ISSN 1009－3095 *Journal of Zhejiang University SCIENCE* V.3，No.4，P.493－496，Sep.－Oct.，2002
http://www.periodicals.com.cn；http://www.zju.edu.cn/English
http://www.zjupress.com；http://lib.zju.edu.cn/eindex.htm；jzu_s@mail.hz.zj.cn

Factors influencing the outcome of embryo freezing and thawing program

YE Ying-hui(叶英辉)†，JIN Fan(金　帆)，XU Chen-ming(徐晨明)，
XING Lan-feng(邢兰凤)

(*Women's Hospital，College of Medicine，Zhejiang University，Hangzhou 310006，China*)

† E-mail：yeyh@telekbird.com.cn

Received June 21，2001；revision accepted Dec.20，2001

Abstract： Objective：To investigate the factors that might influence the success of an embryo freezing and thawing program. Method：The relationship between the pregnancy rate in 73 cycles of embryo freezing and thawing program and the following factors was analyzed：maternal age，E_2 level at the time of HCG trigger，embryo storage time，number of thawed embryos transferred，presence of sponsoring embryos and intact embryos. And the survival rate of thawed embryos with different morphology，cell stage and storage time was evaluated. Result：Transfer with three or more than three thawed embryos resulted in pregnancy rates of 38.5% and 35.7%，respectively，compared with 5.3% for transfer of fewer than three embryos. The presence of sponsoring embryos and intact embryos significantly increases pregnancy rate in embryo freezing and thawing program. No other factor examined had any effect on pregnancy outcome. The survival rate of good morphology embryos was higher than poor ones，but was not influenced by cell stage and storage time. Conclusion：Embryo morphology before freezing，number of thawed embryos transferred and the presence of intact embryos are important to the outcome of embryo freezing and thawing program.

Key words： Embryo freezing and thawing，Embryo morphology，Pregnancy rate，Embryo survival rate
Document code： A　　**CLC number：** R 321－33

INTRODUCTION

The cryopreservation of human embryos provides many clinical benefits，including an increase in the number of pregnancies per stimulation and retrieval cycle，reduction in the risk of multiple pregnancies and the avoidance of ovarian hyperstimulation syndrome（OHSS）in high-risk settings. To optimize the cryopreservation techniques and clinical management，several factors related to a freeze-thaw cycle were examined in this study to determine which factor weighs most heavily on the establishment of pregnancy. These factors include patient age，embryo quality at the time of freezing，cell stage of embryos，storage time of embryo，number of embryos replaced，intactness of embryos，E_2 level at the time of HCG trigger in the original cycle.

MATERIALS AND SUBJECTS

Patients and treatment

From March 1998 until July 2000，a total 73 out of 77 thawing cycles were completed with embryo replacement. The mean age of patients was 30.4（24－38）years.

Patient preparation，oocyte retrieval，sperm preparation，in vitro insemination，embryo culture and replacement were performed according to published protocols（Jin et al.，1998）.

Embryo cryopreservation policy

Two days after insemination，the embryos were evaluated by an experienced embryologist and graded as follows（Kalstrom et al.，1997）：grade Ⅰ＝ even blastomeres without any fragmentation；grade Ⅱ＝ blastomeres of equal or unequal size and minor（＜25% of blastomeres volume）cytoplasmic fragmentation；grade Ⅲ＝ blastomeres of equal or unequal size and medium cytoplamic fragmentation（25－50% of blastomere volume）；grade Ⅳ＝blastomeres of unequal size and major cytoplasmic fragmentation（＞50% of blastomere volume）. Grade Ⅰ and grade Ⅱ were defined as good morphology embryos，grade Ⅲ and grade Ⅳ were poor morphology embryos. We cryopreserved only grade Ⅰ，Ⅱ

and Ⅲ embryos with the permission of patients. A sponsoring embryo was defined as one having grade Ⅰ or Ⅱ appearance, with two to six blastomeres in a two day embryo(Lightman et al., 1997).

Freezing and thawing procedures

Embryo freezing and thawing were performed according to methods described by Testart et al (1987). Briefly, embryos were exposed to a gradient of propanediol in PBS, and then loaded into straws and frozen in a Planer programmable freezer. Patients returned for embryo thawing 1－25 months later.

Thawing of embryos was done by holding the straw in air and immersing it into a 30℃ water bath for 40 seconds sequentially. The embryos were then exposed to a series of solutions with decreasing propanedial concentration. At the completion of thawing, each embryo was examined with inverted microscopy. The surviving embryos contained at least 50% of their initial blastomeres after freezing and thawing. Embryos were replaced after at least two hours of culture. The mean number of embryos transferred per cycle was 3.2(1－5).

Two different regimens were used to prepare recipients for frozen ET (embryo transfer). Normoovulatory women frequently underwent ET in a spontaneous cycle; Oligoovulatory women and donor oocyte recipients generally underwent ET in hormone replacement therapy (HRT) cycles.

