until the endometrium reached a thickness of 10 mm, when the oestradiol was maintained and vaginal progesterone started (Crinone 8%, 90 mg/day; Sero-

São Paulo, Brasil). The transfer was performed 3 days after the start of progesterone administration. The progesterone and oestradiol supplementation was maintained until β HCG was tested, and in cases of pregnancy, it was maintained until 12 weeks' gestation.

Statistical analysis

Categorical data were analysed through chi-squared and Fisher's exact non-parametric test when required. For those variables for which significant differences occurred at the $P = 0.05$ significance level the relative risk was calculated. Continuous data were compared between the two oocyte groups with the Mann-Whitney non-parametric test. All the data were analysed using the Statistics Package for Social Sciences, Version 10.

Results

Comparison of normal fertilization and cleavage rates

Of the 99 frozen-thawed oocytes that survived warming and were inseminated, normal fertilization took place in 61 (61.6%), compared with normal fertilization in 89 of the 135 oocytes that were inseminated while fresh (65.9%) (Figure 1). Normal fertilization rate was not significantly different between the sibling oocyte groups when inseminated after follicle aspiration (while fresh) or after thawing ($\chi^2 = 0.29$; $df = 1$).

First cleavage rates were similar for the two oocyte-treatment groups following fertilization. Of the 61 oocytes fertilized from frozen oocytes, 56 cleaved, representing a cleavage rate of 91.8%; and of the 89 fertilized while fresh, 85 cleaved, representing a cleavage rate of 95.5% ($\chi^2 = 0.35$; $df = 1$) (Figure 1).

Comparison of embryo quality

There was no significant difference in sibling embryo quality, as determined by the number of high quality embryos (morphological

Table 1. Morphological evaluation for human embryos cultured *in vitro*. RED Criteria (Red Latino Americana, 1998).

Embryo grade	Description
I	Embryos with symmetrical blastomeres and no cytoplasmic fragmentation
II	Embryos with symmetrical or asymmetrical blastomeres and/or less than 20% cytoplasmic fragmentation
III	Embryos with symmetrical or asymmetrical blastomeres and/or with 20–50% cytoplasmic fragmentation
IV	Embryos with symmetrical or asymmetrical blastomeres and/or with more than 50% cytoplasmic fragmentation

grades I and II) on the second day of laboratory culture in each of the two treatment groups. Embryos originating from frozen-thawed oocytes (Table 2) presented a high quality frequency of 80.4%, and for embryos originating from fresh oocytes the rate was 89.4% ($\chi^2 = 1.59$; $df = 1$) (Figure 2). Furthermore, there were no significant differences observed for percentage of cytoplasmic fragmentation between the frozen-thawed and fresh oocytes graded as morphological grades I and II on the second day of embryo culture.

However, in a specific analysis of the number of embryos that demonstrated over 20% cytoplasmic fragmentation (low quality embryos according to the morphological grading system), it was observed that embryos originating from thawed oocytes were twice as likely to be of low quality when compared with embryos

from fresh oocytes, although this observational difference was not statistically significant [$\chi^2 = 1.14$; $df = 1$]; (relative risk, RR = 2.12; 95% CI = 0.71–6.36).

Discordant results were observed upon assessment of embryo cleavage rate. The embryos originating from frozen-thawed oocytes cleaved significantly slower than those from fresh oocytes ($\chi^2 = 9.33$; $df = 1$; $P = 0.002$). Of the 56 embryos originating from frozen-thawed oocytes, 25 (44.6%) had between 3 and 5 cells on day 2 (Table 2), whereas 61 embryos (71.8%) of the 85 from fresh oocytes had between 3 and 5 cells on day 2 of culture. Embryos with slower cleavage rates originating from frozen-thawed oocytes featured fewer than three blastomeres. It was observed that embryos originating from frozen-thawed oocytes had almost twice the chance of a lower cell number (RR = 1.96; 95% CI = 1.30–2.96; Figure 3).

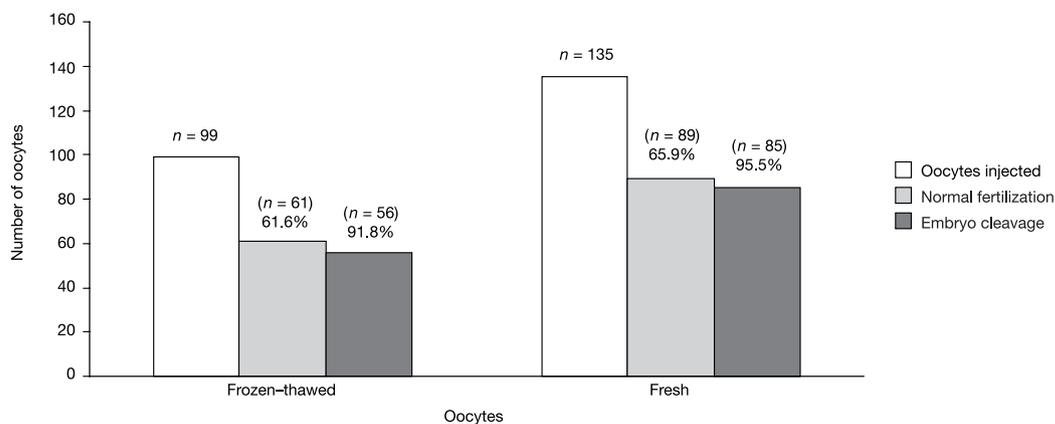


Figure 1. Comparison of normal fertilization and cleavage rates in fresh and frozen-thawed oocytes.

