Blastocyst versus cleavage stage embryo transfer policy. New aspects of an ongoing debate

Laura Dracea[1,2], Dorina Codreanu[1,2], Anca Coricovac[2], Luminița Mirzan[2], Ioan Boleac[2]

1. University of Medicine and Pharmacy "Carol Davila", Bucharest (Romania)
2. Gynera Fertility Clinic, Bucharest (Romania)

Correspondence: Dr. Laura Dracea e-mail: laura.dracea@gynera.ro

Acknowledgements: This paper is supported by the Sectorial Operational Programme Human Resources Development, financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/89/1.5/64109.

Abstract

Objective. *The aim of this study is to analyze the results of a new embryo transfer policy established in order to balance the learning curve effect and the challenges of starting a new in vitro fertilization (IVF) clinic. The ongoing debate regarding blastocyst versus cleavage stage embryo transfer policy becomes more complex when applied in a new clinic that introduced for the first time in Romania new technologies of assisted reproduction: extended culture to blastocyst stage and vitrification of blastocysts.* ***Methods.*** *Patients have been divided into two groups: those with embryos transferred at cleavage stage (group A) and patients with embryos transferred on day 5, at blastocyst stage (group B). Allocation of patients to cleavage stage or blastocyst embryo transfer has been based on a "all cleaved embryos or blastocyst" policy, witch means that we either transferred all cleaved embryos, if up to three, or we cultured the embryos to blastocyst stage, if more than three, including poor quality embryos. This policy comes in contrast to most programs that make a decision based only on good quality embryos. We made a comparative retrospective analyze of our data. We also present our first initial experience with vitrification of blastocysts.* ***Results.*** *Blastocyst formation rate was very high 47%, reflecting the optimal culture conditions. Implantation and pregnancy rates for blastocysts almost doubled when compared with cleavage stage embryos. Pregnancy rate was 62.20% for blastocyst transfers as opposed to 34.03% for cleaved embryos. The pregnancy rate for all cycles was 53.75%, extremely high for a starting program. The number of vitrified blastocysts transfers was not significant. Obtaining a good survival rate of blastocysts and afterward, a good pregnancy rate with vitrified blastocysts are the main outcomes.* ***Conclusions.*** *The policy of transferring either all cleaved embryos up to three or blastocysts is a safe and efficient policy which eliminates the negative effect of the learning curve in a new clinic that introduces new programs. Extended culture of embryos might improve the success rate when using an adequate incubation system. With our policy, the pregnancy rate is not affected by the selection of embryos at cleavage stage and is overall maximized. The significant difference in pregnancy rate between the two groups is related to the better implantation potential of blastocysts, but also to the worst prognosis of the cycles with few than four embryos obtained. Vitrification of blastocysts seems to be a simple, efficient and convenient freezing technique for a starting program. This study showed a pregnancy rate maximisation and cost and time reducing associated with slow freezing of a number of unviable embryos, being one of the first ongoing blastocyst transfers and pregnancies achieved with vitrified blastocysts which could open new future perspective in obstetric field.*

Keywords: *blastocyst, vitrification, in vitro fertilization, extended culture, starting program*

Introduction

In vitro fertilization (IVF) has been widely adopted as a successful infertility treatment, but continuous efforts are needed to further improve the pregnancy rate.

One of the major challenges in IVF has been the ability to select the embryos that are most likely to implant. Certain morphological criteria (blastomere symmetry, absence of fragmentation, normal cleavage rate) correlate with higher implantation rates but a lot of the good quality embryos, as assessed on day 2 or day 3 may have decreased developmental ability; they may not initiate compaction and activation of the embryonic genome and, finally, they may not reach the blastocyst stage[1].

Numerous reports are not consistent in identifying predictors of positive outcome[1,2,3,4]. The "natural" selection of the embryos, by reaching or not the blastocyst stage, along with morphological assessment of the blastocyst could be a method to improve the pregnancy rate[5]. We could also reduce the number of unviable embryos that are cryopreserved and the multiple pregnancy rate (by reducing the number of embryos transferred)[5,6,7].

Cryopreservation of embryos and gametes is an important method to improve the efficiency of assisted reproduction technologies and also, to preserve fertility in oncologic patients and other several circumstances. The method of cryopreservation widely adopted is conventional slow freezing technology, a time consuming technique with a potential risk of various type of injuries. The most damaging injury seems to be caused by intracellular ice formation, not entirely prevented by slow freezing[8].