Pregnancy factors and statistical analysis

Six factors were analyzed for their influences on the overall pregnancy rate (PR): patient age at the time of freezing, embryo quality at the time of freezing (presence or absence of sponsoring embryos), E_2 level at the time of HCG trigger in the original cycle, intactness of post thaw embryos, number of embryos replaced and the storage time of embryos.

Quantitative β-HCG assay was performed 12 to 14 days after ET, and a transvaginal ultrasound examination was performed 4－5 weeks after ET to assess fetal viability. Clinical pregnancy was defined as the presence of an gestational sac on ultrasound examination.

χ_2 statistical analysis was used to analyze the data for significance. A comparison was considered significantly different when $P \leqslant 0.05$.

RESULTS

Of 77 frozen-thaw cycles, 73 resulted in transfer. The overall pregnancy rate was 28.8% (21/73) per transfer. The clinical pregnancy rate was 24.7% (20/73) per completed cycle. Six patients with positive fetal heartbeat miscarried; 2 patients' cases were diagnosed as ectopic pregnancy; 11 patients delivered healthy infants. The incidence of birth per cycle was 15.1% (11/73); 306 embryos were thawed in the 77 cycles; 230 embryos survived the procedure. The overall survival rate was 75.2%.

Differences between the pregnant and nonpregnant groups on the aspects of patient age at the time of freezing, E_2 level at the time of HCG trigger in the original cycle, embryo storage time, were found to be statistically insignificant (Table 1).

In this study, 38.5% (10/26) of the patients who received up to three embryos conceived. When more than 3 embryos were replaced, the PR was 35.7% (10/28; NS). However, when 1 or 2 embryos were replaced, the PR was 5.8% (1/19), which was significantly lower ($P < 0.01$) than that when 3 or more than 3 embryos were replaced.

Significant difference was found between the pregnant and nonpregnant groups with regard to both embryo quality and intactness of thawed embryos. Eighteen of 45 patients who received at least one sponsoring embryo conceived, yielding a success rate of 40%, whereas only 3 of 28 patients who did not have a sponsoring embryo conceived (10.7%, $P < 0.01$). Nineteen of 54 patients who received at least one embryo with all blastomeres intact became pregnant (success rate of 35.2%). Only 2 of 19 patients who transferred all damaged embryos achieved a pregnancy ($P < 0.05$).

No appreciable difference in the survival rate was observed with regard to the storage time and embryo developmental stage (Table 2). The morphology of the embryo prior to freezing was related to embryo survival. Of 229 good morphology embryos, 81.7% survived after thawing, while only 55.8% of 77 poor morphology embryos survived after thawing.

Table 1 Relevance of different factors to thaw cycle success

Variable		Pregnant	Not Pregnant	PR (%)	*P*
Patient age	<35	16	44	26.7	>0.05
Patient age	≥35	5	8	38.5	
Number of embryos replaced	<3	1	18	5.3	<0.05
Number of embryos replaced	=3	10	16	38.5	
Number of embryos replaced	>3	10	18	35.7	>0.05
E_2 level	<14000(pmol/L	8	25	24.2	>0.05
E_2 level	≥14000(pmol/L)	8	24	25.0	
Embryo storage time	<6 months	8	28	22.2	>0.05
Embryo storage time	≥6 months	13	28	31.7	
Sponsoring embryo	present	18	27	40.0	<0.01
Sponsoring embryo	absent	3	25	10.7	
Intact embryo	present	19	35	35.2	<0.05
Intact embryo	absent	2	17	10.5	

Table 2 Comparison of embryo survival rate

	Survival rate	*P*
Good morphology embryos before freezing	81.7% (187/229)	<0.01
Poor morphology embryos before freezing	55.8% (43/77)	
≥4-cell embryos before freezing	73.7% (160/217)	>0.05
<4-cell embryos before freezing	78.7% (70/89)	
Embryo storage time≥6 months	73.2%(104/142)	>0.05
Embryo storage time<6 months	76.8%(126/164)	

DISCUSSION

The average number of embryos per stimulated cycle increased due to ovarian stimulation protocol using gonadotropin-releasing hormone agonist (GnRHa) and improved culture techniques. Embryo freezing permits replacement of a more optimal number of embryos and may avoid ovarian hypersimulation syndrome in high-risk settings by freezing all the embryos (Queenan et al., 1997). The pregnancy rate of 24.7% in our study obtained is similar to the rate of fresh embryo transfer. Cryopreservation not only increases the number of ETs and hence the pregnancies per stimulation cycle but also decreases the expense and necessity for additional stimulation and retrieval cycles.

Embryo quality is important to the success of in vitro fertilization-embryo transfer (IVF-ET). Embryo morphology and developmental stage are two parameters related to embryo quality. The fact that the survival rate of good morphology embryos was higher (81.7%) than poor morphology ones (55.8%) suggested poor-quality embryos are more likely to be damaged during the cryopreservation process. As to the developmental stage (<4cell versus ≥4cell stage), no difference of survival rate was found. It was clear that the morphology, not cell stage, is important in considering which embryos should be frozen. The pregnancy rate is also affected by embryo quality. We found that the presence of sponsoring embryo in a batch of thawed embryos significantly increased pregnancy rate, which corroborate the previous report (Ziebe et al., 1997). We suggest the optimal strategy is to include at least one sponsoring embryo in each batch when possible.