Table 2. Quality of transferred embryos^a originating from frozen-thawed oocytes and the transfer outcome.

Cycle	Embryo cell number (% fragmentation)	Grade	Pregnancy	Outcome
1	2(0), 2(5), 2(5), 3(20)	4-II	Clinical	Born (1)
2	2(0), 3(10), 4(10), 4(10)	1-I, 3-II	Clinical	Born (3)
3	2(10), 2(10), 3(30), 2(0)	3-II, 1-III	Clinical	Born (1)
4	2 (15), 2(40), 3(20)	2-II, 1-IV	Clinical	Abortion
5	4(0), 6(20)	2-II	Biochemical	–
6	3(10)	II	Biochemical	–
7	2(20), 3(5), 4(5), 4 (10)	4-II	No	–
8	2(0), 2(10), 3(10), 5(20)	1-I, 3-II	No	–
9	2(0), 2(0)	2-II	No	–
10	2(15), 2(40), 3(20), 4(10)	3-II, 1-IV	No	–
11	2(0), 2(0), 4(15)	1-II, 2-III	No	–
12	2(5)	II	No	–
13	2(0), 3(0), 4(0), 4(5)	2-II, 2-III	No	–
14	3(0), 3(15), 4 (10)	3-II	No	–
15	2(0), 2(30), 4(20), 4(20), 4(50)	3-II, 1-III, 1-IV	No	–
16	2(5), 3(15)	II, III	No	–

^aSix embryos of the total of 56 originating from frozen-thawed oocytes were not transferred because of poor quality development.

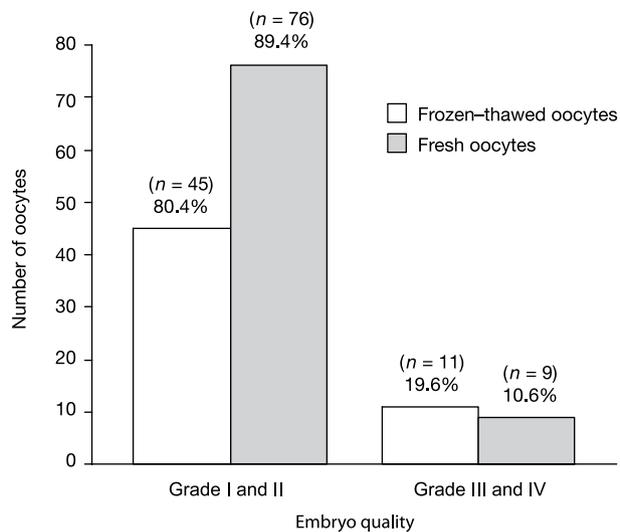


Figure 2. Comparison of embryo quality from fresh and frozen-thawed oocytes on day 2 of culture.

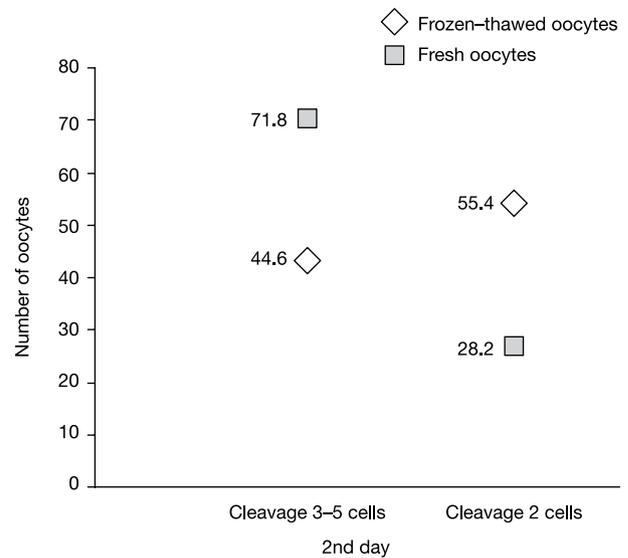


Figure 3. Comparison of embryo cleavage speed from fresh or frozen-thawed oocytes on day 2 of culture.