Vitrification is an ice-free and ultra-rapid technique of cryopreservation witch became very popular in the recent years. It has been used successfully for cryopreservation of embryos, ovarian tissue, oocytes and possibly whole ovaries[8]. Vitrification is even more promising by making cryopreservation simpler and more convenient than conventional freezing methods[8,9] and, for all these reasons, the method of choice for starting programs.

Received. *2nd September 2012*
Revised: *19th October 2012*
Accepted: *4th November 2012*

These two assisted reproduction techniques (extended culture and vitrification) have not been applied in Romania until 2007, probably due to the extra time and costs required by extended culture and the resistance to introducing new techniques associated with unpredictable results.

Although blastocyst transfer has been shown to be beneficial in good prognosis patients, similar benefits were not seen in an unselected group[3,4,5,12]. The advantages of increasing pregnancy rate and decreasing multiple pregnancy rate[6,7] have been opposed to the risks of cancelling the embryo transfer or having fewer embryos to cryopreserve[11,12].

Analyzing the potential of these techniques to improve the results of our IVF program, we decided to establish, since the first IVF cycle in 2007, a policy based on avoiding selection of cleavage stage embryos, going to extended culture for all cycles with more than three embryos, regardless quality, and vitrification of spare blastocysts. The major difference in our program is that we do not have to select embryos at cleavage stage in order to allocate patients to blastocyst culture, as opposed to most programs that extend the culture only if three or more good quality embryos are available.

This study proposes to maximize the pregnancy rate and reduce the cost and time associated with slow freezing of a number of unviable embryos, being one of the first ongoing blastocyst transfers and pregnancies achieved with vitrified blastocysts which could open new future perspective in obstetric field.

Methods

We present a retrospective data analysis of embryology and medical records at Gynera Fertility Clinic, a private IVF clinic started in 2007, over a period of 3 years, from 2007 till 2010.

The whole culture system was designed to enable the creation of an optimal environment for blastocyst formation, including number and type of incubators, gas mixture and sequential culture media. The embryos were cultured in Cook MINC incubators witch provide an individualized place for embryos in a low oxygen environment; the triple gas mixture supply contains 5% O_2, 6% CO_2 and 89% N_2, as opposed to standard CO_2 supply, witch contains 5 to 6% CO_2 in air (with 20% O_2). The incubator was also supplied the ready made sequential culture media, formulated to contribute the appropriate nutrients to match the gamete's an embryo's shifting metabolic requirements. The embryos are cultured in micro droplets culture media, covered by culture oil. The blastocyst media is a bicarbonate-buffered media with an increased glucose concentration, including essential and non-essential amino acids which makes it ideal for use in low oxygen environment, like incubator those we used.

Vitrification and thawing of blastocysts were performed using Cryotop method as described by Kuwayama[8], with freezing and thawing media supplied by Kitazato (Japan). This method is a minimum volume vitrification approach. After a two-step equilibration in a vitrification solution containing ethylene glycol, dimethylsulphoxide and sucrose, embryos are loaded with a narrow glass capillary onto the top of the film strip in a volume of <0.1 ml.

After loading, almost all the solution is removed to leave only a thin layer covering the oocytes or embryos, and the sample is quickly immersed into liquid nitrogen.

For the thawing process the individual Cryotop was immediately and rapidly transferred into the recovery solution. The vitrified sample was melted instantaneously. The embryos were then sequentially pipette through three steps to rinse them. Then were ready to be incubated for transfer (Figure 1).

We define the standard embryo culture as culturing embryos at cleavage stage, up to 72 hours. Extended culture was defined as culturing embryos in blastocyst culture media, up to morula or blastocyst stage.