Generally, thawed embryos that keep at least 50% of their initial blastomeres are considered to have chances for survival (Kalstrom et al., 1997). Some early studies revealed that the embryonic cell survival rate did not affect the ability of a surviving embryo to implant (Tastart et al., 1987). But our study showed that the pregnancy rate was 35.2% when at least one embryo sur-

vived with all blastomeres intact, but was only 10.5% when no embryo survived with all blastomeres intact. This result indicated that the developmental potential was impaired due to diminished embryonic viability, or the inhibitory effect of the damaged blastomeres. Evidence supporting this hypothesis has been provided by experiments with a mouse model. In these experiments, 3/4 and 1/2 embryos were created by micromanipulative mechanical destruction. These embryos generally retained the ability to progress to the blastocyst stage but were significantly impaired in both hatching and implantation capability (Gordon et al., 1993). Other researches suggested that intact thawed embryos have the same implantation potential as equivalent fresh embryos and that the impact of cryopreservation is limited to blastomere loss directly relating to loss of implantation potential (Edgar et al., 2000). The intact thawed embryos can be regarded as one of the prognostic factors for the outcome of freezing thaw embryo replacement.

Generally, there is a positive relationship between the pregnancy rate of embryo replacement and the number of embryos transferred. But too many embryos transferred will increase the risk of multiple pregnancies. In this study, the likelihood of a pregnancy from transferring of three embryos was the same as that of more than three embryos. Thus, the number of embryo frozen and replaced can be reduced when good quality embryos are utilized.

Previous studies also showed that age is important for the pregnancy rate of both fresh cycles and cryopreservation cycles. In this study, the pregnancy rate was not influenced by increased age ($\geqslant$35). This is most likely related to the fact that the patients were relatively young. It was shown that the pregnancy rate of IVF dropped rapidly in patients aged over 40 and that the reproductive ability started to decline in age 35 – 40 patients (Schalkoff et al., 1993). The patients in this study aged 25 to 38, with majority of them below 35 (82%).

High level of E_2 in the original cycle at the time of HCG trigger is one of the high risk factors for ovarian hyperstimulation syndrome. The fact that the high level of E_2 was not found to adversely affect the pregnancy rate provides support for freezing of all embryos to avoid ovarian hyperstimulation syndrome and replacing the embryos in unstimulated cycles.

It was reported that loss of embryonic cells increased with increasing duration of frozen embryo storage (Testart et al., 1987), although other studies showed that longer storage (1 – 2 years) of human embryos did not lower the PR (Kalstrom et al., 1997). Our result was consistent with the latter one. This finding offers patients the convenience of waiting for a reasonable time after stimulated treatment to undergo a subsequent thaw cycle. However, much longer storage time should be avoided because of the possible deleterious effect of the cryoprotectant and the cryopreservation procedure.

References

Edgar, D.H., Bourne, H., Speirs, A.L., et al, 2000. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum Reprod*, **15**(1): 175 – 179.

Gordon, J.W., Dapunt, U., 1993. A new mouse model for embryos with a hatching deficiency and its use to elucidate the mechanism of blastocyst hatching. *Fertil Steril*, **59**(6): 1296 – 1301.

Jin Fan, Shi Yifu, Zhou Fuzhen, et al, 1998. Influences of sperm quality and quantity on fertilization, cleavage rates and quality of embryos in *in-vitro* fertilization. *Chinese Journal of Obstetric and Gynecology*, **33**(1): 28 – 30 (in Chinese, with English abstract).

Kalstrom, P.O., Bergh, T., Forsberg, A.S., et al, 1997. Prognostic factors for the success rate of embryo freezing. *Hum Reprod*, **12**(6): 1263 – 1266.

Lightman, A., Vertman, L.L., Kol, S., et al, 1997. The presence of a sponsoring embryo in a batch of poor quality thawed embryos significantly increases pregnancy and implantation rate. *Fertil Steril*, **67**(4): 711 – 716.

Queenan, J.T.J.R., Veeck, L.L., Toner, J.P., et al, 1997. Cryopreservation of all prezygotes in patients at risk of severe hyperstimulation does not eliminate the syndrome, but the chances of pregnancy are excellent with subsequent frozen-thaw transfer. *Hum Reprod*, **12**(7): 1573 – 1576.

Schalkoff, M.E., Oskowitz, S.P., Douglas PR, 1993. A multifactorial analysis of the pregnancy outcome in a successful embryo cryopreservation program. *Fertil Steril*, **59**(5): 1070 – 1074.

Testart, J., Laddalle, B., Forman, R. et al, 1987. Factors influencing the success rate of human embryo freezing in an in vitro fertilization and embryo transfer program. *Fertil Steril*, **48**(1): 107 – 111.

Ziebe, S., Peterson, K., Lindenberg, S., et al, 1997. Embryo morphology or cleavage stage. How to select the best embryos for transfer after in vitro fertilization. *Hum Reprod*, **12**(7): 1545 – 1549.