Discussion

Fertilization rate

The fertilization rate for oocytes inseminated about 4h after follicular aspiration was 65.9%, whereas for oocytes inseminated after thawing it was 61.6% (**Figure 1**). It was interesting to note that the fertilization rates were not significantly different, since, based upon previous papers (Vincent *et al.*, 1990; Johnson *et al.*, 1988), it was expected that freezing could have deleterious effects on the fertilization process. Boldt *et al.*, (2003) reported 59% fertilization in frozen-thawed oocytes. Also, Quintans *et al.* (2002) inseminated 58 oocytes after thawing, of which 33 (56.9%) were fertilized. These studies (Quintans *et al.*, 2002 and Boldt *et al.*, 2003), which also used a choline-based freezing medium, although different to the medium and protocols used here, obtained similar, although somewhat lower, fertilization rates. These rates, however, are lower than the 100% and 80% observed by Azambuja *et al.* (2002 and 2005b), but quite similar to the 69.2% observed by Azambuja *et al.* (2005a). The results from this study are also similar to those obtained by Porcu *et al.* (1997) (65%) and Fabbri *et al.* (2001) (64%), even though a conventional sodium-based freezing medium was used.

It was observed, however, that the fertilization rate of oocytes submitted to ICSI with no previous freezing was slightly higher (65.9%) than that found in frozen-thawed (61.6%) oocytes, which is similar to the report by Quintans *et al.* (2002) that reported 72% fertilization using ICSI. It is often difficult to make direct comparisons with other reports in the literature due to myriad minute differences in media and protocols used.

Although the global fertilization rate at the Fertilitat-Reproductive Medicine Centre through ICSI from 2001 to 2004 was about 75%, the fertilization rate in this study with oocytes from the same patient was lower (65.9%).

Fabbri *et al.* (2001) reported a 58% fertilization rate for previously frozen oocytes, which was similar to the fertilization rate obtained in their IVF programme. Bos-Mikich *et al.* (2001) also observed a fertilization rate of 62% in their IVF/ICSI programme, whereas Isiklar *et al.* (2002) observed fertilization rates of 74.8% and 77.3% when they compared embryos with and without early cleavage, respectively.

Embryo quality and cleavage rate

It was observed in this study that there was no significant difference in the proportion of high quality embryos (grade I and II) that originated from frozen or fresh oocytes; 80.4% grade I and II embryos from frozen oocytes, versus 89.4% grade I and II embryos resulting from oocytes that were fertilized immediately after follicular aspiration. These data are similar to that observed by Azambuja *et al.* (2005a,b).

It should be noted that embryos originating from frozen-thawed oocytes were transferred on the second day following fertilization. The cleavage rate and subsequent number of cells for embryos maintained *in vitro* has been well documented relative to the transfer day (second or third day post fertilization, **Table 2**). Some reports have considered that a fast cleavage, that is, the first mitotic division, could be related to embryo quality and pregnancy rate (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998a).

Lundin *et al.* (2001) reported that 62.5% of embryos considered of good quality originated from the fertilization process in which the first cleavage took place between 25 h and 27 h after the spermatozoon joined with the oocyte, resulting in embryos with four to six cells on the second day. They also observed a pregnancy rate of 41% for embryos with early cleavage versus 31% for embryos with late cleavage; that is, up to 3 cells in the second day after fertilization. In this study, although a statistical difference in embryo quality was not observed, based on morphology, a statistical difference ($P < 0.002$) was observed in the cleavage rate between embryos originating from frozen-thawed oocytes (44.6%) versus embryos from fresh oocytes (71.8%), as measured by a lower than expected cell number at evaluation on the second day. Since the RED criteria includes blastomere symmetry and fragmentation, and does not include cleavage speed, a statistical difference was observed between the groups in cleavage speed, but not in their morphology.

The reason that embryos resulting from an early mitotic division may be of better quality has not yet been clarified. Perhaps there exists a better synchrony between the cytoplasm and the nucleus in these oocytes, for example a greater competence of availability of ATP, mRNA, mitochondria and other cellular components.

Another theory is related to the spermatozoon. In the human species, the centriole that controls mitotic division of the embryo originates from the sperm (Sathananthan, 1998). Thus spermatozoon quality could be an additional factor for early mitotic division. As a consequence, the spermatozoon DNA may influence the embryo (Sakkas *et al.*, 1998b; Larson *et al.*, 2000). These observations cannot completely explain why embryos in this present study that originated from cryopreserved oocytes produced pregnancies, while embryos from fresh oocytes with early mitotic cleavages did not produce pregnancies. However, there are many factors that contribute to producing a viable pregnancy, some of which are not directly related to the embryo, but which impact on its development. Additionally, it is possible that only patients who failed to become pregnant with fresh eggs would come back for their frozen eggs.

Embryos originating from cryopreserved oocytes in this study had a significantly ($P < 0.002$) slower cleavage rate. It may be that the slower cleavage rate observed could be chromosome related, since the main fear in the use of frozen oocytes is aneuploidy. It is interesting that of the 16 cycles in which there was embryo transfer from fresh oocytes, there were no pregnancies, which suggests that even embryos derived from frozen-thawed oocytes that are fragmented and cleave slower can still be viable embryos capable of producing term pregnancies. Of the 16 patients, four became pregnant and five children were born (originating from the frozen-thawed oocytes), and there was one abortion. One patient gave birth to triplets, and the others produced singletons. All of the children are developing physically and mentally in accordance with their ages.

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