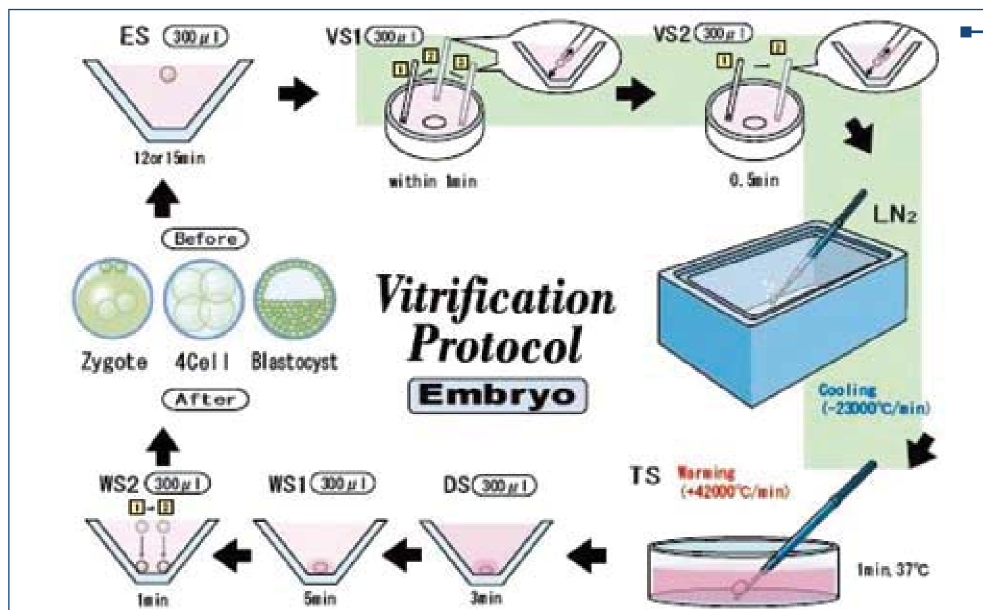

Figure 1. Vitrification protocol

Initially, the general policy was to leave all the embryos for extended culture, only if there were more than 3 good quality cleavage stage embryos, as most of the blastocyst programs do[3,5,7,12]. In several cases we did double transfer, on day 3 and on day 5, for preventing cycle cancellation. After a very short period of time of about two month, we were able to confirm a constantly very good rate of blastocyst formation, even from apparently poor quality embryos. This finding made us change the policy, and culture to blastocyst stage of all the embryos, if they were more than 3, even poor quality embryos. The initial period of time was excluded from the study. The embryo transfer policy became either to transfer all cleaved embryos, if up to three, or to extend the culture to blastocyst stage, if more than three embryos were available, regardless embryo quality.

Ovarian stimulation was similar for all the patients, using a microdose long protocol with daily 0.05 mg triptoreline. The starting dose of the stimulation medication (150 or 225 IU follicle-stimulating hormone) has been established according to our standard protocol, based on antral follicle count, body mass index and previous response to ovarian stimulation; adjustment of the dosage has been performed after 5 days of stimulation, according to ovarian response. Etiology of infertility, age or previous IVF attempts did not affect the laboratory policy. The decision to leave the embryos for extended culture was based only on the number of cleaved embryos available on day 2.

We made a comparative analysis of the results achieved with blastocyst transfers and cleavage stage embryo transfers. The results were analysed using the student t test for independent samples. Statistical significance was set at a two-tailed $p < 0.05$. The main outcomes analyzed were pregnancy rate, implantation rate, multiple pregnancy rate and blastocyst formation.

All the good quality blastocysts available for cryopreservation were vitrified. Good quality blastocysts were defined as embryos with A score and B score cells, according to Gardner's grading system[7]. Assessment of blastocyst morphology after thawing has been done according to the same classification; re-expansion has been assessed following 4 hours of culturing in blastocyst media. Re-expansion represents the sign of viability. An important predictor of successful implantation for vitrified-warmed blastocyst is the blastocyst re-expansion timing. The earlier the blastocyst expands, the better it is expected to perform after transfer.

Fortunately, the majority of couples that have blastocysts vitrified achieved pregnancy in the fresh cycle and they were storing their embryos.

Having in the view that few thawing cycles were done, the survival and pregnancy rates could not be accurately calculated. The fact that we achieved a good survival rate of blastocysts after vitrification and six ongoing pregnancies from the first 16 thawing cycles represents the main outcomes of these data analysis.

Results

We have analyzed 480 IVF cycles performed during a period of 36 months, 144 cycles in group A with cleavage stage embryo transfer (day 2 or day 3) and 336 cycles in group B with blastocyst transfer. The mean age of the patients was 35.22 years old in cleavage stage group and 33.69 years old in blastocyst group. The two groups are also comparable in terms of mean age.

The etiology of infertility is also similar in the two groups and reflects the design of the study, based on unselected patients (Table 1).

Blastocyst formation rate was the first result of the study (Figures 2, 3, 4, 5, 6 and 7). The rate was 47% (1090 out of 2319 embryos), very good when compared to various percentages found in the literature[5,16], ranging from 0 to almost 100%, but mostly between 30 and 50%. The fact that the culture system was designed for optimal blastocyst conditions[13,15], could leave to a better results. The most astonishing finding was the formation of blastocysts from some very poor quality embryos (sometimes with 50% or even higher percentage of fragmentation). In one patient, with 2 previously IVF failures with cleavage stage embryos, such a blastocyst implanted and developed into an ongoing normal pregnancy. On the other hand, a lot of good quality embryos didn't reach the blastocyst stage. These findings made us questioning the efficacy of the selection criteria and encourage the 'natural' selection achieved by extended culture. Reviewing these cycles, we concluded that our policy of avoiding the selection process for cleavage stage embryos has changed the prognostic of a significant number of cycles. Some embryos not suitable for transfer at cleavage stage proved to be able to reach blastocyst stage when better morphological embryos did not.

Table 1 Etiology of infertility

Etiology	A (%) Cleavage stage	B (%) Blastocyst
Tubal factor	29 (20.13 %)	80 (23.80 %)
Male factor	56 (38.88 %)	99 (29.46 %)
Ovarian factor	13 (9.02 %)	16 (4.76 %)
Unexplained	16 (11.11 %)	75 (22.32 %)
Other factors	30 (20.83 %)	66 (19.64 %)
Total	**144**	**336**

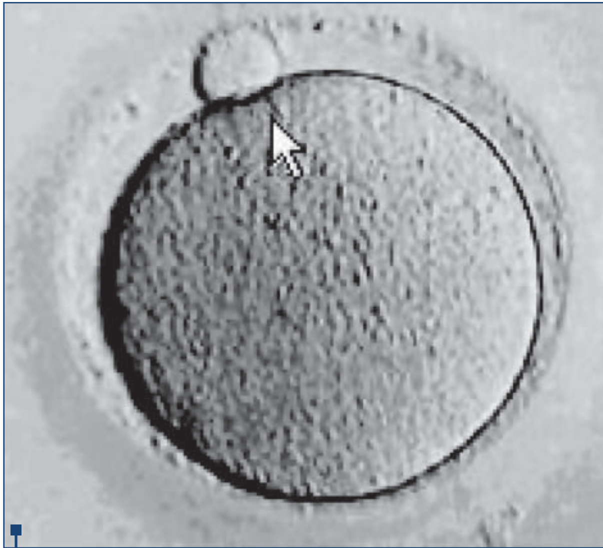

Figure 2. Mature oocyte. Arrow points the polar body

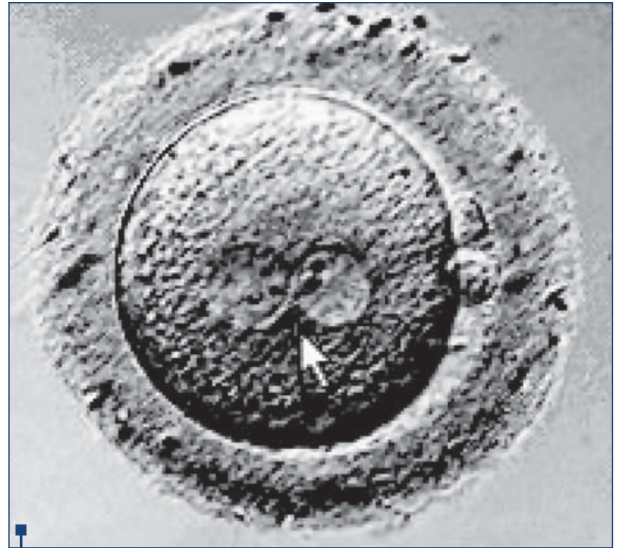

Figure 3. Fertilized egg. Arrow points the two pronuclei (2PN stage)

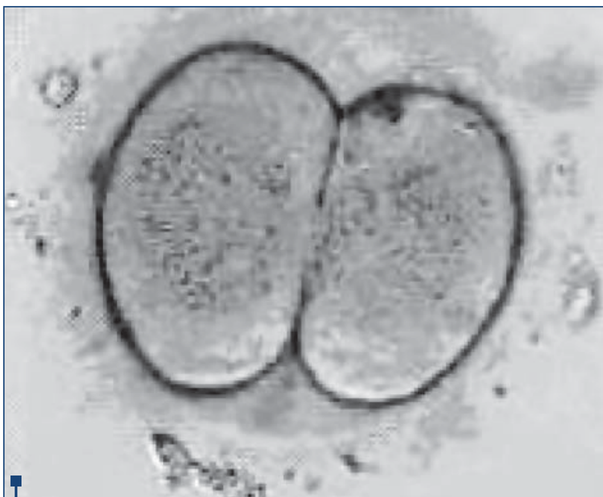

Figure 4. The 2nd day, 2 cell Embryo

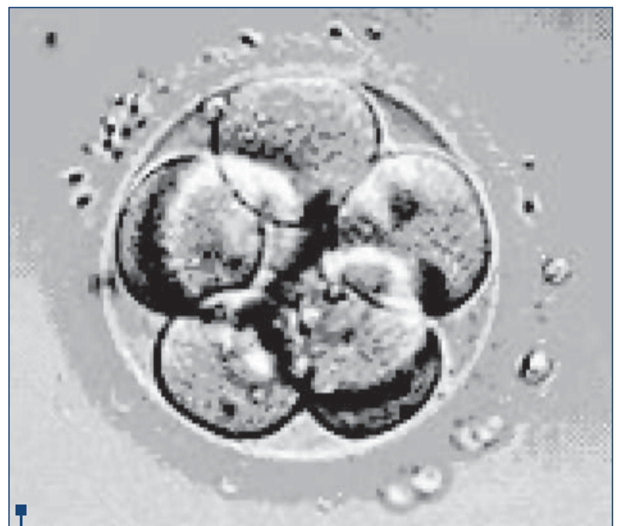

Figure 5. The 3rd day, 8 cell Embryo

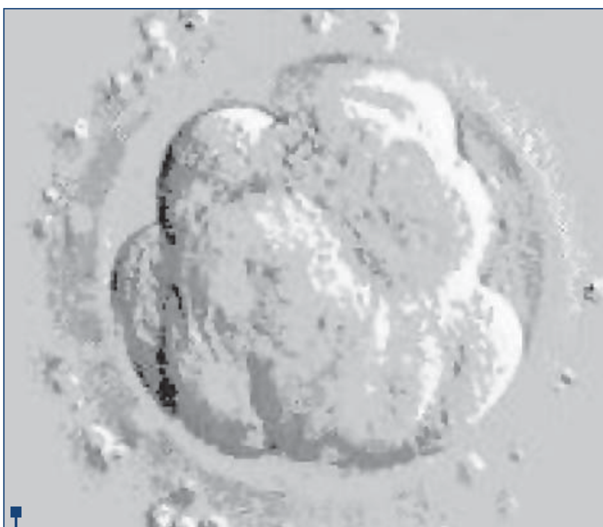

Figure 6. The 4th day, morula stage

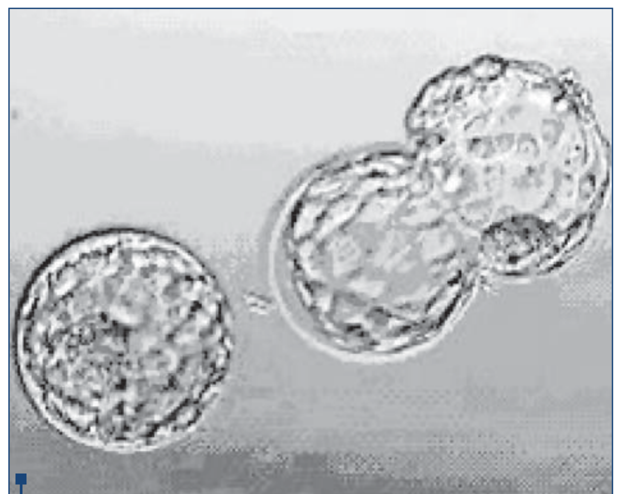

Figure 7. Blastocyst and hatching blastocyst

In only one case we cancelled the transfer in a patient with stage III endometriosis and 6 poor quality embryos on day 3, unable to reach blastocyst stage.

The mean number of embryos transferred was 2.50 in group A and 2.14 in group B. We recommend transferring no more than 2 embryos, but the final decision was taken together with the couple. The high cost of the treatment and the lack of insurance coverage are usually associated with a strong determination of the couple to get the best chance for success. This is why many couples want to have transferred much more embryos that we recommend and we need to find a reasonable solution. A tendency to reduce the number of embryo in blastocyst transfers is observed. This may be due to the clinic policy, but also to the fact that, in some cases, only one embryo reached the blastocyst stage.

Pregnancy rate was 34.03% for cleavage stage group and 62.20% for blastocyst group, a difference that was statistically significant ($p<0.000001$).

The difference in the implantation rate was statistically significant as well ($p<0.000001$). A higher implantation rate of 34.26% for group B, compared to 16.07% for group A reflects the better development potential of blastocysts compared to cleavage stage embryos[17,18,19].

Regarding the vitrification program, we had 252 blastocysts vitrified in 107 freezing cycles, but only 22 thawing cycles, six of them with only one blastocyst.

From a total of 53 blastocysts thawed, 33 of them survived and we performed 16 embryo transfers with those 33 embryos. We showed a total of six clinical pregnancies at 7 weeks. We considered reporting these preliminary data having in the view these firsts pregnancies achieved in our country from vitrified blastocysts are very encouraging for adopting this new technique in a starting program.

Discussion

Blastocysts presents various differences in the cleavage stage embryos. They contain a large number of cells (approximately 100, as opposed to 2-12 cells in 48-72 hours embryos), with two structurally and functionally differentiated cell populations. They have a blastocoele cavity and an activated embryonic genome. The physiological necessities and requirements in order to survive are not the same for embryos at different developmental stage[10,11,12].

Since the development of sequential culture media[13,14], extended culture of the embryos to the blastocyst stage has gained popularity. A low oxygen environment was associated with improved embryo viability and increased clinical outcomes, especially for extended culture[15]. Reducing the oxygen tension in the laboratory could optimize the culture conditions, leading to better embryos.

We decided to use the advantage of high quality extended culture conditions (sequential culture media combined with a low oxygen environment) to determine the optimal embryos for transfer, in order to increase the overall pregnancy rate and avoid unnecessary cryopreservation.

The goal of offering the most cost effective program and the best chances for success might be achieved in a starting program by choosing a safe and efficient embryo transfer policy. The learning curve effects might be minimized when bypassing the subjective selection of embryos at cleavage stage; the costs associated with unnecessary cryopreservation of embryos might be eliminated, without compromising cumulative pregnancy rate.

Regarding the pregnancy rate, our main outcome, the results are very encouraging. Given the retrospective nature of this study, a comparative analyze in terms of groups of age is difficult to do and bias may affect the final result. The overall trend can still be identified, due to the large difference in the results of the two groups (Table 2). Because the blastocyst is better suited to the uterine environment, and blastocyst formation is a form of selection for more viable embryos, we assume that these are the main reasons for better results with blastocysts. The overall pregnancy rate is not affected by a subjective selection of embryos or allocation of patients and seems to be maximized, since we achieved excellent cumulative results for both groups, reaching 53.75% pregnancy rate in unselected patients.

Multiple pregnancy rate could be reduced only for high order multiples, due to the high implantation rate of the blastocysts. Performing single embryo transfer is the only way to further reduce multiple pregnancy rate[5,19]. In one case we confirmed a pregnancy with monozygotic twins, resulted from two blastocyst transferred. Moreover, in another case we have monozygotic triplets resulted, also, from two blastocyst transferred. These data are similar with other results found in literature, the rate of monozygotic twins ranging from 2.7% to 13.2%[20,21]. A number of reports have raised concerns regarding the effects that longer durations of culture, including cryopreservation, may have on the risks of epigenetic mutations in offspring resulting from assisted reproduction, although other studies appear reassuring[22]. All these possible effects of extended culture need to be further assessed to determine the real benefit of a routine blastocyst program[23]. Another study used cryopreservation, viability and quality of umbilical cord blood for successful transplantation. In this regard, the authors showed that sampling conditions procedures are required for pursuing the banking and release of quality umbilical cord blood for successful transplantation[24].

The results may not be similar in respect to other laboratories and/or other methods of prelevation and implantation, taking into account external factors like environment and embryologist skills. Controversy results of different studies may have origin in these differences[25,26,27,28]. From our point of view, the transfer policy based on an almost routine extended culture generated a high overall pregnancy rate, almost unaffected by the learning curve expected for a new clinic and a new program.

Although patients with blastocyst transfers have fewer embryos available for cryopreservation than in cleavage stage embryo programs, a center that performs extended culture should have an established cryopreservation program for surplus blastocysts.

As the limitation of slow rate freezing have become more evident, we have seen in the recent years an increasing inte-

Table 2 Comparative results

Outcome	A Cleavage stage	B Blastocyst	P value
No. of cycles	144	336	
Mean age	35.22	33.69	P = 0.8
Mean no. of embryos transferred	2.50	2.14	P = 0.68
Pregnancies	49 (34.03 %)	209 (62.20 %)	P<0.000001
Implantation rate	16.07 %	34.26 %	P<0.000001
Multiple pregnancy	36.73 %	37.32 %	P = 0.8

rest in the ultra rapid cryopreservation technology[8,29,30,31]. With slow freezing, is difficult to eliminate completely injuries occurring from ice formation and the method requires a long period of time[8]. The superiority of vitrification is not supported by all the studies, but survival and pregnancy rate are extremely high in many vitrification programs[8,32,33]; we might interpret this excellent outcome as the result of a more advanced position on a learning curve. Results with blastocyst slow freezing were inconsistent, so we decided to use exclusively the vitrification method for cryopreservation of blastocysts.

With few thawing cycles we cannot accurately estimate the vitrified blastocyst survival rate; but the very outcome of having most embryos viable after thawing is very promising, considering the expected learning curve.

Conclusions

The preliminary results of our embryo transfer policy for unselected patients strongly recommend extended culture as a good method of increasing success rate. The policy of transferring either all cleaved embryos up to three or blastocysts is simple and safe, eliminating the subjective selection of embryos at cleavage stage and maximizes overall success rates. This policy may not generate the same results in different laboratory conditions.

After our first's experiences using the vitrification method in a starting cryopreservation program, being simple and convenient, the present study showed a pregnancy rate maximisation and cost and time reducing, which could open new future perspective in obstetric field. ■

References

1. Luna M., Copperman AB., Duke M., Ezcurra D., Sandler B., Barrit J. Human blastocyst morphological quality is significantly improved in embryos classified as fast on day 3 (≥10 cells), bringing into question current embryological dogma, Fertil Steril, 2008, 89(2):358–63.
2. Souza Aquitar AP., Herrero L., Pareja S., Alonso M., Losada C., Minguez Y. Human embryo multinucleation on day 2 reduces markedly blastocyst development, Fertil Steril, 2008, 90:341–2.
3. Racowsky C., Jackson KV., Cekleniak NA., Fox JH., Hornstein MD., Ginsburg ES. The number of 8-cell embryos is a key determinant for selecting day 3 or day 5 transfer, Fertil Steril, 2000, 73:558–64.
4. Rienzi I., Ubaldi F., Iacobelli M., Ferrero S., Minasi MG., Martinez F. Day 3 embryo transfer with combined evaluation at the pronuclear and cleavage stages compares favorably to blastocyst transfer, Hum Reprod 2002, 17:1852–5.
5. The Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology, Blastocyst culture and transfer in clinical-assisted reproduction, Fertil Steril, 2008, 90:174–7.
6. Frattarelli JL., Leondires MP., McKeeby JL., Miller BT., Segars JH., Blastocyst transfer decreases the multiple pregnancy rates in in vitro fertilization cycles: a randomized controlled trial, Fertil Steril, 2003, 79:228–30.
7. Gardner DK., Vella P., Lane M., Wagley L., Schlenker T., Schoolxraft WB., Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers, Fertil Steril, 1998, 69:84–8.
8. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The CryoTop method. Theriogenology 67, 73-80, 2007.
9. Baydoun R., Wang B., Al-Qahwaji L., Xu J., Soliman S. Efficiencies of fet cycles using embryos frozen by vitrification vs. programmed slow freezing, Fertil Steril, 2008, 90:291.
10. Behr B. Blastocyst culture without co-culture: role of embryo metabolism, J Assist Reprod Genet 1997, 14(Suppl):13S.
11. Langley MT., Marek DM., Gardner DK., Doody KM., Doody KJ. Extended embryo culture in human assisted reproduction, Hum Reprod, 2001, 16:902–8.
12. Blake D., Proctor M., Johnson N., Olive D. Cleavage stage versus blastocyst stage embryo transfer in assisted conception (review), Cochrane Database Syst Rev, 2005, (2):CD002118.
13. Macklon NS., Pieters MH., Hassan MA., Jeucken PH., Eijkemans MJ., Fauser BC. A prospective randomized comparison of sequential versus monoculture systems for in-vitro human blastocyst development, Hum Reprod 2002, 17:2700–5.
14. Menezo YJ., Hamamah S., Hazout A., Dale B. Time to switch from coculture to sequential defined media for transfer at the blastocyst stage, Hum Reprod, 1998, 13:2043–4.
15. Smuts MP., Miller M., Ramdas-Navale K., Aoki VW. Improved High-Quality Blastocyst Formation Rates Result From In Vitro Embryo Culture in the Cook Minc Incubator Platform, Fertil Steril, 2008, 89–4:21.
16. Gardner DK., Schoolcraft WB., Wagley L., Schlenker T., Stevens J., Hesla J. A prospective randomized trial of blastocyst culture and transfer in invitro fertilization, Hum Reprod, 1998, 13:3434–40.
17. Huisman GJ., Fauster BC., Eijkemans MJ., Pieters MH. Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture, Fertil Steril, 2000, 73:117–22.
18. Karaki RZ., Samarraie SS., Younis NA., Lahloub TM., Ibrahim MH. Blastocyst culture and transfer: a step towards improved in vitro fertilization outcome, Fertil Steril, 2002, 77:114–8.
19. Papanikolaou EG., Camus M., Kolibinakis EM., Van Landuyt L., Van Steirteghem A., Devroey P. In vitro fertilization with single blastocyst stage versus single cleavage-stage embryos, N Engl J Med, 2006, 354:1139–46.
20. Behr B., Fisch JD., Racowski C., Miller K., Pool TB, Milki AA. Blastocyst-ET and monozygotic twinning, J Assist Reprod Genet, 2000, 17:349–51.
21. Milki AA., Jun SH, Hinckley MD., Behr B., Giudice LC., Westphal LM. Incidence of monozygotic twinning with blastocyst compared to cleavage- stage transfer, Fertil Steril, 2003, 79:503–6
22. Lidegaard O., Pinborg A., Andersen AN. Imprinting diseases and IVF: Danish National IVF cohort study, Hum Reprod, 2005, 20:950–4.
23. Kolibianakis EM., Zikopoulos K., Verpoest W., Camus M., Joris H., Van Steirteghem AC. Should we advise patients undergoing in vitro fertilization to start a cycle leading to a day 3 or a day 5 transfer?, Hum Reprod, 2004, 19:2550–4.
24. Marin JA., Calomfirescu M., Bohaltea R., Targoviste Cl., Impcat of maternal and placental pathology on successful umbilical cord blood sampling and cryopreservation, Gineco.ro, 7, 23(1), 2011, 10-14.
25. Hreinsson J., Rosenlind B., Fridstrom M., Ek I., Levkov L., Sjoblom P. Embryo transfer is equally effective at cleavage stage and blastocyst stage: a randomized prospective study, Eur J Obstet Gynecol Reprod Biol, 2004, 117:194–200.
26. Jones GM., Trounson AO., Gardner DK., Kausche A., Lolatgis N., Wood C. Evolution of a protocol for successful blastocyst development and pregnancy, Hum Reprod, 1998, 13:169–77.
27. Levitas E., Lunenfeld E., Har-Vardi I., Albotiano S., Sonin Y., Hackmon-Ram R. Blastocyst-stage embryo transfer in patients who failed to conceive in three or more day 2-3 embryo transfer cycles: a prospective, randomized study, Fertil Steril, 2004, 81:567–71.
28. Papanikolaou EG., D'Haeseleer E., Verheyen G., Van De Velde H., Camus M., Steirteghem A. Live birth rate is significantly higher after blastocyst transfer than after cleavage-stage embryo transfer when at least four embryos are available on day 3 of embryo culture, Hum Reprod, 2005, 20:3198–203.
29. Costigan S., Henman M., Stojanov T. Non-immersion vitrification - a viable alternative to slow freezing of blastocysts, Fertil Steril, 2006, 86–3:208.
30. Fritz RB., Keltz M., Stein D., Gonzales E., Cruz R. A comparison of slow freeze and vitrification in frozen embryo transfer in vitro fertilization, Fertil Steril, 2008, 90:425.
31. Khoury C., Frederick J., Behr B., Potter D. A Comparison of Blastocyst Slow Freeze and Vitrification in Frozen Blastocyst Transfer, Fertil Steril, 2009, 91–3:17.
32. Ozmen B., Schultze-Mosgau A., Youssry M., Diedrich K., Al-Hasani S., Unlu C. Current data on the vitrification of human embryos: witch one is the best; zygote, cleavage or blastocyst stage?, J Turkish-German Gynecol Assoc, 2007, 8(2):218–26.
33. Angle MJ., Shanti A., Smikle C., Kao L. Improved pregnancy rates following the introduction of vitrification for cryopreservation, Fertil Steril, 2008, 90:427–8.